CHEMICALLY-MEDIATED INTERACTIONS IN THE PLANKTON: DEFENSES AGAINST GRAZING AND COMPETITORS BY A RED TIDE DINOFLAGELLATE

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CHEMICALLY-MEDIATED INTERACTIONS IN THE PLANKTON: DEFENSES AGAINST GRAZING AND COMPETITORS BY A RED TIDE DINOFLAGELLATE

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To my family, for not letting me take myself too seriously.

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SUMMARY

The species composition of planktonic communities is determined not only by abiotic factors, such as nutrient availability, temperature, and water column stratification – but also by biotic interactions between hosts and parasites, predators and prey, and among competitors. Blooms of the red tide dinoflagellate, *Karenia brevis*, can dramatically alter the planktonic community, reaching densities of millions of cells per liter and occurring nearly monospecifically. However, *K. brevis* has a relatively low nutrient affinity and growth rate, suggesting that abiotic factors alone cannot explain its dominance of the community. I investigated whether *K. brevis* uses chemical compounds to defend against grazing or to inhibit the growth of competitors. Because *K. brevis* is known to produce brevetoxins, polyketide compounds which act as potent neurotoxins in mammals, I also investigated whether brevetoxins played a role in competition or predator resistance.

Experiments revealed that copepods fed diets rich in *Karenia brevis* experienced lowered survival and fecundity, however, nutritional inadequacy, rather than toxicity, seems to have been responsible for the decrease in grazer fitness. Compounds exuded from natural samples of *K. brevis* blooms did, however, inhibit the growth of four of five model competitors. Compounds exuded from *K. brevis* cultures were similarly allelopathic to competitors. Exposure to these allelopathic compounds resulted in lowered photosynthetic efficiency of all competitors, and decreased cell membrane integrity of three competitors. The allelopathic potency of *K. brevis* blooms was variable between collections and years, but allelopathy did not correlate with bloom density or

concentration of brevetoxins. However, the variability of allelopathy could partially be explained by the presence of specific competitors. The diatom *Skeletonema costatum* reduced the growth-inhibiting effects of *K. brevis* bloom exudates, suggesting that *S. costatum* has a mechanism for undermining *K. brevis* allelopathy. Allelopathic compounds exuded by *K. brevis* that inhibited the growth of the diatom *Asterionellopsis glacialis* were partially characterized. *K. brevis* produced multiple, polar, organic compounds that inhibited *A. glacialis* growth. Two partially characterized allelopathic compounds were found to have molecular weights in the range of 500-1000 Da and to possess aromatic functional groups but no negative charge. Exuded brevetoxins, on the other hand, had no effect on *A.glacialis* growth.

Taken together, these results indicate that *K. brevis* is not chemically defended against grazing, but does produce yet-unidentified allelopathic compounds that inhibit the growth of competing phytoplankton. Brevetoxins had no discernable effect on competitors or grazers, underscoring the important difference between toxicity to mammals and the ecological roles of natural products. Blooms of *K. brevis* may be facilitated by the exudation of potent allelopathic compounds, but the specific phytoplankton assemblage has the potential to alter bloom dynamics.

CHAPTER 1

INTRODUCTION

In marine systems, chemical cues mediate interactions between conspecifics, interspecific competitors, among hosts and their parasites or pathogens, and between predators and their prey. For example, chemical signaling allows some copepods to find and recognize conspecific mates (Yen et al. 1998; Ting et al. 2000). Compounds produced by sponges prevent overgrowth by competitors (Engel and Pawlik 2000; Kubanek et al. 2002), and compounds on the surface of macroalgae prevent fouling (Dworjanyn et al. 2006; Nylund et al. 2006) and infection by pathogens (Kubanek et al. 2003). Some dinoflagellates can detect chemical cues from parasites, and consequently avoid parasitism (Toth et al. 2004). Many seaweeds and benthic invertebrates produce compounds that defend against consumers (reviewed in Hay 1996; Pohnert 2004; Paul et al. 2006). For example, the brown seaweed *Dictyota menstraulis* is chemically defended against mobile herbivores (Duffy and Hay 1994). Chemically-mediated interactions among benthic marine organisms are better characterized than interactions between pelagic marine organisms (McClintock and Baker 2001), but chemically-mediated planktonic interactions have the potential for ecosystem-wide effects, spanning multiple trophic levels and thousands of miles (Hay and Kubanek 2002).

Because grazers regulate the standing crop of phytoplankton during much of the year and can consume up to 60% of the primary production daily (Valiela 1995), selection on phytoplankton to evolve defenses against grazers should be high. However, demonstrating ecologically relevant chemical defenses from single-celled phytoplankton

is a major challenge for researchers (Pohnert et al. 2007). Upon cell lysis, some diatoms have been shown to produce volatile aldehydes which have deleterious effects on grazer reproduction (Pohnert 2000; Ianora et al. 2004a). However, the implications of these findings are still debated; because individual diatoms are killed by grazer attacks, the defense is only advantageous to surviving diatom clones and their descendents (Pohnert 2005). When damaged, the coccolithophore *Emiliania huxleyi*, dimethylsulfoniopropionate is enzymatically cleaved to produce dimethylsulfate (DMS) and acrylic acid, compounds that were initially believed to inhibit grazing by *Oxyrrhis marina* (Wolfe et al. 1997). Further investigation, however, indicated that neither enzymatic product inhibited *O. marina* grazing (Strom et al. 2003). Perhaps the most convincing example of a phytoplankton chemical defense against grazers comes from the dinoflagellates *Alexandrium* spp., which produce paralytic shellfish toxins (PSTs), a group of at least 21 neurotoxic compounds that block mammalian sodium channels (Llewellyn 2006). Recent studies have also indicated that PSTs reduce fitness in bivalves (Bricelj et al. 2005) and copepods (Colin and Dam 2002a; Colin and Dam 2002b). Additionally, the presence of copepods was shown to induce PST production in *Alexandrium minutum*, resulting in selectively reduced copepod grazing on cells with high PST concentrations (Selander et al. 2006).

Phytoplankton have also been shown to kill or inhibit the growth of competitors through the production and exudation of allelopathic compounds (Legrand et al. 2003). For example, compounds exuded by the dinoflagellates *Alexandrium* spp. cause reduced growth, impaired motility, and cell lysis of competitor monocultures and natural competitor assemblages (Tillmann and John 2002; Fistarol et al. 2004b; Tillmann et al.

2007). Planktonic allelopathy can have community-wide effects: allelopathic compounds produced by phytoplankton may alter the species composition of phytoplankton assemblages (Fistarol et al. 2003), influence phytoplankton succession (Keating 1977), and facilitate the spread of invasive species (Figueredo et al. 2007). Allelopathy has also been hypothesized to facilitate the formation of harmful algal blooms (reviewed in Legrand et al. 2003). While some bloom-forming species produce potent neurotoxins which can kill fish, birds, and marine mammals (Landsberg 2002), these toxins have rarely been linked to allelopathy (Sugg and Van Dolah 1999; Tillmann and John 2002; Fistarol et al. 2004b; Lundholm et al. 2005).

Blooms of the red tide dinoflagellate, *Karenia brevis*, occur seasonally in the Gulf of Mexico, sometimes reaching densities of millions of cells per liter (Steidinger et al. 1998). *K. brevis* produces brevetoxins, a group of polyketide neurotoxins that accumulate in shellfish and cause neurotoxic shellfish poisoning in humans (Baden 1989). Brevetoxins also cause massive fish kills (Landsberg 2002). While fish are known to accumulate brevetoxins by ingesting copepods that have fed on *K. brevis* (Tester 2000), fish mortality is suspected to occur primarily by absorption of waterborne brevetoxins directly across gill membranes (Landsberg 2002). Marine mammals including whales, dolphins, and manatees are also suspected to have died from brevetoxin poisoning (Anderson 1992; Trainer and Baden 1999).

The effects of compounds produced by *Karenia brevis* on members of the planktonic community are unclear. *K. brevis* blooms alter the composition of co-occurring phytoplankton species (West et al. 1996), possibly through the production of allelopathic compounds (Kubanek et al. 2005). *K. brevis* has also been reported to

adversely affect zooplankton grazers. Studies have suggested that consumption of *K. brevis* is acutely toxic to some copepods (Sykes and Huntley 1987), however others have reported that grazers avoid consuming *K. brevis* (Huntley et al. 1987; Turner and Tester 1989). *K. brevis* is a useful model organism for understanding chemically-mediated interactions in the plankton, both because *K. brevis* blooms have major ecological and economic effects, and because the influences of *K. brevis* on the planktonic community are largely unknown.

In the following chapters, I will explore the interactions of *Karenia brevis* with zooplankton grazers and phytoplankton competitors. In the first chapter, I consider the effects of *K. brevis* on a grazing copepod, teasing apart the effects of phytoplankton toxicity, nutritional inadequacy, and copepod feeding behavior. In the second chapter, I review the state of the field of allelopathy research in marine systems. In chapters three through five, I report the results of my investigations into the allelopathic effects of *K. brevis* on co-occurring phytoplankton from the Gulf of Mexico, focusing on mechanisms of action of allelopathic compounds, variability in allelopathic effects, competitor responses to allelopathy, and characterization of allelopathic compounds. In the sixth and final chapter, I discuss the methodological limitations that currently hinder research on allelopathy in marine systems and highlight the fruitful areas for future research.

CHAPTER 2

FITNESS CONSEQUENCES FOR COPEPODS FEEDING ON A RED TIDE DINOFLAGELLATE: DECIPHERING THE EFFECTS OF NUTRITIONAL VALUE, TOXICITY, AND FEEDING BEHAVIOR

Abstract

Phytoplankton exhibit a diversity of morphologies, nutritional values, and potential chemical defenses that could affect the feeding and fitness of zooplankton consumers. However, how phytoplankton traits shape plant-herbivore interactions in the marine plankton is not as well understood as for terrestrial or marine macrophytes and their grazers. The occurrence of blooms of marine dinoflagellates such as *Karenia brevis* suggests that, for uncertain reasons, grazers are unable to capitalize on, or control, this phytoplankton growth – making these systems appealing for testing mechanisms of grazing deterrence.

Using the sympatric copepod *Acartia tonsa*, we conducted a mixed diet feeding experiment to test whether *Karenia brevis* is beneficial, toxic, nutritionally inadequate, or behaviorally rejected as food relative to the palatable and nutritionally adequate phytoplankter *Rhodomonas lens*. On diets rich in *K. brevis*, copepods experienced decreased survivorship and decreased egg production per female, but the percentage of eggs that hatched was unaffected. Although copepods showed a 6-17% preference for *R. lens* over *K. brevis* on some mixed diets, overall high ingestion rates eliminated the possibility that reduced copepod fitness was caused by copepods avoiding *K. brevis*, leaving nutritional inadequacy and toxicity as remaining hypotheses. Because egg production was dependent on the amount of *R. lens* consumed regardless of the amount

of *K. brevis* eaten, there was no evidence that fitness costs were caused by *K. brevis* toxicity. Copepods limited to *K. brevis* ate 480% as much as those fed only *R. lens*, suggesting that copepods attempted to compensate for low food quality with increased quantity ingested. Our results indicate that *K. brevis* is a poor food for *A. tonsa*, probably due to nutritional inadequacy rather than toxicity, which could affect bloom dynamics in the Gulf of Mexico where these species co-occur.

Introduction

Herbivores are affected by plant traits, including morphology, nutritional value, and chemical defenses (Fritz and Sims 1992; Rosenthal and Berenbaum 1992). Among terrestrial and marine macrophytes, chemical defenses are known to deter herbivore feeding and/or negatively affect herbivore fitness (Hay and Steinberg 1992). The relative importance of various phytoplankton traits in determining feeding patterns and fitness of zooplankton is less clear, although zooplankton grazers are known to differentiate among prey and are affected by the chemical composition of phytoplankton (e.g., Teegarden 1999).

Several species of marine dinoflagellates, including some that occasionally form dense blooms, produce chemical compounds that are neurotoxic to fish and mammals (Anderson and White 1992). It is hypothesized that these neurotoxins serve as defenses against planktonic grazers, either deterring feeding or killing grazers that do feed, but available data only partially support this hypothesis (Turner et al. 1998). In some cases, grazers such as copepods have suffered negative consequences of feeding on neurotoxic dinoflagellates, with symptoms (e.g., Sykes and Huntley 1987) and lowered fitness (e.g.,

Colin and Dam 2002a) consistent with toxicity. In other cases, copepods have consumed neurotoxic dinoflagellates without obvious adverse effects (e.g., Teegarden and Cembella 1996), whereas dinoflagellates that do not produce known neurotoxins can negatively impact copepod fitness (Ianora et al. 2004b). It is now generally acknowledged that there is no single obvious relationship between phytoplankton neurotoxicity and consequences for planktonic grazers and that experiments involving sympatric grazer and prey species are necessary to understand trophic interactions in the plankton (Smayda 1997; Turner and Tester 1997). Studies of plant-herbivore interactions in the marine plankton may also provide insights into ecosystem-level (or broader) processes, since phytoplankton blooms can move hundreds to thousands of kilometers and affect pelagic, benthic, and coastal communities (Hay and Kubanek 2002).

Karenia brevis (ex *Gymnodinium breve*, ex *Ptychodiscus brevis*), an unarmored pelagic dinoflagellate, has bloomed periodically in the Gulf of Mexico for at least hundreds of years (Steidinger et al. 1998). Ecological effects of *K. brevis* blooms occur at several levels. The phytoplankton community is altered (West et al. 1996), possibly due to allelopathic effects of *K. brevis* (Kubanek et al. 2005). Mass mortalities of clams, crabs, polychaetes, and amphipods have been documented in response to *K. brevis* blooms (Simon and Dauer 1972; Tiffany and Heyl 1978). Fish are known to accumulate brevetoxins produced by *K. brevis*, by ingesting copepods that have fed on *K. brevis* (Tester et al. 2000), but fish mortality is suspected to occur primarily via absorption of waterborne brevetoxins directly across gill membranes (Landsberg 2002). Marine mammals including whales, dolphins, and manatees are suspected to have died from

brevetoxin poisoning (Anderson and White 1992; Trainer and Baden 1999; Flewelling et al. 2005).

How *Karenia brevis* affects sympatric zooplankton grazers is not well understood, and may differ significantly among grazers. When five co-occurring copepod species were presented with *K. brevis* collected from a monospecific bloom, two copepod species ingested *K. brevis* at very low rates, whereas three others, including *Acartia tonsa*, ate increasing amounts of *K. brevis* with increasing availability (Turner and Tester 1989). When these authors offered copepods mixed phytoplankton from a bloom where a common diatom outnumbered *K. brevis*, copepods preferentially ate diatoms, suggesting that *K. brevis* somehow avoids or deters grazers. In addition, the copepod *Acartia tonsa* fed only *K. brevis* suffered lower egg production than *A. tonsa* fed other foods in lab experiments, which could have been due to poor nutritional quality or toxicity of *K. brevis* (Turner et al. 1996). The copepod *Calanus pacificus* rejected *K. brevis* as food (Huntley et al. 1986) and suffered loss of motor control, accelerated heart beats, low survivorship, and interrupted development when exposed to *K. brevis* (Huntley et al. 1987; Sykes and Huntley 1987); however, the geographic range of *C. pacificus* does not overlap with *K. brevis*, which reduces the ecological relevance of these findings. Overall, previous work indicates that both grazer behavior and physiology are affected by *K. brevis*, and that mechanisms could include avoidance, deterrence, nutritional inadequacy, and toxicity.

Deciphering the relative importance of feeding preferences, prey nutritional quality, and prey toxicity is possible if fitness parameters and consumption rates are considered in mixed diet experiments (Jonasdottir et al. 1998). Comparisons of ingestion

rates among grazers offered prey choices can reveal prey deterrence or avoidance strategies that may be based upon prey size, motility, feeding stimulants, and feeding deterrents (e.g., Wolfe et al. 1997). If ingestion rates, grazer survivorship, and grazer reproduction on a suspect phytoplankton food is similar or higher than survivorship and reproduction on a control food known to be healthy for grazers, then the suspect food is likely to be a non-toxic, nutritionally adequate food. If grazer survivorship or reproduction on a suspect food is decreased relative to controls even when the suspect food is consumed at similar rates, then the suspect food may be either toxic or nutritionally inadequate for the grazer. If the suspect food is nutritionally inadequate, consuming it in a mixture with control food should offset the deleterious effects of the suspect food, in proportion to the amount of control food consumed. If the suspect food is toxic, however, it should detract from the beneficial effects of the control food, and so mixing the suspect food with control food will not offset the deleterious fitness effects of the suspect food. In addition, grazers may compensate for low nutritional value by feeding at higher rates when confined to low value, but non-toxic, foods (e.g., Cruz-Rivera and Hay 2000). Colin and Dam (2002a; 2003) successfully applied a mixed diet approach to establish that a marine dinoflagellate, *Alexandrium* sp., with high saxitoxin content was toxic to some copepods of the genus *Acartia* and that copepods became physiologically incapacitated, although this approach could not reveal which compounds (whether specific saxitoxins or other *Alexandrium* natural products) were the direct cause of negative fitness effects. Using the same approach, *Prorocentrum minimum* was found to be nutritionally inadequate, rather than toxic, to a co-occurring copepod (Dam and Colin 2005).

In this study we used a mixed diet approach, in laboratory experiments, to determine whether the red tide dinoflagellate, *Karenia brevis*, is an adequate, toxic, or nutritionally inadequate food, and/or whether it is rejected by the calanoid copepod *Acartia tonsa*. *A. tonsa* was chosen because it is an abundant generalist feeder, occurs throughout the range of *K. brevis*, is present during *K. brevis* blooms, will consume *K. brevis* (Turner and Tester 1989), and bioaccumulates brevetoxins (Tester et al. 2000). Clarifying the interaction between this important phytoplankter and one of the major members of the co-occurring zooplankton should enable a better understanding of the community-level interactions involving harmful algal blooms, and plant-herbivore interactions in general.

Materials and Methods

Organisms

Phytoplankton strains were obtained from the Provasoli-Guillard Center for the Culture of Marine Phytoplankton. *Karenia brevis* (CCMP 2228, cell biovolume 4.0×10^3 μm^3 calculated from dimensions [mean $\pm$ SD] 24.4 $\pm$ 4.3 µm X 24.4 $\pm$ 2.0 µm X 12.9 $\pm$ 1.3 µm, $n = 5$, measured using an Olympus IX50 inverted microscope) originally isolated off Sarasota, Florida, in August 2001, was maintained in a Percival environmental chamber at 22 ^{o}C with a 12:12 h light:dark cycle with irradiance of 100-145 µmol m^{-2} s^{-1} (PAR measurement using Biospherical Instruments Inc light meter). *K. brevis* culture medium was autoclaved L1 (Guillard and Hargraves 1993) prepared with filtered 36 psu Maine seawater. Stock cultures of *Rhodomonas lens* (CCMP 739, cell biovolume 2.9×10^2 μm^3 calculated from dimensions 12.4 $\pm$ 0.7 µm X 6.7 $\pm$ 0.7 µm X 6.7 $\pm$ 0.7 µm, $n = 5$),

originally isolated from Bahamian Gulf Stream waters, were maintained at 25 ^{o}C with a 20:4 h light:dark cycle. *R. lens* culture media was L1 media prepared with filtered artificial seawater (32 psu). Total carbon and nitrogen for *R. lens* and *K. brevis* cells were analyzed by Micro-Dumas combustion at the University of Georgia Institute of Ecology.

Acartia tonsa was originally collected off Skidaway Island, Georgia, USA, in the summer of 2002. Stock cultures were maintained in a 20 ^{o}C incubator with a 12:12 h light:dark cycle, in 2 L bottles with 32 psu filtered artificial seawater with aeration and monthly water changes. *A. tonsa* were maintained with a mixed diet of *Rhodomonas salina* (CCMP 1319; cultured as *R. lens* above) and *R. lens*. The sex ratio in stock cultures was 60:40 female:male (n = 50 copepods sexed). Typical egg production rates for our *A. tonsa* population were 34 $\pm$ 8 (mean $\pm$ SD) eggs per female per day at density of 5 copepods per 150 mL; 20 $\pm$ 3 eggs per female per day at density of 10 copepods per 150 mL; and 14 $\pm$ 4 eggs per female per day at density of 30 copepods per 150 mL when feeding on 100% *R. lens* at concentrations used in grazing experiments (1.89 x 10^4 cells mL^{-1}, n = 5 for all). The highest density of copepods fed *R. lens* at fives times the concentration used in grazing experiments suffered high mortality (61 $\pm$ 28 %) over two days (suggesting that variation in egg production rates were due to crowding and not food limitation). To keep the variance in sex ratio low across experimental beakers without determining the sex of all 1050 copepods needed for the grazing experiment, a density of 30 copepods per 150 mL was used, despite the lower egg production rates and higher mortality rates.

Grazing experiments

Sixteen hours prior to the start of the experiment, *Acartia tonsa* stock cultures were filtered to remove algae and the copepods were transferred to filtered seawater and starved overnight, then randomly sorted into groups of 30 adults. Each group was placed within a "basket" inside in a 250 mL beaker containing 150 mL of 32 psu filtered artificial seawater. Baskets consisted of plexiglass tubing (10 cm length X 5.5 cm diameter) with 160 μm nylon mesh covering one end. Mesh size was chosen to be large enough for eggs but too small for copepods to pass through. Beakers were randomly assigned to one of seven treatments: starvation control, 100% *Karenia brevis,* 100% *Rhodomonas lens*, or a mixture of the two species with 34% $\pm$ 9%, 63% $\pm$ 10%, 81% $\pm$ 4%, or 93% $\pm$ 2% *K. brevis* (percentages calculated by cell biovolume, not cell number, n = 5). In all treatments except starvation controls, total biovolume of phytoplankton ranged from of 5.9 to 9.6 $x10^6$ μm^3 per mL (i.e., for 100% *K. brevis* diet, this corresponds to 1500-2400 cells mL^{-1} and for 100% *R. lens*, 18,100-20,500 cells mL^{-1}). For each treatment, there were five replicate beakers covered with a plexiglass petri dish. Replicates of a treatment were haphazardly interspersed among other treatments in an incubator at 20 ^oC with 12:12 h light:dark cycle.

Phytoplankton were replaced within experimental beakers each day for five days. Immediately after each addition, a 10 mL sample was withdrawn from within each basket and fixed with Lugol's solution. Visual cell counts were performed using 2-5 mL of fixed sample with a Hydrobios settling chamber and an Olympus IX50 microscope. We ran copepod-free controls with mixtures of *Karenia brevis* and *Rhodomonas lens* in seawater prior to the grazing experiment, which indicated that cell densities of these two species

did not significantly change either through growth or settling over 24 h in the absence of copepods (*K. brevis* $p = 0.99$, $n = 4$; *R. lens* $p = 0.95$, $n = 4$; 2-tailed paired t test). Thus, we decided to forgo interspersed copepod-free controls during the grazing experiment in order to maximize the number of experimental treatments and replicates.

Survivorship, ingestion rates, and reproduction were assessed each day, for five days, after the copepods had been allowed to feed for 18 h. The basket containing the copepods was removed from the beaker and placed in a petri dish containing artificial seawater. Live and dead copepods were counted and dead ones removed. The contents of each beaker (minus the basket with copepods) were filtered through a 40 μm mesh in order to collect eggs, and 10 mL of remaining sample was fixed with Lugol's solution for phytoplankton cell counts, as described above. Daily ingestion rates on each phytoplankter were calculated as: $\frac{(C_1 - C_2)(V)}{N} * \frac{24}{18}$ where C_1 is the cell concentration immediately after addition of phytoplankton, C_2 is the cell concentration after 18 h, V is the cell biovolume, and N is the number of live copepods at the end of 18 h. The equation is adjusted for a 24 hour day by dividing by 18 and multiplying by 24 (from Frost 1972 with cell growth term negligeable as determined above). Eggs were transferred to a vial filled with filtered seawater. After one, three, and five days, eggs were fixed immediately with Lugol's solution and counted. After two and four days, live eggs were collected, counted, and incubated for two days at 20 ^{o}C in artificial seawater, after which time nauplii were counted, to calculate egg hatching success. The experimental beakers were filled with 150 mL of fresh filtered seawater and the live copepods in baskets were returned to their beakers, and fresh food of the appropriate mixed composition was added.

To directly test whether compounds exuded by *Karenia brevis* (including brevetoxins) directly affected copepod survivorship, we conducted an additional experiment using the same basket design and feeding protocol described above. Survivorship of *Acartia tonsa* was assessed following exposure to one of four treatment diets: 1) *Rhodomonas lens*; 2) *R. lens* + extracellular *K. brevis* extract; 3) no food (starvation control); 4) no food + extracellular *K. brevis* extract (starting with 8-11 adults per replicate beaker; n = 4 beakers). Extracellular *K. brevis* extracts were prepared from *K. brevis* stock cultures (13,500 cells mL^{-1}), which were incubated for 18 h with a mixture of three adsorbent resins (Diaion HP-20 (Supelco), Amberlite XAD-7-HP (Acros Organics), Amberlite XAD-16 (Supelco)) designed for extraction of lipophilic compounds from aqueous media. Preliminary experiments in our lab confirmed that extracellular brevetoxins were efficiently adsorbed by this method, without lysing live *K. brevis* cells. Lipophilic compounds were eluted from the resin with methanol followed by acetone, and solvents removed by rotary evaporation. The extracellular extract was administered to treatments 2 and 4 each day at the time that food was added, using a concentration of extract equivalent to 1450 cells mL^{-1} of *K. brevis*, first re-dissolving the extract in 100 µl dimethyl sulfoxide (DMSO) per beaker. DMSO was added to treatments that did not receive extract.

Brevetoxin analysis

Karenia brevis stock culture and extracellular filtrate of this stock culture (generated using an Amicon ultrafiltration apparatus with 5 µm Millipore membrane to separate cells from media without lysing cells) were frozen and sent to the University of

North Carolina at Wilmington for type II brevetoxin analysis by competitive ELISA (enzyme linked immunosorbent assay) (Naar et al. 2002). Intracellular brevetoxin concentration was calculated by subtracting the brevetoxin concentration of the filtrate (extracellular brevetoxins) from the brevetoxin concentration in stock cultures, and then dividing by the number of *K. brevis* cells in the sample ($n = 3$).

Statistical analyses

Copepod survivorship and per capita total ingestion rates, egg production rates, and egg hatching success on different experimental diets were compared by ANOVA (SYSTAT 9). Pair-wise comparisons between treatments were made using a Tukey-Kramer post hoc test. Survivorship was compared among treatments after five days. In order to compare total ingestion rates among treatments for the whole experiment, we first averaged feeding data over the five-day period for each replicate, then statistical analyses among treatments were performed.

For each treatment, the percent of *Karenia brevis* ingested (relative to the total ingested biovolume of *K. brevis* and *Rhodomonas lens*) was compared with the percent of *K. brevis* offered in the diet using a 1-sample 2-tailed t-test. In this case, results were deemed significant when $p < 0.013$ (sequential Bonferroni adjustment for multiple comparisons) (Rice 1989).

To determine if *Karenia brevis* undermined, supplemented, or had no effect upon egg production, a reference line was constructed connecting the mean egg production rates for copepods fed 100% *R. lens* and 100% *K. brevis*, and then egg production rate data for copepods fed mixed diets was compared with hypothetical values predicted by a

linear relationship along the reference line (adapted from Jonasdottir et al. 1998). In order to make this comparison, the egg production rate data were fit with both linear regression and a second-order polynomial equations, and the two models were compared by an extra sum-of-squares F test (using GraphPad Prism 4.0), to determine whether the more complicated model (second-order polynomial) was necessary to explain the data. This method is similar to isobolographical analysis, which tests for non-linear interactions in chemical defense and pharmaceutical research (Nelson and Kusar 1999).

Whether the presence or absence of *Karenia brevis* affected egg production as a function of biovolume of *Rhodomonas lens* ingested was assessed by comparison of slopes and Y intercepts from linear regression on two groups of data: egg production rates for copepods that only had *R. lens* to eat – i.e., copepods that were part of the 100% *R. lens* treatment, vs. egg production rates for copepods in the mixed diet treatments that had access to both *K. brevis* and *R. lens* (using GraphPad Prism 4.0).

Results

Survivorship

Survivorship of *Acartia tonsa* declined as the proportion of *Karenia brevis* increased in experimental diets (Figure 1.1A). Measured over a period of five days, survivorship of copepods offered 93-100% *K. brevis* was 29-40% of the survivorship of copepods fed only *Rhodomonas lens* ($p < 0.001$); whereas there were no significant differences in survivorship among copepods offered 34-81% *K. brevis* and only *R. lens*. All starved copepods were dead after five days. Survivorship of starved copepods was only marginally different from copepods fed only *K. brevis* ($p = 0.063$), but was

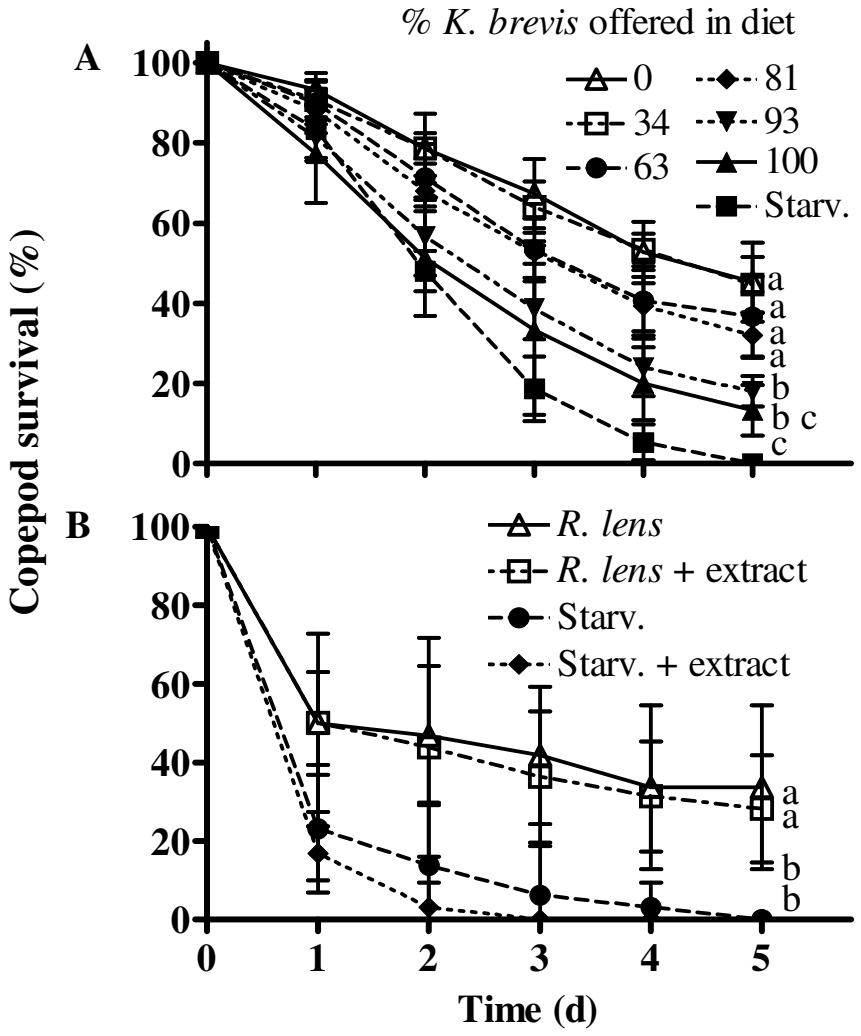

Figure 2.1. Survivorship (mean ± SD) of copepods (*Acartia tonsa*): (A) fed combinations of *Karenia brevis* (0, 34, 63, 81, 93, 100% of diet) and *Rhodomonas lens* for five days or a starvation control (abbreviated Starv.) (n = 5; starting with 30 copepods per replicate) and (B) fed *R. lens* with or without the addition of extracellular extracts of *K. brevis* for five days (n = 4; starting with 8-11 copepods per replicate). Letters indicate significant grouping of treatments (ANOVA and Tukey-Kramer test) at $p \leq 0.05$.

significantly lower than survivorship for copepods on other diets ($p \leq 0.005$ for all) (Figure 1.1A).

Extracellular extracts of *Karenia brevis* did not directly affect *Acartia tonsa* survivorship (Figure 1.1B) or consumption rates (data not shown), when copepods were exposed to extracts while feeding on *Rhodomonas lens* ($p = 0.92$ comparing 5-day survivorship of copepods exposed to *R. lens* vs. *R. lens* + *K. brevis* extracellular extract).

Ingestion rates and feeding preferences

Copepods did not stop feeding when offered *Karenia brevis* – in fact, ingestion rates were highest for copepods on diets rich in *K. brevis* (Figure 1.2). Averaged over 5 days, copepods offered only *K. brevis* consumed 4.8 times as much phytoplankton biovolume as copepods fed only *Rhodomonas lens* ($p < 0.001$). Copepods offered 93% *K. brevis* consumed 3.4 times as much phytoplankton biovolume as copepods fed *R. lens* ($p = 0.007$). This enhanced ingestion rate for copepods fed all or mostly *K. brevis* was most pronounced on Days 3-5 (data for individual days not shown).

To assess whether *Karenia brevis* was rejected by copepods relative to *Rhodomonas lens* within each mixed diet treatment, we compared the actual proportion of *K. brevis* removed by consumption to the proportion of *K. brevis* available in each experimental diet. Copepods discriminated significantly against *K. brevis* when offered 63, 81, and 93% *K. brevis*, consuming 53, 72, and 87% *K. brevis,* respectively, in those treatments ($p = 0.002$-0.011), but did not discriminate significantly against *K. brevis* in the 34% *K. brevis* treatment ($p = 0.25$) (Figure 1.3).

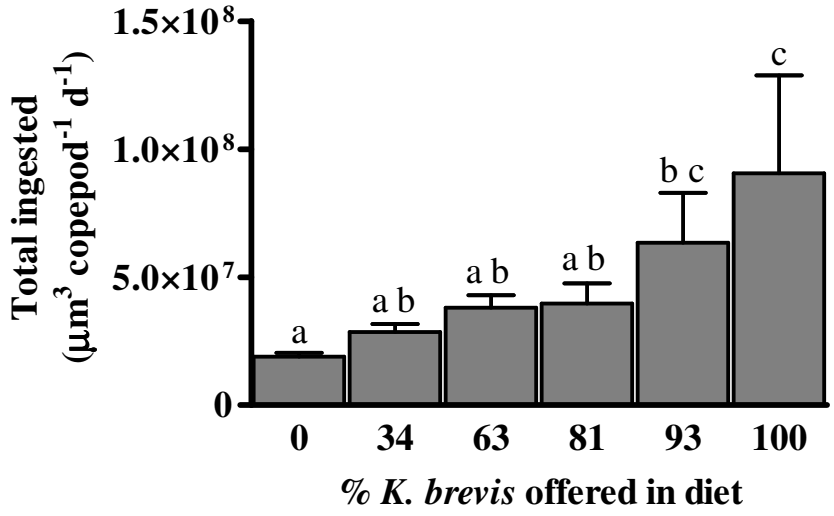

Figure 2.2. Ingestion of experimental diets by copepods (*Acartia tonsa*) (mean ± SD, n = 5). Total daily ingestion rates expressed as phytoplankton biovolume, for copepods fed combinations of *Karenia brevis* and *Rhodomonas lens* each day for five days. Letters indicate significant grouping of treatments (ANOVA and Tukey-Kramer test) at $p \leq 0.05$.

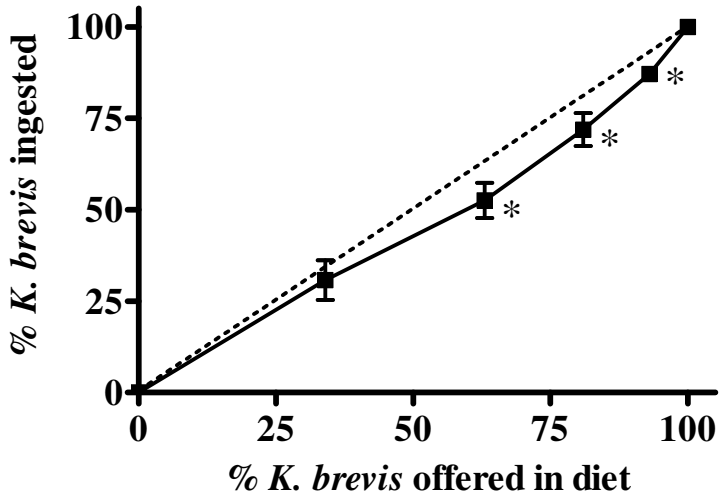

Figure 2.3. Feeding discrimination by copepods (*A. tonsa*). The percent of *Karenia brevis* ingested (—) as a function of the diet offered and the expected proportion of phytoplankters ingested (---) if copepods showed no feeding preferences. Asterisks (*) indicate data that significantly differed from non-discriminatory behavior (1-sample 2-tailed t test) at $p < 0.013$ (determined by a sequential Bonferroni adjustment for multiple comparisons).

Egg production

Egg production was lower for copepods on diets richer in *Karenia brevis* (Figure 1.4A). By the fifth day, copepods offered 81-100% *K. brevis* produced only 25-42% as many eggs per day as copepods fed only *Rhodomonas lens* ($p = 0.005$-0.042) (Figure 1.4A). Egg production by starved copepods was significantly lower than for copepods fed only *R. lens* ($p < 0.001$), but was not significantly different from copepods offered 81-100% *K. brevis* ($p = 0.49$-0.99) (Figure 1.4A). A similar but less pronounced effect of *K. brevis* on egg production also occurred on Days 1 and 3 (data not shown).

Because proportions of *Karenia brevis* ingested did not exactly match proportions of *K. brevis* available in mixed diets, we also tested whether there was a significant relationship between egg production rates and the proportion of *K. brevis* ingested on Day 5, when the compounded effects of several days of exposure to experimental diets could be observed. We found that egg production rates decreased linearly with the proportion of *K. brevis* ingested ($r^2 = 0.57$; $p < 0.001$; Figure 1.4B solid line). When a reference line was drawn connecting the mean egg production rate for copepods fed 100% *R. lens* and 100% *K. brevis* (Figure 1.4B dashed line), and actual egg production rate data were compared with the reference line, we found that the reference line fell within the 95% confidence intervals predicted by the linear regression of the egg production rates. In addition, comparison of linear and non-linear (second-order polynomial) models using an extra sum-of-squares F test resulted in acceptance of the simpler, linear model, rather than the non-linear one ($p = 0.34$).

In order to test whether egg production rates could be predicted by the amount of *Rhodomonas lens* consumed by copepods regardless of the presence of *Karenia brevis*,

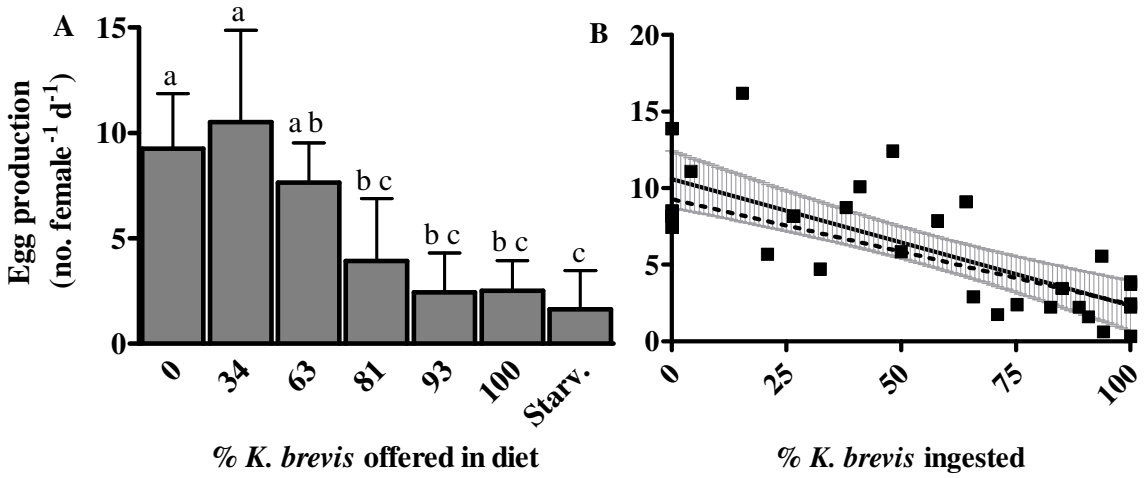

Figure 2.4. Egg production rates for the copepod *Acartia tonsa* (A) fed combinations of *Karenia brevis* and *Rhodomonas lens*, measured on Day 5 (mean $\pm$ SD, $n = 5$) and (B) as a proportion of *K. brevis* in the diet. Letters indicate significant grouping of treatments (ANOVA and Tukey-Kramer test) at $p \leq 0.05$. The solid line in (B) represents linear regression of all data ($r^2 = 0.57$, $p < 0.0001$), with the shaded area representing 95% confidence intervals for this regression. The dashed line connects mean egg production of copepods fed 0% *K. brevis* to mean egg production of copepods fed 100% *K. brevis*.

we compared the relationship between amount of *R. lens* ingested and egg production rates, when *K. brevis* was present (i.e., mixed diets) vs. absent from the diet (i.e, 100% *R. lens* diet) (Figure 1.5). For copepods fed mixed diets, egg production rates increased linearly with consumption of *R. lens* ($r^2 = 0.55$; $p = 0.0002$), whereas for copepods fed only *R. lens*, this relationship was not significant ($r^2 = 0.24$; $p = 0.41$). Nevertheless, egg production appeared to be independent of *K. brevis* ingestion, since slopes and Y intercepts did not significantly differ for copepods fed mixed diets vs. *R. lens* only (p = 0.32, 0.44 for comparisons of slope and Y intercept, respectively, for treatments with vs. without *K. brevis*).

Egg hatching

Egg hatching rates were statistically indistinguishable across treatments, except for starvation controls (Figure 1.6). By Day 4, the hatching rate for eggs produced by starved copepods was significantly lower than for copepods offered 34% *Karenia brevis* in their diet ($p = 0.036$), but indistinguishable from other treatments. It should be noted that by the last three days of the experiment, starved copepods were producing few eggs, approximately 20% as many as copepods fed *Rhodomonas lens* (Figure 1.4A).

Brevetoxin and nutrient content of phytoplankton

As measured by ELISA, our *Karenia brevis* stock cultures contained 12.0 $\pm$ 8.7 pg cell^{-1} (mean $\pm$ SD) intracellular type II brevetoxins and 3.6 $\pm$ 2.1 pg cell^{-1} exuded brevetoxins (n = 3 stock cultures). Total carbon was 510 $\pm$ 70 pg cell^{-1} and total nitrogen was 95 $\pm$ 10 pg cell^{-1} for *K. brevis;* total carbon was 44.3 $\pm$ 3.3 pg cell^{-1} and total nitrogen

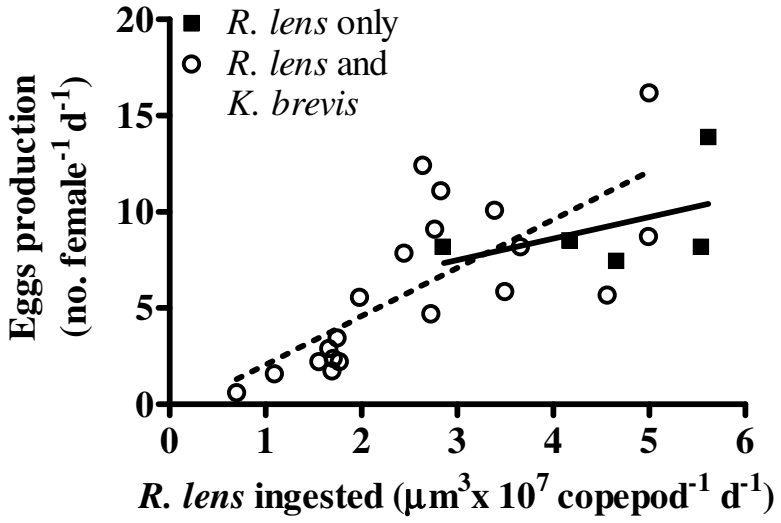

Figure 2.5. Egg production rates on Day 5 for the copepod *Acartia tonsa* as a function of *Rhodomonas lens* ingestion rates. The solid line represents linear regression of data from copepods fed 100% *R. lens* (*R. lens* only, ■) (r^2 = 0.24). The dashed line represents linear regression of data from mixed diet treatments (*R. lens* and *K. brevis*, ○) (r^2 = 0.55) (p = 0.32, 0.44 for comparison of slopes and Y intercepts of two lines, respectively).

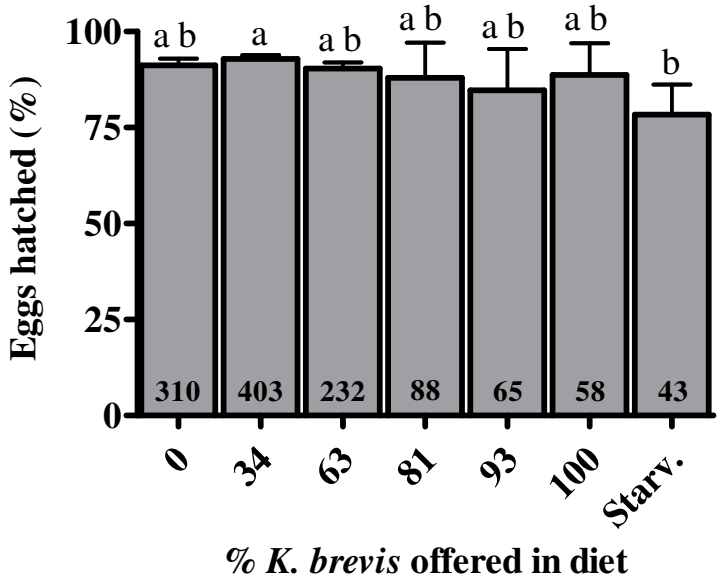

Figure 2.6. Egg hatching success for the copepod *Acartia tonsa* fed combinations of *Karenia brevis* and *Rhodomonas lens*, measured from eggs laid on Day 4 (mean ± SD, n = 5). Letters indicate significant grouping of treatments (ANOVA and Tukey-Kramer test) at $p \leq 0.05$. The total number of eggs used to determine hatching success is shown within the bars.

was 9.92 $\pm$ 0.66 pg cell^{-1} for *Rhodomonas lens* (n = 5 for each species). The C/N ratios for *K. brevis* and *R. lens* were 5.33 $\pm$ 0.24 and 4.46 $\pm$ 0.08, respectively. Thus, in the 100% *K. brevis* diet, copepods were exposed to (1.22 $\pm$ 0.19) x 10^3 μg carbon L^{-1}; in the 100% *R. lens* diet, copepods were exposed to 874 $\pm$ 54 μg carbon L^{-1}; and for mixed diets, carbon levels fell between these two values.

Discussion

Phytoplankton compounds that are toxic to vertebrates may or may not affect the fitness and behavior of invertebrate grazers (Turner and Tester 1997; Turner et al. 1998). Historically, assessing the mechanisms by which zooplanktonic grazers are affected by phytoplankton food quality has not been straightforward. Toxicity may be confounded with starvation, if grazers do not ingest the suspect food. Previous studies that have attempted to assess grazer fitness based on egg production or egg hatching have sometimes not measured ingestion rates or have assessed toxicity by exposing copepod eggs directly to phytoplankton extracts in ways that would not normally occur in the field (see discussions in Hay 1996; Jonasdottir et al. 1998). This may have led to over-reporting of toxic effects of phytoplankton on grazers. On the other hand, we and others may have missed toxic effects of phytoplankton by studying single grazer populations, if effects on grazers are variable among populations due to evolved resistance (e.g., Colin and Dam 2002b) or if lab rearing of copepods affects feeding behavior or food assimilation. The copepods used in the current study came from a population with little historic exposure to *Karenia brevis* blooms, making it less likely that our copepods had evolved resistance to any putative *K. brevis* toxins.

The effects of the red tide dinoflagellate *Karenia brevis* on zooplankton grazers have been unclear, with some studies suggesting acutely toxic effects of consuming *K. brevis* (Sykes and Huntley 1987) and others suggesting avoidance by grazers (Huntley et al. 1986; Turner and Tester 1989). In our experiments, *Acartia tonsa* significantly favored *Rhodomonas lens* over *Karenia brevis* by 6-17% when *K. brevis* made up 63-93% of the available food (Figure 1.3). However, more strikingly, copepods that were offered only or almost only *K. brevis* consumed 340-480% as much phytoplankton biovolume, overall, as copepods fed only *R. lens* (Figure 1.2). The highest total ingestion rates occurred for copepods fed only *K. brevis*.

Combining the evidence for high ingestion rates with reduced copepod survivorship and reproduction, we established that *Karenia brevis* is not an adequate food for the calanoid copepod *Acartia tonsa*. Consuming large quantities of *K. brevis*, even in mixed diets with 7-19% *Rhodomonas lens*, reduced *A. tonsa* egg production rates by 57-74% relative to copepods fed only *R. lens* (Figure 1.4A), although egg hatching success remained unaffected (Figure 1.6). Because we did not measure the lifetime reproduction of individual copepods, nor did we assess survivorship of juvenile copepods or reproduction of distinct age classes of copepods, estimates of fitness calculated from our data should not be generalized to natural copepods populations. Yet, using a basic population growth rate model (Ricklefs and Miller 2000), with birth rates approximated from egg production rates and hatching success, and death rates approximated from adult survivorship data, by Day 5 the instantaneous rate of population increase (r) for copepods fed only *R. lens* was approximately 3.5 times greater than r for copepods fed 93-100% *K. brevis.* Compounding this difference with the already-diminished population size for

copepods consuming *K. brevis* for five days (Figure 1.1A) resulted in population growth rates that differed among our treatments by more than 10-fold. This ought to translate to substantial fitness costs for copepods exposed to *K. brevis* in the field.

Despite eating 3.4-4.8 times as much phytoplankton biovolume (Figure 1.2), copepods offered diets of 93-100% *Karenia brevis* had only 29-40% as much likelihood of surviving 5 days as copepods fed only *Rhodomonas lens* (Figure 1.1A). Thus, the observed negative fitness effects could not have been caused by decreased feeding rates when faced with *K. brevis*, but these data could be explained by either toxicity or nutritional inadequacy of *K. brevis.* The high ingestion rates we observed for copepods on diets rich in *K. brevis* (Figure 1.2) suggested that copepods did not find *K. brevis* toxic but instead found it nutritionally inadequate, and tried to compensate for low quality by consuming greater quantities (see also Cruz-Rivera and Hay 2000; 2003). It remained possible that copepods were dealing with toxic prey by expending energy in detoxification mechanisms, which may have required additional consumption to offset detoxification costs. To further differentiate between nutritional inadequacy and toxicity of *K. brevis*, we analyzed egg production rates as a function of ingestion on mixed diets, as recommended by Jonasdottir et al. (1998).

Because copepod egg production rates (1) decreased with the proportion of *Karenia brevis* ingested (Figure 1.4B, $p < 0.001$); (2) were similarly dependent on the amount of *R. lens* consumed, whether *K. brevis* was present or absent from copepod diets (Figure 1.5); and (3) did not differ significantly from hypothetical rates predicted linearly for mixed diet treatments from the 100% *Rhodomonas lens* and 100% *K. brevis* treatments (Figure 1.4B); and (4) because copepod survivorship was not directly affected

by the presence of *K. brevis* extracellular extracts (Figure 1.1B), we suggest that there is little evidence that *K. brevis* is toxic to *Acartia tonsa*. If *K. brevis* was toxic or reduced assimilation efficiency in this copepod, we would expect that ingesting *K. brevis* would detract from the beneficial effects of eating *R. lens,* such that the number of eggs produced on mixed diets of *K. brevis* and *R. lens* would be lower than the number of eggs predicted solely as a function of the proportion (Figure 1.4B) or quantity (Figure 1.5) of *R. lens* ingested, as previously observed with a high-saxitoxin strain of *Alexandrium* sp. (Colin and Dam 2002a). This did not occur in our experiments, even though our strain of *K. brevis* produced typical concentrations of brevetoxins (Baden and Tomas 1988). The most likely explanation is that *K. brevis* is nutritionally inadequate, with survivorship and egg production for copepods fed 100% *K. brevis* not differing significantly from starvation controls (Figures 1.1A, 1.4A). The attempt by copepods to feed compensatorily on diets rich in *K. brevis* (Figure 1.2) is consistent with the conclusion of nutritional deficiency rather than toxicity. This pattern has also been observed for other marine consumers, as one lowers food quality without the addition of toxins (Cruz-Rivera and Hay 2000; 2003).

Low nutritional value has been suggested to deter consumers (Feeny 1976; Price et al. 1980; Augner 1995). However, when food is nutritionally inadequate, herbivores may consume more in order to compensate for poor quality (Price et al. 1980). In choice feeding experiments, amphipods chose a more nutritious plant food over a less nutritious plant food, but also demonstrated compensatory feeding behavior when presented only with nutritionally poor food (Cruz-Rivera and Hay 2000; 2003). Copepods are also known to compensate for low quality by increasing the quantity consumed (Paffenhofer

1976; Koski et al. 1999), but only with some food sources (Koski and Breteler 2003). We found that *Acartia tonsa* did increase its feeding rate on *Karenia brevis* in the absence or near absence of a more desirable alternative after three to five days of receiving the same diet (Figure 1.2), although this compensatory feeding behavior did not translate into recovered fitness (Figures 1.1A, 1.4A). However, when presented with mixed diets rich in *K. brevis*, *A. tonsa* demonstrated significant preferences for *Rhodomonas lens* (Figure 1.3). The magnitude of discrimination against *K. brevis* (6-17%) was small relative to the magnitude of compensatory feeding (340-480% on diets rich in *K. brevis*), or to the loss of fitness (>50% for copepods fed diets rich in *K. brevis*), suggesting that feeding preferences may not play a dominant role in this grazer-prey system.

Several factors may determine the nutritional value of a phytoplankter to grazers, including protein and carbohydrate concentrations (Jonasdottir 1994). *Rhodomonas lens* is reported to have more protein per biovolume than *Karenia brevis*, but lower levels of carbohydrates (data from Jonasdottir 1994 and Kamykowski et al. 1998), and we found that *R. lens* had a slightly lower C/N ratio than *K. brevis*. However, another important predictor of nutritional quality may be specific fatty acids or fatty acid ratios (Jonasdottir 1994; Kleppel et al. 1998). Giner et al. (2003) hypothesized that *K. brevis* may be nutritionally inadequate because of its unusual sterol composition, such that copepods may be unable to convert these steroids to cholesterol, the principle steroid used by animals, rendering *K. brevis* nutritionally inadequate for copepods. However, this hypothesis has not yet been tested. It is not currently possible to assess which of many possible nutritional factors account for the poor food value of *K. brevis* for *Acartia tonsa.*

Our egg production rates for copepods fed only *Rhodomonas lens* (Figure 1.4A) were 30-77% lower than previously reported for *Acartia* spp. (Jonasdottir 1994; Dutz 1998), which we attribute to crowded conditions in our grazing experiment. Separate experiments indicated that reducing copepod density, but not adding more food, returned egg production to more typical rates (see Materials and Methods). However, the reduction in overall fecundity caused by crowding was not likely to have misled our conclusions, as dramatic differences were observed between copepods fed *R. lens, Karenia brevis*, and mixed diets, all with the same initial copepod densities. In fact, the low survivorship of copepods fed mostly or all *K. brevis* (Figure 1.1A) should have reduced the effects of crowding late in the experiment, which could have led to enhanced egg production rates in those treatments. Yet, we observed significantly lower egg production rates among copepods fed more *K. brevis* (Figure 1.4A). For treatments in which copepods were fed only *R. lens*, copepods had access to 874 $\pm$ 54 μg carbon L^{-1}, which is below the saturation concentration for ingestion and egg production reported for congener *A. clausi* feeding on *R. baltica* (Dutz 1998). Saturation on *K. brevis* has not been measured for *Acartia* copepods; however, in our experiments *A. tonsa* had access to 1215 $\pm$ 186 μg carbon L^{-1} in 100% *K. brevis* treatments, which is similar to saturation levels reported for *Acartia* spp. on other phytoplankton foods (Berggreen et al. 1987; Dutz 1998). Thus, in all treatments, copepods had sufficient carbon for reasonable egg production, but not so much that egg production would be expected to be independent of ingestion rates.

The current study contributes to our understanding of the complexity of relationships between primary producers and consumers, and demonstrates ecological

strategies which are employed by, but not limited to, harmful algal species. Prey may benefit by being nutritionally inadequate to consumers either because consumers preferentially feed on more nutritious prey or by lowering consumer fitness (Cruz-Rivera and Hay 2000; 2003), both of which were observed in our study. However, the adaptive benefits of reducing consumer fitness, post-ingestion, should be minimal for single-celled prey unless prey populations are genetically homogeneous, which has yet to be shown for harmful phytoplankton. Therefore, while low prey nutritional value may negatively affect consumer distribution and abundance, consumer pressure is unlikely to select for low nutritional value among single-celled prey such as phytoplankton. In contrast, consumer pressure ought to strongly select for deterrence mechanisms among prey in general, including phytoplankton – especially if alternative prey are available to consumers. Yet, in our experiments, copepods discriminated only modestly (6-17%) against *Karenia brevis* in mixed diets (Figure 1.3), and in fact ate the most when they had access to high proportions of *K. brevis*. In the field, copepods would typically be exposed to several phytoplankton species as potential prey, except during nearly monospecific blooms of phytoplankton species like *K. brevis*. Overall, it seems likely that the fitness of *Acartia tonsa* would decline if they occurred within *K. brevis* blooms. Although we did not test whether other zooplankton grazers suffer similar negative fitness effects, *A. tonsa* is itself an abundant member of the zooplankton grazing community in the Gulf of Mexico, co-occurring with *K. brevis* (Gunter et al. 1948; but see Sutton et al. 2001), and so the failure of this copepod to capitalize on phytoplankton growth may impact plankton community structure. It is possible that reductions in copepod populations could contribute to the formation or maintenance of *K. brevis* blooms. However, testing this using additional

grazers and phytoplankton species, and under field conditions, is expected to bring additional insight.

CHAPTER 3

ALLELOPATHY IN MARINE SYSTEMS: STATE OF THE FIELD

Introduction

The field of community ecology was strongly influenced by studies of interspecific competition (e.g., MacArthur 1958; Connell 1961; Hutchinson 1961), and researchers continue to consider competition an important factor in structuring communities (Morin 1999). Competition for limiting resources (e.g., nutrients, space) can occur through the differential exploitation of these resources, or through direct interference among competitors (Birch 1957). Allelopathy, a form of interference competition, occurs when chemical compounds produced by one species inhibit or kill another species (Lambers et al. 1998; Morin 1999). While numerous examples of allelopathy in marine systems exist, the role of allelopathic interactions in structuring marine communities is less well known. Few studies have investigated either whether allelopathy is common among marine organisms or the relative importance of allelopathy compared to other mechanisms of competition.

Because several reviews dealing with allelopathy in marine systems have been published in recent years (Gross 2003; Legrand et al. 2003; Graneli & Pavia 2007), we do not provide a comprehensive review of marine allelopathic interactions. Instead we focus on specific studies and systems that best allow us to focus on the limitations of current methods and to emphasize those that have overcome these limitations. For the sake of brevity we have chosen to limit the scope of this review mostly to marine systems but the principles governing allelopathy in freshwater systems are similar and our conclusions

are likely applicable to freshwater environments. We have divided this review into two parts. In this introductory chapter, we provide a brief review of the state of the field, synthesizing progress made in benthic and pelagic marine systems, highlighting the historic differences in research, and focusing on similarities between these environments. In the conclusion chapter, we will explore in more detail the current limitations of allelopathy research, drawing attention to possible solutions to experimental problems, as well as to the most important unanswered questions and fruitful areas for future research.

Allelopathy in benthic and pelagic marine systems

Little is known about how common or how important allelopathic interactions are in marine systems, either in benthic or pelagic communities. In benthic systems, one study indicated that 30% of sponges contained compounds inhibitory to the growth of other sponges, suggesting that allelopathy among sponges may be relatively common (Engel & Pawlik 2000). However, no similar studies have been conducted for other benthic organisms. Likewise, while researchers have detected production of allelopathic compounds from a variety of planktonic organisms (reviewed in Legrand et al. 2003), researchers have conducted no surveys to assess whether allelopathy is a strategy employed by most, or only a few, planktonic organisms. The relative importance of allelopathy in competitive interactions is also largely unknown, in both planktonic and benthic systems. Models suggest that allelopathy could change the outcome of competitive interactions in some systems (Hulot & Huisman 2004), while in other systems models indicate that allelopathy is less important than other factors (Mulderij et al. 2007). Assessing the relative importance of allelopathy is difficult both because

allelopathic interactions are difficult to separate from exploitation competition in the field, and because the effects of allelopathy may be variable. For example, experimental evidence suggests that, at least in planktonic systems, allelopathy may be more important when organisms are nutrient limited (Von Elert & Juttner 1997; Uronen et al. 2005). While examples of allelopathic interactions between pairs of species exist in both benthic and pelagic systems, the processes shaping these interactions and their ecological implications are often considered separately by scientists. However, the factors influencing allelopathic interactions in the two systems are often similar, and exploring differences between environments has the potential to raise important questions and inform future research directions.

Because competitive environments should select for allelopathy, it is important to understand the factors that create intense competition in benthic and pelagic systems in order to understand under which conditions allelopathy will be favored. For example, nutrients are often the major limiting resource in planktonic communities (Tilman 1982; Valiela 1995) and so allelopathy has been hypothesized as a mechanism to prevent nutrient limitation by weak exploitation competitors. In contrast, settlement space is often limiting in benthic communities (Porter & Targett 1988; Menge & Farrell 1989), leading to tests of allelopathic interactions as mechanisms to acquire or retain space (Graneli & Pavia 2007). Alternative hypotheses (e.g., that nutrients are limiting in benthic systems or space in pelagic systems) are rarely tested. However, several studies suggest that phytoplankton do compete for the space that has light of appropriate wavelength and intensity necessary for growth (e.g., Huisman et al. 2004; Berger et al. 2006; Stomp et al. 2007), and that allelopathic interactions may play a role in this competition (Pouvreau et

al. 2007). Likewise, nutrient availability may influence allelopathic interactions in benthic systems (Suzuki et al. 1998; McCook et al. 2001). Knowledge of the limiting resource in a given system (i.e., nutrients or space) may allow scientists to better predict the conditions under which allelopathic interactions will occur. For example, in some cases the production of allelopathic compounds is induced when a specific nutrient are limiting (Von Elert & Juttner 1997; Uronen et al. 2005) indicating that low nutrient conditions promote allelopathy and consequences of allelopathic interactions will be more likely to be observed under these conditions.

Allelopathic compounds have been shown to be exuded into the water column by planktonic organisms, whereas in benthic systems, allelopathy has usually been shown to be mediated by direct contact between organisms co-occurring on the ocean floor. However, some phytoplankton compete through direct contact (e.g., Uchida et al. 1995; Yamasaki et al. 2007b) and some benthic organisms can inhibit the settlement and growth of competitors through exuded compounds (e.g., Dobretsov et al. 2006), indicating that in both systems allelopathic interactions may occur through either mechanism. Some differences between allelopathy in benthic and pelagic systems may be real, but researchers may also be predisposed towards generalizations because of the chosen experimental design, for example by working with exudates of cultured planktonic organisms vs. whole tissue samples of field-collected benthic organisms. In general, allelopathic interactions may not be seen, or may be misinterpreted if researchers fail to observe interactions outside of the prevailing paradigm, fail to devise and test alternative hypothesis, or incorrectly interpret the results of studies.

Identity of allelopathic compounds

The identity of allelopathic compounds is largely unknown in pelagic systems, however, in benthic systems several allelopathic compounds have been identified from whole tissue extracts or surfaces of allelopathic organisms. For example, the red alga *Plocamium hamatum* caused tissue necrosis to the coral *Sinularia cruciata*, which was explained by the monoterpene, chloromertensene (de Nys et al. 1991). The defensive compound 7-deacetoxyolepupuane also allowed the sponge *Dysidea* sp. to overgrow a competing sponge, *Cacospongia* sp. (Thacker et al. 1998). Similarly, researchers have found that triterpene glycosides, which also act as antipredator defenses, prevented the sponge *Ecctyoplasia ferox* from being overgrown by competing sponges and another sponge, *Erylus formosus,* from being fouled by propagules (Kubanek et al. 2002). In addition, several well-characterized furanones from the surface of the red algae *Delisea pulchra* deterred settlement of fouling organisms at ecologically relevant concentrations (Dworjanyn et al. 2006).

In pelagic systems, studies have rarely identified pure compounds with allelopathic effects at their natural exuded concentrations. Kubanek et al. (2005) found that a mixture of pure brevetoxins produced by the dinoflagellate *Karenia brevis* modestly inhibited the growth the diatom *Skeletonema costatum*, but unidentified compounds, rather than brevetoxins, were primarily responsible for the allelopathic effect on *S. costatum* (Prince et al. submitted). In some cases pure compounds that inhibited the growth of competitors have been identified (Kajiwara et al. 1992; Windust et al. 1996; Perez et al. 1997; Ribalet et al. 2007), but either these compounds were not allelopathic at natural concentrations (Kajiwara et al. 1992; Sugg & Van Dolah 1999) or allelopathic

effects were not tested at natural concentrations (Perez et al. 1997). Polyunsaturated aldehydes have been shown to inhibit the growth of phytoplankton species from several different genera (Ribalet et al. 2007), but since these compounds are produced by diatoms upon cell lysis (Pohnert 2005) it is not clear whether they are allelopathic under ecologically relevant conditions. In other cases, researchers have partially characterized allelopathic compounds, but the structures were not determined. For example, polar lipids have been isolated from the exudates of dinoflagellate *Karlodinim micrum* (Deeds et al. 2002), and have been shown to inhibit the growth of some co-occurring phytoplankton (Adolf et al. 2006). Additionally, a polyphenolic pigment has been isolated from exudates of the diatom *Haslea ostrearia*, which inhibits the growth of competing phytoplankton (Pouvreau et al. 2007).

Physiological effects of allelopathic compounds on competitors

The outcomes of allelopathy are usually reported as inhibition of competitor growth among phytoplankton (e.g., Schmidt & Hansen 2001; Kubanek et al. 2005; Yamasaki et al. 2007a) and tissue necrosis among benthic organisms (e.g., de Nys et al. 1991; Aceret et al. 1995; Thacker et al. 1998). In addition, allelopathic compounds have been shown to prevent organisms from being overgrown (e.g., Engel & Pawlik 2000; Kubanek et al. 2002) or to inhibit fouling and settlement of competitor larvae (e.g., Maida et al. 1995; Koh & Sweatman 2000; Dobretsov et al. 2006). Only a few studies have considered mechanisms of action of allelopathic compounds, but from these studies some physiological targets have been identified, including inhibition of photosynthesis,

inhibition of specific enzymes, cell membrane disruption, loss of cell motility, and life history changes.

Several studies involving both benthic and pelagic systems have reported decreased photosynthetic efficiency (usually measured as the maximum quantum yield of photosystem II [Parkhill et al. 2001]) of organisms exposed to allelopathic compounds. For example, Prince et al. (2008) reported that compounds exuded by *Karenia brevis* blooms and cultures inhibited the photosynthetic efficiency as well as growth of five competing phytoplankton species. Similarly, polyunsaturated aldehydes, compounds produced by some diatoms upon cell lysis, resulted in chlorophyll degradation several phytoplankton including the dinoflagellate *Alexandrium carterae* (Ribalet et al. 2007). In a benthic system, caulerpenyne, a terpenoid compound produced by the invasive seaweed *Caulerpa racemosa*, inhibited the photosynthesis of native seagrass (Raniello et al. 2007). Because corals contain symbiotic zooxanthellae, corals may also be subject to decreased photosynthetic efficiency due to allelopathy. Pawlik et al. (2007) found that sponges had two mechanisms to decrease coral photosynthesis. Compounds extracted from some sponges negatively affected the hard coral *Diploria labyrinthiformis* by decreasing total number of zooxanthellae within coral tissue (i.e., coral bleaching) while compounds extracted from other sponges decreased the photosynthetic efficiency of zooxanthellae. Aceret et al. (1995) found that the hard corals *Acropora formosa* and *Porites cylindrical* expelled zooxanthellae in response to flexibilide, a terpene extracted from competing soft corals; however it is not clear whether the hard corals would be exposed to this compound at allelopathic concentrations in the field.

In pelagic systems, the effects of allelopathic compounds on a few specific enzymes have been described. For example, okadaic acid, a diarrhetic shellfish toxin produced by several dinoflagellates including *Prorocentrum lima*, inhibited protein phosphatases *in vitro* (Bialojan & Takai 1988), and also suppressed growth of a variety of phytoplankton species (Windust et al. 1996). Okadaic acid is hypothesized to enter competitor cells in the form of okadaic acid diol-ester, (Windust et al. 1997; Windust et al. 2000), where it may be converted to the more polar and more toxic okadaic acid (Hu et al. 1995; Sugg & Van Dolah 1999). The mechanism of action has been characterized for allelopathy among planktonic organisms in the freshwater Sea of Galilee. Compounds exuded by *Microcystis* sp. which inhibited the growth of the dinoflagellate *Peridinium gatunense* (Vardi et al. 2002) also inhibited photosynthesis in *P. gatunense*. Because photosynthesis inhibition occured with a build-up of internal carbon stores, the authors suggested that it was caused by suppression of carbonic anyhydrase in *P. gatunense* (Sukenik et al. 2002).

Studies of allelopathy in pelagic systems have occasionally reported damage to competitor cell membranes. For example, compounds exuded by blooms and cultures of the red tide dinoflagellate, *Karenia brevis*, increased the membrane permeability of three of five competitor species, indicating that compounds exuded by *K. brevis* damage the cell membrane integrity of competing phytoplankton (Prince et al. 2008). Since an increase of membrane permeability was observed before competitors died, it is likely that this represents a mechanism of allelopathy rather than a side effect of cell death. Additionally, the freshwater dinoflagellate *Peridinium aciculiferum* may cause membrane damage in competitor cells; Rengefors & Legrand (2001) observed that filtrates of *P.*

aciculiferum caused blisters on the surface of *Rhodomonas lacustris* and eventually cell death. Exposure to polyunsaturated aldehydes also resulted in the membrane disruption of several other phytoplankton species, including the prymnesiophyte *Isochrisis galbana* and the prasinophyte *Micromonas pusilla* (Ribalet et al. 2007).

Exposure to allelopathic compounds may also change the behavior or life history of competitor species. In co-incubation experiments, several strains of dinoflagellates *Alexandrium* spp. inhibited the cell motility of the heterotrophic dinoflagellates *Oblea rotunda* and *Oxyrrhis marina* and autotrophs *Dunaliella salina*, and *Thalassiosira weisflogii* (Tillmann & John 2002). Exposure to *Alexandrium ostenfeldii* caused *Rhodomonas* sp. cells to swim irregularly (Tillmann et al. 2007). The autotrophic dinoflagellate *Scrippsiella trochoidea* underwent a life history change in response to allelopathy, forming temporary cysts in response to compounds produced by *Alexandrium spp., Karenia mikimotoi*, and *Chrysomulina polylepsis* (Fistarol et al. 2004a; Tillmann et al. 2007). Although no experiments were performed to determine if *S. trochoidea* cysts grew vegetatively after compounds were removed, the authors suggested that cyst formation might be a mechanism that allows *S. trochoidea* to escape allelopathic compounds and then to re-emerge when conditions have improved. In benthic communities, larval recruits may also have strategies to avoid negative fitness effects of allelopathy. For example, compounds produced by *Caulerpa racemosa* prevented settlement of polychaete and bryozoan larvae (Dobretsov et al. 2006).

Conclusions

Research on allelopathy in marine communities has provided many examples of allelopathic interactions between pairs of species, indicating that benthic invertebrates, macroalgae and plankton use compounds that kill or inhibit the growth of competitors. At the sub-cellular level, the physiological consequences to target organisms are varied, but may include reductions in photosynthesis (Raniello et al. 2007), inhibition of specific enzyme activities (Windust et al. 1996), and damage to cell membranes (Prince et al. 2008). Competitors may avoid exposure to allelopathic compounds with behavioral (Tillmann et al. 2007) or life history changes (Fistarol et al. 2004a). With increasing examples of allelopathic interactions in marine systems, role of allelopathy in structuring marine communities can be elucidated.

CHAPTER 4

EFFECTS OF HARMFUL ALGAL BLOOMS ON COMPETITORS: ALLELOPATHIC MECHANISMS OF THE RED TIDE DINOFLAGELLATE *KARENIA BREVIS*

Abstract

Because competitive interactions may have led to adaptations enabling bloom-forming phytoplankton to dominate pelagic communities, we explored the allelopathic effects of one red tide dinoflagellate, *Karenia brevis,* on competing phytoplankton species. Exposure to waterborne compounds from natural *K. brevis* blooms resulted in growth inhibition or death for four out of five co-occurring species tested, whereas compounds exuded by *K. brevis* cultures suppressed three of these same competitors (the diatoms *Asterionellopsis glacialis* and *Skeletonema costatum* and the dinoflagellate *Prorocentrum minimum*) plus one additional species (the dinoflagellate *Akashiwo* cf. *sanguinea*) that was unaffected by bloom exudates. *K. brevis* exudates lowered photosynthetic efficiency and damaged cell membranes of competing phytoplankton, but had no effect on competitor esterase activity, nor did they limit competitor access to iron. Overall, during blooms, *K. brevis* exudes potent allelopathic compounds, competitors vary in their susceptibility to *K. brevis* allelopathy, and *K. brevis* may achieve nearly monospecific blooms by lowering the photosynthetic efficiency of competitor species and increasing competitor membrane permeability, eventually resulting in competitor growth suppression or death.

Introduction

Because the composition of phytoplankton communities is determined by a wide variety of abiotic and biotic factors, the plankton environment has been used as a model system to understand species interactions and diversity through the lens of disturbance (Hutchinson 1961), predator-prey interactions (Leibold 1989), and resource competition (Tilman 1982). In addition to competing for limiting resources, phytoplankton may exclude each other more directly. The inhibition of competitors by the release of compounds, a process known as allelopathy, may be important in planktonic systems (reviewed in Legrand et al. 2003). Allelopathy has been hypothesized to play a role in species succession (Keating 1977), the formation of harmful algal blooms (Smayda 1997), and the establishment of invasive species (Figueredo et al. 2007).

Despite its likely importance, our understanding of allelopathy is still in the early stages. Allelopathy is difficult to conclusively demonstrate in the field, and responsible compounds have rarely been identified. Co-culturing experiments and observations of phytoplankton dynamics in the field have supported the possibility of allelopathy (e.g., Schmidt and Hansen 2001; Vardi et al. 2002), but have not definitively separated its effect from exploitative competition. Although lab experiments using high nutrient concentrations have helped to shed light on the process of allelopathy, their value has often been undermined by the use of whole cells extracts rather than exudates (Freeburg et al. 1979), which places phytoplankton in contact with a suite of compounds not usually waterborne. We need to simultaneously better understand how allelopathy happens, and what its consequences are in the field (e.g., Fistarol et al. 2003).

Although allelopathic compounds remain mostly unidentified, mechanisms for allelopathy have been proposed in some cases. Possible modes of action include oxidative damage, loss of competitor motility, inhibition of photosynthesis, inhibition of enzymes, and membrane damage (reviewed in Legrand et al. 2003). For example, Vardi et al. (2002) found that the presence of the cyanobacterium *Microcystis* sp. caused a build-up of apoptosis-inducing reactive oxygen species in the competing dinoflagellate *Peridinium gatunense*. Further, compounds produced by two dinoflagellate species have been reported to cause loss of motility in competitor cells, although there are likely multiple mechanisms. Cell contact with dinoflagellate *Heterocapsa* sp. was required for a loss of competitor motility (Uchida et al. 1995), whereas cell-free filtrates of the toxic dinoflagellate *Alexandrium* spp. caused loss of motility in the heterotrophic dinoflagellate *Oxyrrhis marina* (Tillmann and John 2002).

Several studies have indicated that photosystem II (PSII) may be a target for allelopathy. Unknown compounds produced by the cyanobacterium *Trichormus doliolum* inhibited PSII in other cyanobacteria (Von Elert and Juttner 1997). However, a decrease in photosynthetic efficiency may be a symptom of allelopathy even if PSII is not the target. For example, Sukenik et al. (2002) found that compounds produced by the cyanobacterium *Microcystis* sp. inhibited carbonic anhydrase activity of the dinoflagellate *Peridinium gatunense*, leading to CO_2 limitation and inhibition of photosynthesis. A decrease in PSII efficiency has also been reported for a variety of general cell stresses, including nutrient stress (Parkhill et al. 2001), temperature stress (Lesser and Gorbunov 2001), and metal toxicity (Miller-Morey and Van Dolah 2004).

In addition to carbonic anhydrase inhibition observed by Sukenik et al. (2002), other studies have reported enzyme inhibition as a mechanism for allelopathy. When tested as a pure compound, okadaic acid, produced by the dinoflagellate *Prorocentrum lima*, was shown to inhibit the growth of three microalgal species (Windust et al. 1996), perhaps because okadaic acid is a potent phosphatase inhibitior (Bialojan and Takai 1988). However, compounds other than okadaic acid appeared to be responsible for allelopathic effects of *P. lima* filtrates (Sugg and Van Dolah 1999). Microcystins, produced by the cyanobacterium *Microcystis aeruginosa,* were also shown to inhibit phosphatases (Dawson 1998), but no studies have demonstrated that microcystins are allelopathic at ecologically relevant concentrations (Babica et al. 2006). As with a decrease in photosynthetic efficiency, phytoplankton enzyme activity may be a general indicator of cell stress. For example, esterase activity in several species of marine and freshwater algae can be either enhanced or suppressed by copper toxicity (Franklin et al. 2001).

Damage to cell membranes is another proposed mechanism for allelopathy (Legrand et al. 2003). Microalgal compounds have been shown to damage red blood cell membranes which suggests that competing phytoplankton could be similarly affected (Igarashi et al. 1998), and fatty acids potentially produced by microalgae have been shown to increase permeability of the plasma membranes of chlorophytes and cyanobacteria (Wu et al. 2006). The freshwater dinoflagellate *Peridinium aciculiferum* may cause membrane damage in competitor cells; Rengefors and Legrand (2001) observed that filtrates of *P. aciculiferum* caused blisters on the surface of *Rhodomonas lacustris* and eventually cell death. However, more research is needed to definitively

establish that compounds exuded by phytoplankton at realistic waterborne concentrations cause membrane damage in competing phytoplankton.

Blooms of *Karenia brevis*, a red tide dinoflagellate occurring in the Gulf of Mexico and occasionally along the Southeastern coast of the United States (Tester et al. 1991), cause ecosystem-wide effects, mostly due to the production of neurotoxic compounds (brevetoxins) that kill fish and accumulate in shellfish (Landsberg 2002). Like many dinoflagellates, *K. brevis* has a low nutrient affinity and growth rate compared to most diatoms and other phytoplankton taxa (Smayda 1997; Steidinger et al. 1998). This suggests that *K. brevis* does not achieve densities of millions of cells L^{-1} (Steidinger and Haddad 1981), altering the phytoplankton community (West et al. 1996), by exploitation competition. One possible explanation for its occasional dominance is that *K. brevis* uses allelopathy to out-compete other phytoplankton species, as previously demonstrated using cultured strains of *K. brevis* (Kubanek et al. 2005).

Herein, we tested whether natural *Karenia brevis* blooms are allelopathic, and we investigated allelopathic mechanisms of action using both field samples and cultures of *K. brevis*. In order to better understand the mode of action of *K. brevis* allelopathy, we tested whether compounds exuded by *K. brevis* inhibit the photosynthetic efficiency or esterase activity of competitor species, damage competitor cell membranes, or limit competing species' access to iron.

Materials and Methods

Collection of field samples

Three field samples of *Karenia brevis* (referred to as bloom samples) were collected approximately 10 m apart during a red tide at Long Boat Key beach, near the city of Bradenton on the west coast of Florida, in September 2006. One natural seawater sample containing no *K. brevis* (referred to as a non-bloom sample) was collected from the nearby beach at Green Key, near New Port Richey, on the same day. *K. brevis* and other phytoplankton species were identified from samples stained with Lugol's solution, counted by light microcopy using an Olympus IX-50 inverted microscope with a Palmer-Maloney settling chamber, and found to be present at $(3.2 \pm 1.8) \times 10^2$ cells mL^{-1} in the bloom samples, considered to be a medium-density bloom according to criteria set by the Florida Fish and Wildlife Research Institute (FWRI; http://research.myfwc.com/). Because the bloom began several weeks before samples were taken, and persisted several weeks after collection, we designated the bloom to have been in maintenance stage (described in Steidinger et al. 1998). Although in our hands *K. brevis* could not be conclusively distinguished from morphologically similar congeners such as *K. mikimotoi*, reports by FWRI stated that *K. brevis* constituted the majority of *Karenia* spp. present during this bloom. Each bloom and non-bloom sample was maintained at approximately 25 °C for 36 h with a 12 h light:dark cycle, then extracted using the method described below.

Phytoplankton culturing

Experiments were performed using five species of phytoplankton whose growth was previously shown to be suppressed by *Karenia brevis* live cells or filtrates (Kubanek et al. 2005). The following non-axenic clones were obtained from the Provasoli-Guillard National Center for Culture of Marine Phytoplankton (CCMP): the dinoflagellates *Akashiwo* cf. *sanguinea* (CCMP 1740) and *Prorocentrum minimum* (CCMP 695), and the diatoms *Amphora* sp. (CCMP 129), *Asterionellopsis glacialis* (CCMP 137), and *Skeletonema costatum* (CCMP 775). All species are known to co-occur with *K. brevis* in the Gulf of Mexico, were isolated from the Gulf of Mexico or the Caribbean, and to tolerate similar light, nutrient, and temperature conditions. Cultures were maintained at 22 °C with a 12 h light:dark cycle in a Percival incubator with Philips F32T8/TL741 Universal/Hi-Vision fluorescent bulbs mounted vertically, producing irradiance of 100-145 ❍mol m^{-2} s^{-1} throughout the incubator (measurement using Biospherical Instruments Inc light meter model QSL2100), in F/2 + silicate media (Sigma-Aldrich Co) made with filtered Maine seawater (salinity 36). Cultures of *K. brevis* (CCMP 2228) used to generate extracellular extracts were grown in 2.5 L Fernbach flasks in L1 + silicate media (CCMP) made with filtered Maine seawater (36). Growth curves were generated by visual cell count data using an Olympus IX-50 inverted microscope with a Palmer-Maloney settling chamber on culture samples preserved with Lugol's solution.

Generation of extracellular extracts

Exuded organic compounds were extracted from each field sample and *Karenia brevis* culture by adding a mixture of three adsorbent resins (Diaion HP-20 (Supelco),

Amberlite XAD-7-HP (Acros Organics), Amberlite XAD-16 (Supelco)) optimized for extraction of lipophilic compounds from aqueous media (Prince et al. 2006). Preliminary experiments in our lab confirmed that organic compounds with a range of polarity were extracted by this method, without lysing live *K. brevis* cells. We determined that a small proportion of inorganic nutrients contained in L1 media (6% of nitrate and 8% of phosphate) was also extracted from media using this method, but this did not cause an increase in the growth of any of the phytoplankton species relative to a solvent control (data not shown; $p = 0.31\text{-}1.00$ for all species). The ability to largely separate inorganic nutrients from organic exudates reduces the problem of nutrient artifacts in experiments, and is an advantage over traditional methods of generating cell-free filtrates. The resin was left in live field samples and cultures for 12-15 h, then recovered by slow filtration through 100 μm Nitex mesh and rinsed with deionized water. Lipophilic compounds were eluted from the resin with methanol followed by acetone, and solvents removed by rotary evaporation. Extracts of L1 + silicate media were generated in the same way, except without the presence of live cells. Brevetoxin B (PbTx-2) was quantified from *K. brevis* extracts by liquid chromatography-mass spectrometry (LC-MS) as described in (Kubanek et al. 2007). No other brevetoxins were present at concentrations above the detection limit.

Experiments

Experiment 1 tested whether compounds exuded by a bloom of *Karenia brevis* inhibit the growth of competing phytoplankton. We combined the three field sample extracts and determined that the combined extract contained 2.7 ng PbTx-2 mL^{-1} of

bloom water (i.e., from (3.2 + 1.8) x 10^2 *K. brevis* cells mL^{-1}; representing an average of 8.5 pg of PbTx-2 exuded by each live *K. brevis* cell). We used a 48 h assay to test the effect of this combined *K. brevis* bloom extract on the growth of four competing phytoplankton species: *Amphora* sp.*, Asterionellopsis glacialis, Prorocentrum minimum,* and *Skeletonema costatum*, and a 96 h assay to test its effect on the slower-growing dinoflagellate *Akashiwo* cf. *sanguinea.* For all species except *A.* cf. *sanguinea,* a 200 µL inoculum of the competitor species and 2.8 mL of L1 + silicate media were added to each of 14 small culture tubes. Tubes with *A.* cf. *sanguinea* contained a 1.0 mL inoculum and 2.0 mL of L1 + silicate media. All tubes were placed haphazardly in an incubator at 22 °C until competitor species reached exponential growth stage (approximately 3-5 d; cell densities of competitors in exponential growth ranged from 3.3 x 10^3 to 1.9 x 10^5 cells mL^{-1}). At that time, tubes were paired based on similar *in vivo* fluorescence measurements and assigned arbitrarily to be either treatments or controls. In order to approximate natural concentrations of bloom and non-bloom waterborne compounds, we added an amount of extract equivalent to that generated from 3.0 mL of *K. brevis* bloom or non-bloom sample (dissolved in 3.3 µL dimethyl sulfoxide (DMSO); n = 7 replicate culture tubes for bloom treatment and non-bloom control). Using a Turner Designs TD-700 fluorometer calibrated with chlorophyll *a* (for all species used in these experiments, relationships between visual cell counts and fluorescence were linear; r^2 = 0.75-0.99), *in vivo* fluorescence (used as a proxy for relative cell concentrations) was assessed immediately before addition of extracts. Pairs were placed randomly in the incubator at 22 ºC after extract addition, and 48 or 96 h later the percent change in *in vivo* fluorescence was determined by the Equation 1:

$\frac{final\ \ fluorescence - initial\ \ fluorescence}{initial\ \ fluorescence} \times 100\%$. In order to normalize the percent change in *in vivo* fluorescence by species we also calculated the percent reduction in growth of treatments relative to controls using Equation 2:

$\frac{\Delta control - \Delta treatment}{\Delta control} \times 100\%$, where Δ control and Δ treatment are the percent changes in *in vivo* fluorescence for the control and treatment, respectively, over the course of the experiment.

Because laboratory cultures provide a renewable source of allelopathic material to compare with bloom samples, we tested the effects of 16 independently generated extracts of *Karenia brevis* cultures in exponential growth stage on the growth of one susceptible competitor, *Asterionellopsis glacialis*, using the 48 h assay described above (with DMSO as control). The five *K. brevis* extracts that resulted in the strongest allelopathic effects against *A. glacialis* were pooled, and the effects of this pooled extract were tested on the growth of *Amphora* sp.*, Akashiwo* cf. *sanguinea, A. glacialis, Prorocentrum minimum,* and *Skeletonema costatum* (competitor cell density at onset of experiment ranged from 3.6 x 10^3 to 3.7 x 10^5 cells mL^{-1}), using five to ten replicates with the assay method described above. The combined extract came from cultures with an average concentration of 3.02 x 10^4 *K. brevis* cells mL^{-1}, and contained 55 ng PbTx-2 mL^{-1} of culture (i.e., representing an average of 1.8 pg PbTx-2 exuded by each live *K. brevis* cell).

Experiment 2 tested whether *Karenia brevis* exudates reduce competitor photosynthetic efficiency as a potential mechanism for allelopathy. For each of the same five competitor species used in Experiment 1, 5-6 pairs of 10 mL culture tubes containing

2.8 mL of L1 + silicate media were inoculated with 200 µL of phytoplankton culture. When the competitor species reached exponential growth stage, bloom or non-bloom extract (from Experiment 1, generated from 3.0 mL of field sample) was dissolved in 5.0 µL DMSO and added to each tube. All tubes were incubated in the dark for 1 h. For each species, we determined photosynthetic efficiency of photosystem II, measured as F_v:F_m, where F_v=F_m-F_0 (e.g., Parkhill et al. 2001; Miller-Morey and Van Dolah 2004). F_0 (initial fluorescence) was determined using a Turner Designs TD-700 fluorometer. F_m (maximal fluorescence) was determined by adding 9.6 µL of 10 mol μL^{-1} aqueous 3'-(3,4-dichlorophenyl)-1',1'-dimethylurea (DCMU), waiting 30 s, and measuring the fluorescence.

To determine the competitor growth stage at which photosynthetic efficiency is most inhibited, we repeated this experiment using extracts of *Karenia brevis* cultures, adding extracts during competitor lag, early exponential growth, and stationary growth stages. Sixty culture tubes for each species were prepared by inoculating 2.8 mL of L1 + silicate media with 200 µL of phytoplankton culture. For each species, 20 tubes were removed from the pool at each of three separate growth stages; the remaining tubes were allowed to grow until reaching later growth stages. *K. brevis* extract (combined allelopathic extract used in Experiment 1, generated from 3.0 mL of culture) dissolved in 5.0 µL DMSO was added to each of ten tubes and 5.0 µL DMSO (control) was added to the other ten tubes. Preliminary experiments indicated no significant difference between solvent controls and media extract controls for any of the species considered (data not shown; p = 0.31-1.00; Wilcoxon sign-rank test). Five pairs of tubes were then incubated in the dark for 1 h, and F_v:F_m was determined as described above. The other five pairs

were allowed to grow for 23 h and then incubated in the dark for 1 h before F_v:F_m was determined. This process was repeated with competitor cultures in early exponential growth and stationary stages.

Because enzyme activity has been reported to be a target of allelopathy (Windust et al. 1996; Sukenik et al. 2002) and active esterases are indicative of healthy, functional cells (Agusti et al. 1998; Agusti and Sanchez 2002), Experiment 3 assessed whether *Karenia brevis* exudates inhibit competitor esterase activity as a potential mechanism for allelopathy, using the fluorescent dye fluorescein diacetate (FDA) (Agusti and Sanchez 2002). This dye passes freely through the membrane of phytoplankton cells; once inside, cells with active esterases hydrolyze the ester bonds of FDA. The hydrolyzed dye is fluorescent, and cannot exit the cell. Thus, cells with active esterases fluoresce, and cells without active esterases do not. For each competitor species, 10 mL tubes containing 2.0 mL of phytoplankton culture were incubated at 22 °C with *K. brevis* extract (from Experiment 1, combined allelopathic extract generated from 2.0 mL of *K. brevis* culture) in 3.3 µL DMSO (or 3.3 µL DMSO for control tubes) (n = 7). Preliminary experiments indicated no differences in the esterase activity of cultures exposed to DMSO or extract of L1 + silicate media in DMSO (data not shown; p = 0.50-1.00; Wilcoxon sign-rank test). After 2 h, 500 µL of each culture was stained with 2.5 µL of 25 mol μL^{-1} FDA in DMSO (to assess the number of cells with active esterases) and 500 µL of each culture was stained with Lugol's solution (to determine the total cell concentration). Samples stained with FDA were kept in the dark for 10-30 min before excitation at 451 nm. FDA-stained cells emitting light at 510 nm were counted using fluorescence microscopy on an Olympus IX-50 inverted microscope with a Palmer-Maloney settling chamber. Samples

preserved with Lugol's solution were counted with light microscopy using the same instrument. We assessed the proportion of cells with active esterases by dividing the number of cells stained with FDA by the number of cells stained with Lugol's solution for each sample.

Experiment 4 determined the effect of *Karenia brevis* allelopathic compounds on competitor cell membrane permeability using the fluorescent dye SYTOX green (Brussaard et al. 2001). This dye binds to cellular nucleic acids but cannot permeate healthy membranes; thus only cells with damaged membranes fluoresce. The combined allelopathic *K. brevis* culture extract from Experiment 1 was added to each competitor species as described for Experiment 3 (n = 7-8). Preliminary experiments indicated no differences in membrane permeability from cultures exposed to DMSO or extract of L1 + silicate media in DMSO (data not shown; p = 0.25-1.00; Wilcoxon sign-rank test). After 2 h, 500 µL of each culture was stained with 5.0 µL of 0.50 mol μL^{-1} SYTOX green in DMSO (to assess the number of cells with permeable membranes) and 500 µL was stained with Lugol's solution (to determine the total cell concentration). Samples stained with SYTOX green were kept in the dark for 10-30 min before excitation at 504 nm. SYTOX green-stained cells emitted light at 523 nm. Cells stained with SYTOX green and Lugol's solution were counted and the proportion of cells with permeable membranes was assessed as in Experiment 3. In addition to the total proportion of cells with permeable membranes, we also assessed the fraction of stained cells that were motile (actively swimming) for the two dinoflagellates (*Prorocentrum minimum* and *Akashiwo* cf. *sanguinea*). Motility could not be assessed by microscopy for the three diatom species.

Experiment 5 tested whether *Karenia brevis* exudates strongly chelate iron, potentially limiting the access of other phytoplankton species to this essential nutrient. We analyzed *K. brevis* extracellular extracts for the presence of siderophores, compounds that bind to iron, using a universal chemical assay, in which siderophores compete with chrome azurol S (CAS) for Fe(III) (Schwyn and Neilands 1987). When bound to iron, CAS absorbs strongly at 630 nm. A decrease in absorption at this wavelength indicates iron is bound to a siderophore. Made according to Schwyn and Neilands (1987), 1.5 mL of the CAS shuttle solution was combined with extract of *K. brevis* generated from 3.0 mL of culture dissolved in 1.5 mL of deionized (DI) water, extract of *K. brevis* generated from 30 mL of culture (i.e., 10X natural concentration) dissolved in 1.5 mL of DI water, or extract of L1 + silicate generated from either 3.0 mL or 30 mL of media (control) prepared the same way (n = 3 each). All samples were equilibrated for 4.5 h after which the absorbance of each sample was measured at 630 nm using a Spectronic 21D spectrophotometer. This experiment was performed both for the allelopathic combined extract of *K. brevis* culture (used in Experiment 1), and non-allelopathic extract of *K. brevis* (from a non-allelopathic culture generated in Experiment 1; 1.52 x 10^4 cells mL^{-1}, containing 17 ng PbTx-2 mL^{-1}, representing an average of 1.1 pg PbTx-2 exuded by each *K. brevis* cell). The relative abundance of siderophores normalized to media controls was calculated using Equation 3: % siderophore bound iron =

$$\frac{A(media) - A(K.brevis)}{A(media)} \times 100\%$$

where A(media) is the absorbance of media extracts combined with the CAS shuttle solution and A(*K. brevis*) is the absorbance of *K. brevis* extracts combined with the CAS shuttle solution.

Statistical analyses

Preliminary analysis using the Kolmogorov-Smirnov statistical test determined that the percent growth of all phytoplankton species used in our experiments was normally distributed (Zar 1999). Therefore, Experiment 1 was analyzed using parametric tests. However, we were unable to perform similar analyses for the data acquired in Experiments 2-5, because sample sizes were too small; therefore these experiments were analyzed using non-parametric tests.

To analyze the Experiment 1 data testing allelopathic effects of *Karenia brevis* bloom and culture extracts, the percent change in fluorescence was compared for treatments vs. controls of each species using a paired two-tailed *t*-test (GraphPad Prism 4.0). To analyze data testing allelopathic effects of several extracts of *K. brevis* cultures assayed concurrently, the percent growth of *Asterionellopsis glaclialis* after 48 h was compared to controls using a one-way ANOVA with Tukey post-hoc test (SYSTAT 9). Differences were accepted as significant when $p \leq 0.05$.

For Experiment 2, treatments and controls were compared by two methods (GraphPad Prism 4.0). We used a one-tailed Wilcoxon sign-rank test to test whether the photosynthetic efficiency of treatments was lower than that of controls ($p \leq 0.05$ deemed significant). However, non-parametric tests are less powerful than their parametric counterparts, so no adjustment could be made for multiple comparisons without losing the ability to detect significant differences. In order to limit the possibility that we committed a type I error, we also analyzed data using a paired two-tailed *t*-test with a Bonferroni adjustment (thus, $p \leq 0.017$ was deemed significant).

Experiments 3 and 4 were analyzed using a two-tailed Wilcoxon sign-rank test (GraphPad Prism 4.0). We compared the effect of *Karenia brevis* extracts vs. controls for each species, for which differences were deemed significant when $p \leq 0.05$. Because several comparisons were made for each species, statistical tests comparing the proportion of stained cells that were motile between treatments and controls were analyzed using a Wilcoxon sign-rank test (GraphPad Prism 4.0) with a Bonferroni adjustment for multiple comparisons such that differences were accepted as significant when $p \leq 0.017$.

Experiment 5 was analyzed by comparing results of siderophore activity tests for an allelopathic vs. non-allelopathic *Karenia brevis* extract with a Mann Whitney *U*-test. Differences were accepted as significant when $p \leq 0.05$.

Results

Bloom composition

Approximately 80% of the cells in the three field samples collected from a single Florida bloom in 2006 were *Karenia* species. However, we estimate that low concentrations of at least 15 other species were also present. The most abundant included *Oxyphysis oxytoxoides*, and *Skeletonema costatum*, as well as species from the *Chaetoceros*, *Baceriastrum*, and *Scrippsiella* genera. Several unidentified dinoflagellates and pennate diatoms were also present. The composition of the non-bloom sample collected on the same day was quite different, containing only a few of the species also present in bloom samples. More than half of cells present in the non-bloom sample were pennate diatoms, including *Nitzchia longissima*. One dinoflagellate, *Prorocentrum*

mexicanum, made a significant contribution to the non-bloom community. Several other species, including the dinoflagellate *Ceratium furca* and a small number of unidentified centric diatoms, were also present.

Allelopathic effects of extracellular extracts from *Karenia brevis* bloom samples and cultures

Compounds exuded by a *Karenia brevis* bloom inhibited the growth of four of five competitor species when compared to effects of extracts of non-bloom seawater, but the degree of inhibition varied among species (Figure 3.1A). Three competitor species, *Asterionellopsis glacialis*, *Prorocentrum minimum*, and *Skeletonema costatum*, were strongly inhibited by the *K. brevis* bloom extract, such that their cell concentrations were lower after 48-96 h than at the start of the experiment when they were in exponential growth stage ($p \leq 0.001$ for all). The growth of one competitor, *Amphora* sp., was inhibited by $16 \pm 9\%$ ($p = 0.007$), and another species, *Akashiwo* cf. *sanguinea* was not inhibited ($p = 0.40$).

Seven of 16 *Karenia brevis* cultures were allelopathic to *Asterionellopsis glacialis*. Five of these were strongly allelopathic, suppressing *A. glacialis* growth by $\geq 90\%$ in 48 h ($p \leq 0.001$), and two were moderately allelopathic (22-41% suppression; $p \leq 0.05$). Nine extracts did not suppress *A. glacialis* growth at all ($p = 0.13$-0.94; data not shown). The five most allelopathic extracts were combined, and found to adversely affect four of five competitor species tested (Figure 3.1B). This combined extract killed one species, *Akashiwo* cf. *sanguinea*, and reduced the growth of three species by 60-97%: *A.*

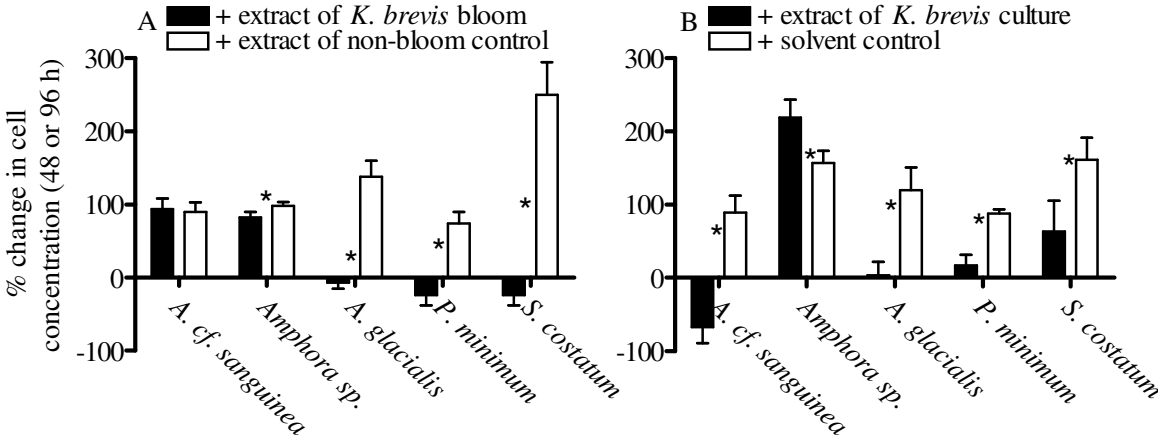

Figure 4.1. Effects of extracellular extracts of (A) *K. brevis* bloom or (B) cultures on the growth of 5 competing phytoplankton species (Experiment 1). Changes in cell concentration were assessed by *in vivo* fluorescence after 48 h for all species except the slow-growing *A.* cf. *sanguinea* whose final concentration was measured after 96 h. Error bars in this and subsequent figures indicate one standard deviation. Significance is marked with an asterisk (*) ($p \leq 0.05$; n = 5-10).

glacialis, *Prorocentrum minimum*, and *Skeletonema costatum* (all $p < 0.001$). One species, *Amphora* sp., was stimulated by *K. brevis* extracts ($p < 0.001$).

Reduction of competitor photosynthetic efficiency by *Karenia brevis* allelopathy

All five competitor species experienced decreased photosynthetic efficiency within 1 h of exposure to *Karenia brevis* bloom extract (Figure 3.2A). *Skeletonema costatum* was intensely affected, with a 68% reduction in photosystem II efficiency. The photosynthetic efficiencies of *Akashiwo* cf. *sanguinea, Amphora* sp., *Asterionellopsis glacialis*, and *Prorocentrum minimum* were inhibited by 19-43% (Wilcoxon sign-rank test: $p \leq 0.031$ for all; *t*-test: $p \leq 0.016$ for all).

Consistent with effects of the bloom extract, extracellular extracts of cultured *Karenia brevis* inhibited the photosynthetic efficiencies of all five competitor species during at least one stage of their growth (Figure 3.2B). *A. glacialis, P. minimum,* and *S. costatum* photosystem II was inhibited during their lag, exponential growth, and stationary stages ($p = 0.031$ for each species, Wilcoxon sign-rank test; $p \leq 0.01$ for each species, *t*-test). The photosynthetic efficiency of *S. costatum* was consistently strongly suppressed by 65-77%, whereas *A. glacialis* and *P. minimum* were most inhibited during lag stage (49-67%), rather than in exponential growth (39-48%) or stationary stage (20%). *A.* cf. *sanguinea's* photosynthetic efficiency was lowered by 33-53% by *K. brevis* extracts generated in lag and exponential growth stage ($p = 0.031$ in both cases, Wilcoxon sign-rank test; $p < 0.01$, *t*-test) but not in stationary stage ($p = 0.41$, Wilcoxon sign-rank test). Interestingly, *Amphora* sp., whose growth over 48 h was not suppressed by cultures of *K. brevis* (Figure 3.1B), suffered a 65% reduction of photosynthetic efficiency when

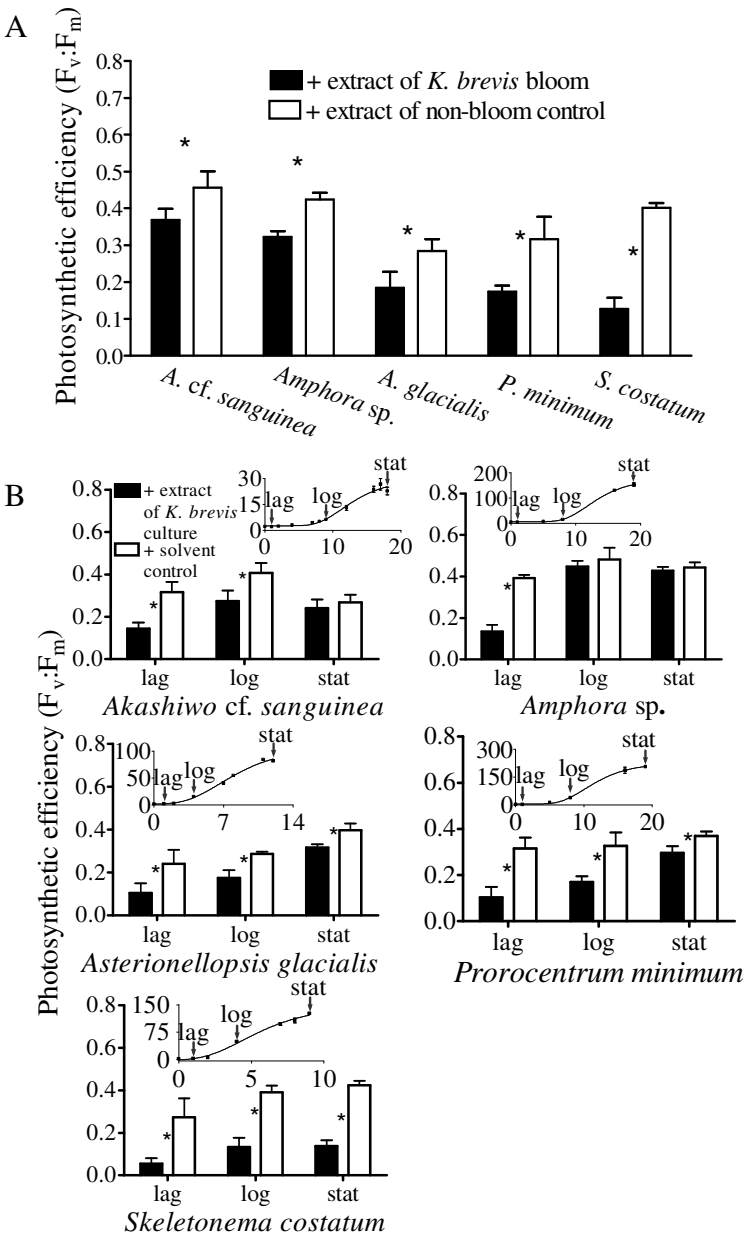

Figure 4.2. Effects of *K. brevis* allelopathy on competitor photosynthetic efficiency (Experiment 2). (A) During exponential growth stage, competitor species were treated with extracellular extracts of either *K. brevis* bloom or non-bloom sample. (B) Competitor species were treated with an allelopathic extracellular extract of cultured *K. brevis* or a solvent control at three different competitor growth stages (lag, exponential growth (log), or stationary (stat) phases). Insets show growth curve of competitors, where number of days is on the x axis, and fluorescence (µg Chl *a* L^{-1}) is on the y axis. Arrows indicate the place in the growth curve where competitors were harvested for this experiment. Significance is marked with an asterisk (*) ($p \leq 0.017$, *t*-test; $p \leq 0.05$, Wilcoxon sign-rank test; $n = 5$).

exposed to *K. brevis* extracts for 1 h in lag phase ($p = 0.031$, Wilcoxon sign-rank test; $p < 0.001$, *t*-test), but photosynthetic efficiency was unaffected if *K. brevis* extracts were added at later growth stages (exponential growth stage, $p = 0.16$; stationary stage, $p = 0.31$; Wilcoxon sign-rank test) (Figure 3.2B). Additional measurements of photosynthetic efficiency generated 24 h after the addition of extract were similar for all species except *A.* cf. *sanguinea* and *S. costatum,* which showed even more dramatically reduced photosynthetic efficiency after 24 h (data not shown) than after 1 h (Figure 3.2B).

Effects of *Karenia brevis* allelopathy on competitor esterase activity

Karenia brevis extracellular extracts had no effect on the esterase activity of any competitor species tested (Figure 3.3). For each species, no significant difference was found in the proportion of competitor cells with active esterases for those exposed to cultured *K. brevis* extract vs. those exposed to a solvent control ($p = 0.11$-0.81, Wilcoxon sign-rank test).

Effects of *Karenia brevis* allelopathy on competitor membrane permeability

Exposing competitors to cultured *K. brevis* extracellular extracts increased the proportion of cells with permeable (damaged) membranes for three species: *Akashiwo* cf. *sanguinea* ($p = 0.016$; Wilcoxon sign-rank test), *Asterionellopsis glacialis* ($p = 0.016$), and *Prorocentrum minimum* ($p = 0.008$) (Figure 3.4A). The effects on membrane permeability of two species, *Skeletonema costatum* and *Amphora* sp., were insignificant ($p = 0.47$ and 0.38, respectively). For *A.* cf. *sanguinea* and *P. miniumum*, whose motility could be directly observed by light microscopy, 2 h exposure to allelopathic extracts

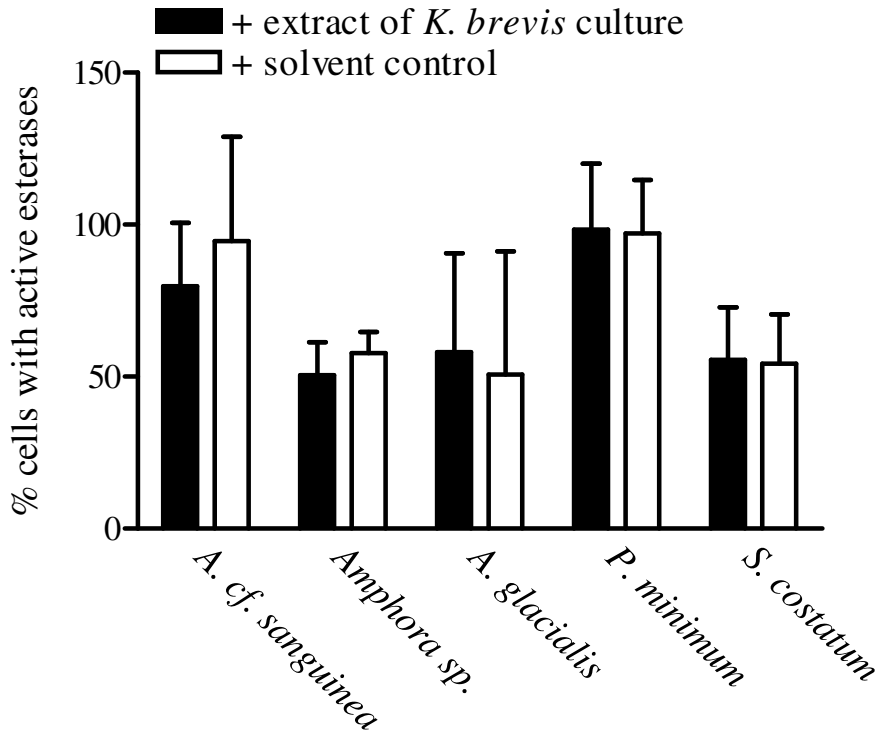

Figure 4.3. Allelopathic effect of cultured *K. brevis* extracellular extract on the esterase activity of five competing phytoplankton species measured with fluorescein diacetate (FDA) (Experiment 3). For all five species, contrasts between treatments and controls yielded $p > 0.10$ ($n = 7$).

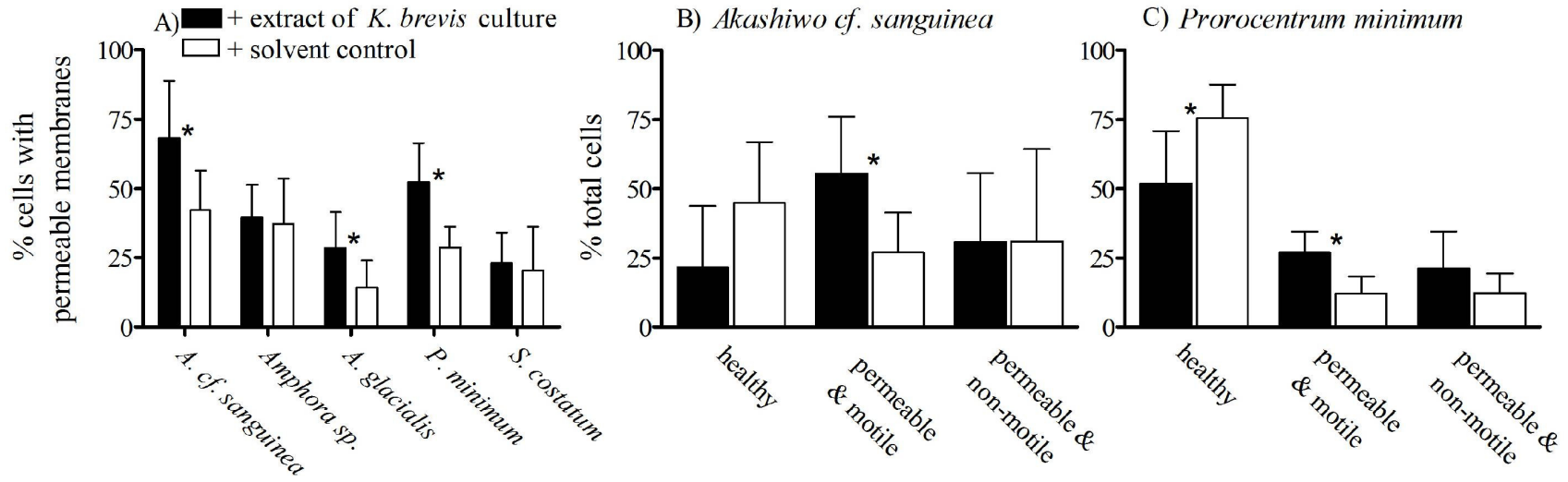

Figure 4.4. Allelopathic effect of cultured *K. brevis* extracellular extract on the membrane permeability of five competing phytoplankton species using SYTOX green to assess damaged (permeable) cells (Experiment 4). (A) Cells with damaged membranes for all species tested ($p \leq 0.05$; n = 7-8). (B) *Akashiwo* cf. *sanguinea* and (C) *Prorocentrum minimum.* Motile (swimming) cells with damaged membranes ($p \leq 0.017$; n = 8). Significant difference between treatments and controls is marked with an asterisk (*).

increased the proportion of motile cells with damaged membranes ($p = 0.016$ and 0.008, respectively) (Figure 3.4B). The proportion of membrane-permeable non-motile *A.* cf. *sanguinea* cells was not significantly different between cells exposed to extracts vs. those exposed to a solvent control ($p = 0.31$), and was only marginally significant for *P. minimum* ($p = 0.042$).

Siderophore activity of *Karenia brevis* extracts

No significant differences were detected in iron-chelating activity between *Karenia brevis* allelopathic and non-allelopathic extracts (Figure 3.5), regardless of the concentration at which the extracts were tested ($p = 0.40$ for 1X natural concentration and $p = 0.70$ for 10X natural concentration of cultured *K. brevis* extract).

Discussion

Karenia brevis blooms are allelopathic

The growth of three Gulf of Mexico phytoplankton species was strongly inhibited by compounds exuded during blooms of the red tide dinoflagellate, *Karenia brevis,* and the growth of one species was very slightly inhibited, providing strong evidence that *K. brevis* is allelopathic in the field (Figure 3.1A). We found that cultures of *K. brevis* also inhibited the growth of competitor species (Figure 3.1B), consistent with previous studies using cultured strains of *K. brevis* (Freeburg et al. 1979; Kubanek et al. 2005). Thus, allelopathy may play a role in enabling *K. brevis* to out-compete other phytoplankton species in the field and to dominate the plankton community during blooms.

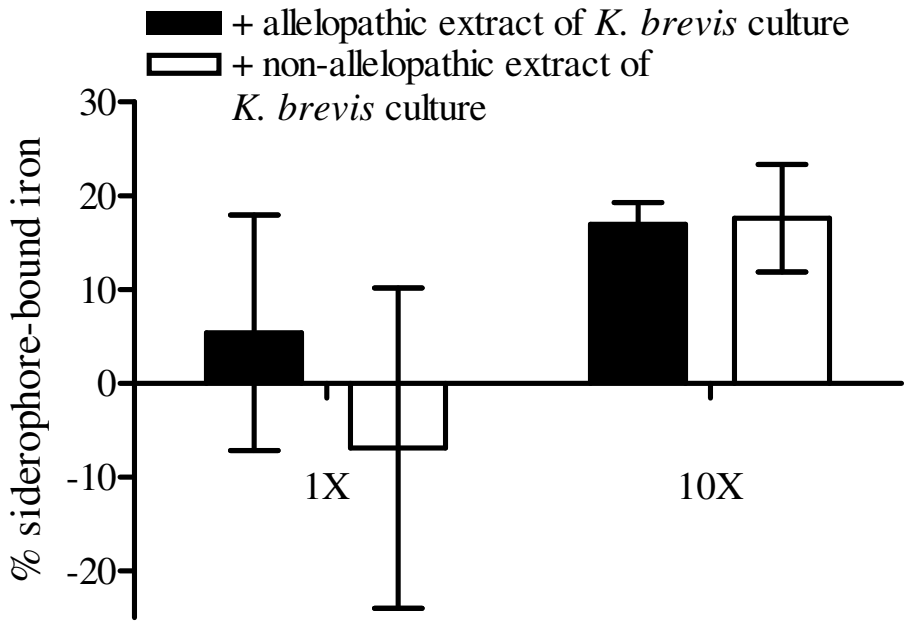

Figure 4.5. Test of siderophore activity for cultured *K. brevis* extracellular extracts (Experiment 5). The presence of iron binding compounds in *K. brevis* extracellular extracts was tested at natural (1X) and ten times natural (10X) concentration using a chrome azurol S (CAS) shuttle solution (Schwyn and Neilands 1987). Data depict the proportion of siderophore-bound iron relative to the appropriate media control. Contrasts between allelopathic and non-allelopathic extract treatments yielded $p \geq 0.40$ at both concentrations ($n = 3$).

Three competitor species strongly inhibited *K. brevis* cultures were at least as inhibited by compounds exuded by *Karenia brevis* blooms (Figure 3.1), even though *K. brevis* cell concentrations in cultures were almost 100 times greater than in natural bloom samples. Waterborne concentrations of PbTx-2, the major brevetoxin present in bloom waters (Pierce et al. 2005), were nearly 20 times higher in *K. brevis* cultures than in field samples, supporting the previous finding that brevetoxins are unlikely to be responsible for most cases of *K. brevis* allelopathy (Kubanek et al. 2005).

The composition of the plankton community is likely to play a role in the allelopathic potency of *K. brevis* blooms. Approximately 20% of cells in bloom samples belonged to non-*Karenia* taxa, and the representation of these community members differed between bloom and non-bloom samples collected on the same day (*see* Results). Therefore, it is possible that species other than *K. brevis* contributed to bloom allelopathy, possibly accounting for the observation that growth of *Amphora* sp. was modestly suppressed by bloom but not cultured *K. brevis* exudates (Figure 3.1). However, since none of the non-*Karenia* species made up more than 5% of the bloom community, it is difficult to predict which other species may be involved in bloom allelopathy. Because allelopathic effects of cultured *K. brevis* and bloom samples were similar for three of five species tested (*Asterionellopsis glacialis, Prorocentrum minimum, Skeletonema costatum*; Figure 3.1), it appears likely that *K. brevis* is responsible for most of the allelopathic potency of bloom samples.

It is not clear why one competitor, *Akashiwo* cf. *sanguinea*, was inhibited by *Karenia brevis* cultures but not by waterborne compounds from *K. brevis* blooms (Figure 3.1). It is possible that *A.* cf. *sanguinea* is less sensitive to *K. brevis* allelopathy than other

species, and that the 2006 bloom we sampled *K. brevis* was not dense enough to inhibit this competitor's growth. Kubanek et al. (2005) reported that *A*. cf. *sanguinea* was not inhibited by filtrates of cultured *K. brevis*. However, in that case, filtrates were tested at lower concentrations than extracellular extracts used in this study, supporting the hypothesis that *A*. cf. *sanguinea* may be suppressed only by compounds from high concentrations of *K. brevis* cells. It is also possible that *A.* cf. *sanguinea* was not susceptible to the *K. brevis* strains present in the 2006 bloom. Our laboratory cultures of *K. brevis* consisted of a single strain, whereas the field population was likely more diverse. No studies have addressed the genetic diversity of *K. brevis* blooms, but phytoplankton blooms are often genetically complex (e.g., John et al. 2004; Wilson et al. 2005). *A.* cf. *sanguinea* may be unaffected by bloom extracts because the allelopathic potency of *K. brevis* varies among strains, possibly in a species-specific manner. *K. brevis* also may produce compounds allelopathic to *A.* cf. *sanguinea* only under certain conditions which may have been absent during field sampling (e.g., under a particular nutrient regime or in the presence of particular plankton community members).

Production of allelopathic compounds and their effects on competitors are variable

Both the susceptibility of competitor species to allelopathy and the production of allelopathic compounds by *Karenia brevis* were variable. Of the 16 extracts generated from exponential growth stage cultures of a single *K. brevis* strain, seven were allelopathic to the diatom *Asterionellopsis glacialis* (*see* Results). Allelopathic effects also varied for natural *K. brevis* bloom samples; allelopathic effects of ten bloom samples collected during two separate years ranged from complete suppression of competitor

growth to no suppression at all, although the pooled data indicate that *K. brevis* blooms are significantly allelopathic (Figure 3.1A; Prince et al., unpublished).

Variability of allelopathic effect among cultures may be influenced by nutrient limitation, associated bacteria, and lack of selection pressure, as well as other factors such as growth stage and pH (Schmidt and Hansen 2001). Previous research on *Karenia brevis* indicated that allelopathic effects of cultures varied among strains and growth stages (Kubanek et al. 2005). Although attempts were made to consistently control growth conditions, it is likely that *K. brevis* cultures had small differences in pH, were extracted at slightly different growth stages and cell densities, were inoculated with cultures at different growth stages, or had accumulated mutations over time due to lack of selective pressure. Thus, we are currently unaware of which factors control the production of allelopathic compounds by *K. brevis*, although this issue begs further investigation.

Even when allelopathic compounds were produced by *Karenia brevis*, they did not affect all species to the same extent (Figure 3.1). *Akashiwo* cf. *sanguinea* was more susceptible to *K. brevis* culture than bloom exudates, whereas *Amphora* sp. was slightly suppressed by bloom exudates but actually stimulated by *K. brevis* culture exudates, suggesting that these two competitor species are affected by different *K. brevis* compounds. Our finding of the species-specific nature of allelopathy is consistent with results from previous studies involving *K. brevis* (Freeburg et al. 1979; Kubanek et al. 2005) and other bloom-forming dinoflagellates (Arzul et al. 1999; Sugg and Van Dolah 1999). Kubanek et al. (2005) and Freeburg (1979) found that compounds produced by *K. brevis* inhibited the growth of the diatoms *Asterionellopsis glacialis* and *Skeletonema costatum*, species that we also report to be susceptible to *K. brevis* allelopathy. In general,

however, we saw stronger allelopathic effects than Kubanek et al. (2005), probably because of the enhanced efficiency and avoidance of inorganic nutrient artifacts of the extracellular extraction method used in the current study.

The observed variability of *Karenia brevis* allelopathy may have implications for bloom dynamics. *K. brevis* blooms typically occur in four general stages: initiation, growth, maintenance, and termination (Steidinger et al. 1998). Because our *K. brevis* extracts came from exponential growth stage cultures with high cell concentrations reminiscent of a dense bloom, our data (Figure 3.1B) support the hypothesis that *K. brevis* uses allelopathy to maintain blooms, but we cannot assess whether allelopathy is important during bloom initiation. Extracts of a *K. brevis* bloom sampled during maintenance stage were strongly allelopathic (Figure 3.1A), further supporting the idea that allelopathy may allow *K. brevis* to maintain blooms. Future studies should consider the allelopathic activity of *K. brevis* blooms at different cell concentrations and bloom stages.

Karenia brevis lowers photosynthetic efficiency of competitors

Compounds exuded by *Karenia brevis* inhibited photosynthesis of competing phytoplankton, suggesting a potential mechanism for allelopathy (Figure 3.2). The maximum quantum yield of photosystem II (PSII) is frequently used as a proxy for photosynthetic efficiency and consequently as a general measure of cell stress (e.g., Parkhill et al. 2001; Miller-Morey and Van Dolah 2004); a decrease in PSII efficiency can result from a variety of factors including nutrient limitation (Parkhill et al. 2001), shading (Villareal and Morton 2002), metal toxicity (Miller-Morey and Van Dolah 2004),

and allelopathy (Figueredo et al. 2007). However, several studies have warned about the limitations of this method, claiming that nutrient limitation cannot be detected with balanced growth conditions (Parkhill et al. 2001), that a decrease in maximal quantum yield is specific to some stressors but others (e.g., heat shock) cannot be detected (Miller-Morey and Van Dolah 2004), and that nutrient stress may be missed due to the time required for a fluorescence response to nutrient starvation (Singler and Villareal 2005). These studies indicate that results of experiments measuring photosynthetic efficiency should be interpreted with care, and a lack of inhibition of PSII may not indicate that cells are not stressed. However, in our experiments, compounds exuded by *K. brevis* inhibited the PSII of competitors within 1 h, strongly indicating competitor stress (Figure 3.2).

Inhibition of competitor photosynthesis by *Karenia brevis* extracellular extracts appeared to parallel the negative effects on competitor growth (Figures 3.1-3.2). Species whose growth was strongly suppressed by bloom extracts (*Asterionellopsis glacialis, Prorocentrum minimum*, and *Skeletonema costatum*) also experienced substantial reduction in photosynthetic efficiency (Figures 3.1A and 3.2A). Parallels between reduced photosynthesis and growth inhibition were not as strong when competitor species were exposed to *K. brevis* culture extracts. However, the species least sensitive to general allelopathic effects, *Amphora* sp., appeared most resistant to PSII inhibition (Figures 3.1B and 3.2B). The efficiency of PSII measured after 1 h exposure to allelopathic extracts may be a more sensitive indicator of competitor stress than effects on growth measured over 2-4 d, since *K. brevis* bloom extracts slightly inhibited PSII of *Akashiwo* cf. *sanguinea* but did not significantly suppress *A.* cf. *sanguinea* growth (Figures 3.1A

and 3.2A). Because of the sensitivity and speed of this assay, decreases in PSII efficiency may also provide information about when competitors are most susceptible to allelopathy. We found that *K. brevis* compounds most strongly suppressed competitor photosynthetic efficiency when competitor cell concentrations were low (i.e., lag stage prior to exponential growth) (Figure 3.2B), although we did not determine if this effect is due to low cell concentrations or increased physiological susceptibility during lag stage. Thus, one way *K. brevis* may be able to maintain nearly monospecific blooms is by preventing competitors from reaching high cell densities.

Inhibition of PSII is commonly proposed as a mechanism for allelopathy; however, it is difficult to determine whether reduced efficiency of PSII is a direct target or a symptom of allelopathy-induced stress. Both bloom and culture filtrates of the cyanobacterium *Cylindrospermopsis raciborskii* inhibited the photosynthetic efficiency of competing cyanobacteria (Figueredo et al. 2007). Other studies have further elucidated the mechanism by which photosynthetic efficiency is inhibited. Von Elert and Juttner (1997) showed that while photosystem I of a cyanobacterial competitor was unaffected by compounds produced by the cyanobacterium *Trichormus doliolum*, overall oxygen evolution was reduced due to inhibited electron flow in PSII. In addition, Srivastava et al. (1998) found when the cyanobacterium *Anabaena* P9 was exposed to fishcherellin A, electron transport in PSII decreased, followed by inactivation of reactive centers, then grouped units within PSII were separated. However, neither our nor any of these studies have yet shown that PSII is the primary target of allelopathy rather than a secondary effect of another target.

Karenia brevis reduces competitor membrane integrity but not cellular esterase activity

Using the fluorescent stain SYTOX green, we found that allelopathic compounds exuded by *Karenia brevis* increased the membrane permeability of three competitor species (Figure 3.4). SYTOX green is usually used to distinguish between live and dead cells (Brussaard et al. 2001), but we found that many dinoflagellate cells with membranes permeable to this dye were swimming and therefore certainly not dead after 2 h exposure to *K. brevis* extracts (Figure 3.4B). In fact, *K. brevis* exudates primarily increased the proportion of motile cells with damaged membranes indicating that allelopathic compounds increased membrane permeability before competitor cells died or ceased swimming. This suggests that membrane damage is not a side effect of cell death, but rather a likely target for allelopathic compounds. We therefore propose that *K. brevis* may out-compete other phytoplankton by producing allelopathic compounds that damage the cell membranes of competitors.

We did not detect significant differences in esterase activity for competitor species exposed to allelopathic compounds vs. controls, using the fluorescent stain fluorescein diacetate (FDA) (Figure 3.3), suggesting that compounds produced by *Karenia brevis* do not initially target esterases as a mechanism for allelopathy. In other systems, esterase activity is used as a proxy for "metabolic vigor" (Dorsey et al. 1989), and has been shown to decrease due to viral infection (Lawrence et al. 2006), nutrient limitation (Brookes et al. 2000), and metal toxicity (Franklin et al. 2001). Although no previous studies have investigated whether esterase activity is a target for allelopathic compounds, other enzymes can be affected including phosphatases (Windust et al. 1996)

and carbonic anhydrase (Sukenik et al. 2002). Thus, establishing that *K. brevis* did not target the esterases of competitors ruled out a potentially important allelopathic pathway.

Karenia brevis allelopathy is not mediated by removal of waterborne iron

Iron availability is an important factor governing phytoplankton abundance, especially in high nutrient, low chlorophyll areas in the ocean (Hutchins et al. 1999) and may play a role in triggering harmful algal blooms (Wells et al. 1991). Several species of the bloom-forming diatom genus, *Pseudo-nitzchia*, have been shown to increase toxin production in response to iron limitation (Wells et al. 2005), indicating that harmful algal bloom species may exude compounds in order to compete for iron. More than 99% of iron in oceanic waters is chelated by organic ligands (Rue and Bruland 1995), some of which are siderophores, iron binding compounds produced by marine bacteria and cyanobacteria and utilized by eukaryotic phytoplankton in response to iron limitation (Wilhelm and Trick 1994; Hutchins et al. 1999). We hypothesized that *Karenia brevis* cultures may be allelopathic because *K. brevis* or its symbiotic bacteria produce iron-binding compounds that competitors were unable to access. However, we found no differences in iron-chelating activity between allelopathic and non-allelopathic *K. brevis* extracellular extracts (Figure 3.5), demonstrating that removal of iron from cultures could not be the mechanism *K. brevis* uses to suppress competitor growth.

Overall, we found that *Karenia brevis* blooms were allelopathic to four of five competing phytoplankton tested, and that *K. brevis* cultures exuded compounds with similar inhibitory effects. Competitor species whose growth was most inhibited by *K. brevis* bloom exudates also suffered substantial decreases in photosynthetic efficiency. In

addition, compounds exuded by *K. brevis* damaged cellular membranes of three competitor species, indicating a potential mechanism for *K. brevis* allelopathy. Our results suggest that *K. brevis* exudes allelopathic compounds that may facilitate its occasional dominance of the phytoplankton community, potentially leading to red tides.

CHAPTER 5

COMPETING PHYTOPLANKTON UNDERMINES ALLELOPATHY OF A BLOOM-FORMING DINOFLAGELLATE

Abstract

Abstract

Biotic interactions in the plankton can be both complex and dynamic. Competition among phytoplankton is often chemically-mediated, but no studies have considered whether allelopathic compounds are modified by biotic interactions. Here, we show that compounds exuded during *Karenia brevis* blooms were allelopathic to the cosmopolitan diatom *Skeletonema costatum*, but that bloom allelopathy varied dramatically among collections and years. We investigated several possible causes of this variability and found that neither bloom density nor concentrations of waterborne brevetoxins correlated with allelopathic potency. However, when we directly tested whether the presence of competing phytoplankton influenced bloom allelopathy, we found that *S. costatum* reduced the growth-inhibiting effects of bloom exudates, suggesting that *S. costatum* has a mechanism for undermining *K. brevis* allelopathy. Additional lab experiments indicated that competitor-mediated changes to *K. brevis* allelopathy were restricted to two diatoms among ten phytoplankton species tested. Our results suggest that *S. costatum* possesses an effective and previously undescribed method for resisting *K. brevis* allelopathy, potentially influencing community structure and altering bloom dynamics.

Introduction

Competition, one of the dominant processes structuring ecological communities, can occur through differential exploitation of limiting resources, but also through direct inhibition of competing organisms (Morin 1999; Krebs 2000). The direct inhibition of competitors via chemical compounds, allelopathy (Rice 1974; Lambers et al. 1998), can influence community-wide processes. For example, in microbial communities, allelopathic interactions can promote biodiversity if weak exploitation competitors persist in a community by directly inhibiting strong exploitation competitors (Czaran et al. 2002). Allelopathy has also been implicated in patterns of succession in both terrestrial (Gant & Clebsch 1975) and planktonic communities (Keating 1977; Vardi et al. 2002). Recent studies have indicated that allelopathy may also facilitate the spread of invasive species (Bais et al. 2003; Figueredo et al. 2007), if naïve native competitors have not evolved resistance to allelopathic compounds of exotic competitors (Vivanco et al. 2004).

Recent studies have shown that phytoplankton allelopathy can alter aquatic community structure, including patterns of species dominance. For example, while allelopathic compounds produced by the haptophyte *Prymnesium parvum* suppressed the overall phytoplankton assemblage, dinoflagellates and cyanobacteria increased in relative abundance (Fistarol et al. 2003). Similarly, after exposure to allelopathic compounds exuded by the dinoflagellate *Alexandrium tamarense*, the dominant species within a natural phytoplankton community changed from the dinoflagellate *Scrippsiella trochoidea* to the diatom *Leptocylindrus* sp. (Fistarol et al. 2004b). However, how some phytoplankton resist allelopathic compounds is largely unknown.

Although rarely studied, competitors may fight back against allelopathy. For example, upon exposure to the allelopathic plant *Centaurea maculosa*, two resistant plants produce oxalate, which prevents oxidative damage from the allelopathic compound (+)-catechin by scavenging the reactive oxygen species it produces (Weir et al. 2006). Resistant phytoplankton may also respond to allelopathic compounds from competitors; however, reports of such reciprocal interactions are rare, possibly because these interactions are difficult to detect in laboratory experiments. If we are to understand the dynamic process and consequences of chemical signaling in competition, we need to design experiments that allow detection of reciprocal interspecific interactions.

In the Gulf of Mexico, the red tide dinoflagellate *Karenia brevis* can form nearly monospecific blooms of thousands to millions of cells per liter (Tester & Steidinger 1997). *K. brevis* produces brevetoxins, polyketide-based natural products that cause neurotoxic shellfish poisoning in humans and massive fish kills (Landsberg 2002). Previous studies with *K. brevis* have shown that although both blooms and cultures exude compounds that inhibit the growth of competing phytoplankton, brevetoxins are rarely responsible for these effects (Prince et al. unpublished; Kubanek et al. 2005; Prince et al. 2008). Only the diatom *Skeletonema costatum* was modestly but significantly suppressed by a mixture of three common brevetoxins at natural concentrations (Kubanek et al. 2005). However, no previous studies have considered how the presence of competitors changes the allelopathic effects of *K. brevis* on those competitors, or indeed whether competitors alter allelopathy in any planktonic system.

Materials and Methods

Phytoplankton culturing

Experiments were performed using ten species of phytoplankton whose growth was previously shown to be affected by *Karenia brevis* live cells or filtrates (Kubanek et al. 2005). The following non-axenic (i.e., bacteria-containing) clones were obtained from the Provasoli-Guillard National Center for Culture of Marine Phytoplankton (CCMP): the dinoflagellates *Akashiwo* cf. *sanguinea* (CCMP 1740), *Peridinium* sp. (CCMP 626), *Prorocentrum mexicanum* (CCMP 687) and *Prorocentrum minimum* (CCMP 695); the diatoms *Amphora* sp. (CCMP 129), *Asterionellopsis glacialis* (CCMP 137), *Rhizosolenia* cf. *setigera* (CCMP 1694), *Skeletonema costatum* (CCMP 775), and *Thalassiosira* sp (CCMP 1055); and the cryptophyte *Rhodomonas lens* (CCMP 739). All species are known to co-occur with *K. brevis* in the Gulf of Mexico, were isolated from the Gulf of Mexico or the Caribbean (except *Rhizosolenia* cf. *setigera* available as an isolate from the Arabian Sea), and tolerate similar light, nutrient, and temperature conditions. Cultures were maintained as described in Kubanek et al. (2005). Experiments were also conducted in L1 + silicate media (CCMP) made with filtered Maine seawater (36 ppt).

Collection of field samples

Field samples of *Karenia brevis* blooms ("bloom samples") and nearby field samples containing no *K. brevis* ("non-bloom samples") were collected during 2005 and 2006. In October 2005, three bloom samples (3 l each) were collected during a red tide from St. Joseph's Peninsula near the town of Port St. Joe in Florida, USA. Three non-bloom samples (3 l each) were collected on the same day from Apalachicola Bay near the

town of Apalachicola, FL. In September 2006, eight bloom samples (2 l each) were collected during a red tide at Long Boat Key beach, near the city of Bradenton, FL. Seven non-bloom samples (2 l each) were collected on the same day from the nearby beach at Green Key, near New Port Richey, FL. Collections at each site were approximately 10 m apart. *Karenia brevis* concentrations, assessed as described in Prince et al. (2008), ranged from (2.1-4.4) x 10^2 cells ml^{-1} in bloom samples. *K. brevis* was not detected in non-bloom samples, indicating concentrations less than 7 x 10^{-2} cells ml^{-1}, the limit of detection for this study. We also identified common species from aliquots using samples stained with Lugol's solution. We allowed 5 ml of each of three bloom and three non-bloom samples from each year to settle in a Palmer-Maloney settling chamber and identified all morphologically distinct species to the lowest taxonomic level possible by comparison with descriptions, pictures, and drawings available in several taxonomic guides (Tomas et al. 1997; Horner 2002). Exuded organic compounds were extracted from each sample using the method described in Prince et al. (2008). Type II brevetoxins (e.g., PbTx-2,-3, and -9) in *K. brevis* cultures were quantified by competitive ELISA (Naar et al. 2002) using the method described in Kubanek et al. (2005), and brevetoxin B (PbTx-2) was quantified from all field samples using liquid chromatography/mass spectrometry (LC-MS) (Kubanek et al. 2007).

Variability of Bloom Allelopathy (Experiment 1)

We assessed the variability of allelopathic effects of *Karenia brevis* blooms on the growth of *Skeletonema costatum*, by exposing cultured *S. costatum* to extracellular extracts of bloom and non-bloom field samples. The experimental design and extract

addition was conducted as described in Prince et al. (2008) except that extracts were added on the day the cultures were inoculated and again after 7 d to counteract the probable decomposition of allelopathic compounds. In 2005, culture tubes of *S. costatum* initially contained 6.0 ml of media and were inoculated with 250 µl of *S. costatum* culture and in 2006 culture tubes initially contained 5.0 ml of media. Final concentration of *S. costatum* in each tube was (1.8 $\pm$ 0.3) x 10^4 (n = 10 in 2005; n = 3 in 2006). One additional 2006 bloom sample was tested using six replicates. Relative cell growth of each culture was assessed as described in Kubanek et al. (2005).

Effect of *Skeletonema costatum* on Bloom Allelopathy (Experiment 2)

We tested how the competitor *Skeletonema costatum* affected *Karenia brevis* bloom allelopathy by exposing field bloom samples to *S. costatum* before extractions. After collection, half of each bloom sample was exposed to live cultured *Skeletonema costatum* cells (filtered onto GF/F filter paper and re-suspended in filtered seawater to avoid adding nutrients to field samples), and the other half was exposed only to filtered seawater (collected from Boothbay Harbor, Maine). In 2005, 100 ml of *S. costatum* was added to each of three bloom samples and three non-bloom samples. In 2006, 50 ml of *S. costatum* was added to each of seven bloom samples and seven non-bloom samples (controls received the same volume of filtered seawater). The final density of *S. costatum* in treated field samples was (1.9 $\pm$ 0.8) x 10^4 cells ml^{-1} in 2005 and (1.6 $\pm$ 0.2) x 10^4 cells ml^{-1} in 2006. Samples to which *S. costatum* was added were referred to as either "bloom + *S. costatum* samples" or "non-bloom + *S. costatum* samples". Because one 2006 bloom sample (number 11) was not exposed to *S. costatum*, the total number of "bloom + *S.*

costatum" samples was 10, even though the total number of "bloom" samples was 11. After 36 h of exposure to *S. costatum*, samples were extracted using the methods previously described.

The growth of *Skeletonema costatum* exposed to bloom extracts, bloom + *S. costatum* extracts, non-bloom + *S. costatum*, and non-bloom extracts was measured using methods described in Experiment 1. Each treatment was normalized to non-bloom controls using the following equation: % growth of *S. costatum* relative to non-bloom control = $(1-\frac{[S.c.]ctrl-[S.c.]trt}{[S.c.]ctrl})$ x 100%, where [*S. c.*] *ctrl* is the average maximum fluorescence of *S. costatum* exposed to non-bloom controls and [*S. c.*] *trt* is the maximum fluorescence of *S. costatum* exposed to each of the other treatments. Cultures of *S. costatum* exposed to non-bloom + *S. costatum* extracts were never significantly different from the maximum density growth of *S. costatum* exposed to non-bloom extract controls (data not shown; non-linear regression, $p = 0.88$ in 2005, and $p = 0.47$ in 2006).

Effect of Competitors on Allelopathy of *Karenia brevis* Cultures (Experiment 3)

Experiment 3 was conducted with cultured phytoplankton to determine whether the ability to alter *Karenia brevis* allelopathy was common among phytoplankton, or specific to *Skeletonema costatum*. The experimental design is presented in supplementary material. Initially, two 1 l cultures were inoculated with *K. brevis*, and two 1 l seawater control flasks were set-up on the same day for each of ten phytoplankton competitor species. Once the *K. brevis* cultures reached exponential growth stage (usually after 5-10 d), 150 ml of either a target competitor culture or seawater was added to each culture. All flasks remained in the incubator for 36 hours, and then were extracted using the method

described above. Concentrations of *K. brevis* cells in cultures were (7.4-17) x 10^3 cells ml^{-1}, and the competitor cell concentrations ranged from 3.6 x 10^2 cells ml^{-1} for the slow growing *Akashiwo* cf. *sanguinea* to 6.4 x 10^4 cells ml^{-1} for *Thalassiosira* sp.

To test the allelopathic activity of each extract, ten species of competing phytoplankton (*Akashiwo* cf. *sanguinea, Amphora* sp., *Asterionellopsis glacialis*, *Peridinium* sp., *Prorocentrum mexicanum*, *Prorocentrum minimum*, *Rhizosolenia* cf. *setigera*, *Rhodomonas lens*, *Skeletonema costatum*, and *Thalassiosira* sp.) were each grown in 60 ml culture tubes. For each species except *A*. cf. *sanguinea* and *P. mexicanum*, tubes containing 29.0 ml of L1 + silicate media were inoculated with 1.0 ml of phytoplankton. A 2.5 ml inoculum plus 27.5 ml of media was used for slow growing *A*. cf. *sanguinea* and *P. mexicanum*. Each tube received extracts generated from 30.0 ml of one of the four treatments (each dissolved in 50 µl of DMSO) (n = 8). Extracts were added initially on the day the experiment began and again after 7 d. Relative cell concentration of each tube was measured as described above. Experiment 3 was repeated five additional times for *S. costatum* at a smaller scale (using the same experimental design used in experiment 1, 2005; n = 5), with independent extracts generated for each experiment.

We normalized allelopathic effects using an equation similar to the equation used in Experiment 1: % Growth relative to control = $(1-\frac{[C.P]ctrl-[C.P.]trt}{[C.P.]ctrl})$ x 100%, where [*C.P.*] *ctrl* is the average maximum fluorescence of the competing phytoplankter exposed to the seawater extract control and [*C.P.*] *trt* is the maximum fluorescence of the competing phytoplankter exposed to either *K. brevis* extracts, *K. brevis* + competitor extracts, or competitor extracts.

Statistical analysis

Before testing for differences in allelopathic effects among treatments and among collections, data was tested for normality using both a Kolmogorov-Smirnov and a D'Agostino-Pearson test (Dagostino et al. 1990). Although the Kolmogorov-Smirnov test suggested that data from all experiments was distributed normally, the D'Agostino-Pearson test indicated results of experiment 2 had a non-normal distribution. Experiments 1 and 2 were analyzed using nested ANOVA (SYSTAT 9), with the random effects of collection nested within the fixed effects of treatment (Zar 1999). We analyzed the results of 2005 and 2006 experiments separately, as well as together. Pair-wise comparisons between treatments were made using a Tukey post-hoc test. However, because we cannot be sure that data from experiment 2 was normally distributed, the results (i.e., the average effect of each collection) were analyzed using the non-parametric Mann-Whitney test (GraphPad Prism 4.0). Differences were accepted as significant when $p \leq 0.05$.

For Experiment 3, growth curves were compared among treatments using nonlinear regression with an *F* test (GraphPad Prism 4.0). A Gompertz (bacterial growth) equation successfully fit the data in most cases. All treatments were compared to the "seawater" control, and "*K. brevis*" treatments were compared to "*K. brevis* + target competitor" treatments with respect to maximum cell concentration. Differences were accepted as significant when $p \leq 0.05$. In the cases where nonlinear regression failed to fit the data, the maximum density data were compared for treatment vs. seawater control via 2-tailed *t*-tests. When this led to two contrasts using a single control dataset, we used a Bonferroni adjustment to determine that differences were accepted as significant when $p \leq 0.025$.

We used regression analysis to test for a correlation between concentrations of brevetoxins and allelopathic potency of *Karenia brevis* blooms and cultures as well as for a correlation between *K. brevis* cell concentration in blooms and cultures and allelopathic potency of blooms and cultures (GraphPad Prism 4.0). We accepted a trend as significantly non-zero when $p \leq 0.05$.

Results

Phytoplankton community composition of bloom and non-bloom samples

In both 2005 and 2006, the vast majority of phytoplankton cells present in bloom samples were *Karenia brevis*, and no species other than *K. brevis* made up more than 5% of phytoplankton cells in the community. In 2005, the remainder of the bloom community was largely composed of unidentified species of pennate diatoms. Other dinoflagellate species, including *Ceratium furca* and *Prorocentrum mexicanum*, were also present in low numbers. In contrast, the 2005 non-bloom community did not have a single dominant species, and included at least 20 morphologically distinct species, including nearly all of the species present in the bloom community except *K. brevis*. The centric diatom, *Skeletonema costatum*, was not observed in 2005 bloom nor 2005-2006 non-bloom samples. The 2006 bloom samples contained dinoflagellates *Oxyphysis oxytoxoides* and *Scrippsiella* sp., and diatoms of the *Chaetoceros* and *Baceriastrum* genera. *S. costatum* was present at 67 ± 25 cells ml^{-1} in 2006 bloom samples ($n = 8$). The composition of the 2006 non-bloom community was quite different from the 2006 bloom community; nearly half of cells present belonged to one of a few species of pennate diatoms, including *Nitzchia longissima*. One dinoflagellate, *Prorocentrum mexicanum*,

also made a significant contribution to the 2006 non-bloom community, as did several other species at lesser abundance, including the dinoflagellate *Ceratium furca* and a small number of unidentified centric diatoms.

Variability of Bloom Allelopathy (Experiment 1):

Skeletonema costatum exposed to extracellular extracts of *Karenia brevis* blooms reached a significantly lower final cell density than when exposed to non-bloom extract controls ($p < 0.001$; Fig. 1). However, the allelopathic effects of *K. brevis* blooms were highly variable. Although on average, *S. costatum* exposed to bloom extracts grew to 73% of the density of controls, final densities ranged from <1% to 122% of controls. Allelopathy varied not only among bloom collections ($p < 0.001$), but also across years: in 2005, the overall allelopathic effect was quite strong, with bloom extracts suppressing *S. costatum* by an average of 66% relative to non-bloom controls ($p < 0.001$; $n = 3$), whereas in 2006 allelopathic effects of the *K. brevis* bloom samples were only marginally significant, with bloom extracts suppressing *S. costatum* by 13% relative to non-bloom controls ($p = 0.063$; $n = 8$).

Effects of *Skeletonema costatum* on Bloom Allelopathy (Experiment 2):

Allelopathic effects of *Karenia brevis* blooms decreased when exposed to live *Skeletonema costatum* (Fig. 2). Extracts of five allelopathic bloom samples from 2005-2006 (samples 2-3 and 8-10; Fig. 1) all inhibited the growth of *S. costatum* cultures by more than 30%, with an average suppression of 62%. When each of these five bloom samples was exposed for 36 h to *S. costatum* (i.e., "bloom + *S. costatum*" samples), the

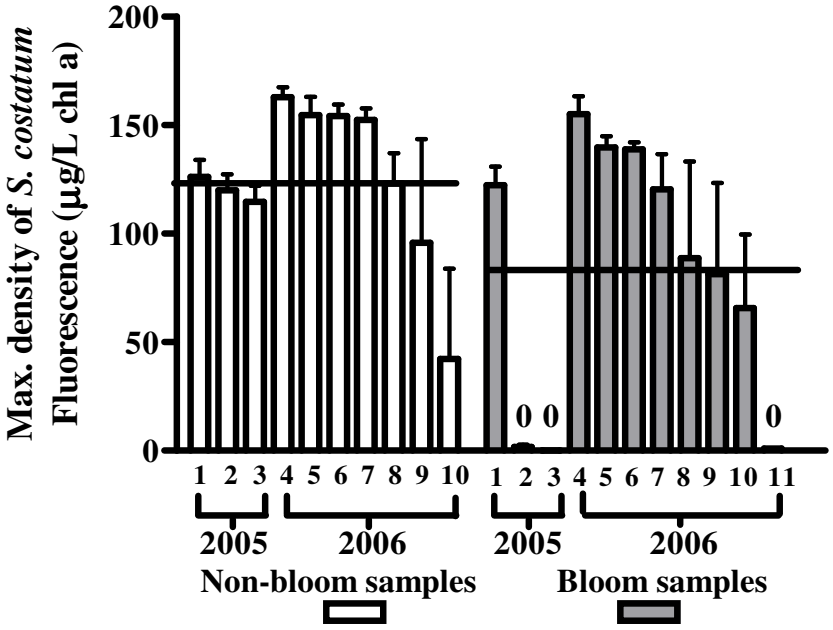

Figure 5.1. Effects of extracellular extracts of *Karenia brevis* blooms and non-bloom controls on the growth of *Skeletonema costatum* (Experiment 1). Each bar represents the average maximum growth of *S. costatum* treated with extract of an independent field sample and measured after 14-18 d. Black lines indicate the grand mean of each treatment. Error bars indicate standard error. The grand mean is significantly different between treatments ($p < 0.001$; $n = 10\text{-}11$), and the effect of sample within treatments is significant ($p < 0.001$; $n = 3\text{-}10$).

resulting extracts suppressed the growth of *S. costatum* by only 6%, significantly less than extracts of bloom samples that were incubated without *S. costatum* for this same period of time ($p < 0.001$, nested ANOVA; $p = 0.032$, Mann-Whitney test; Fig. 2). In 2005, reduction of *K. brevis* allelopathic potency by *S. costatum* was dramatic, with the allelopathic potency of bloom samples reduced by >99% (samples 2-3, Fig 2; $p < 0.001$, nested ANOVA). When the 2006 bloom data were considered alone (i.e., samples 8-10; Fig. 1), the trend was towards a reduction in allelopathy, with the allelopathic potency of bloom samples reduced by 53%, however, the effect was not significant ($p = 0.29$).

Effects of Competitors on Allelopathy of *Karenia brevis* Cultures (Experiment 3):

Of the ten competitor species tested, only *Asterionellopsis glacialis* and *Skeletonema costatum* significantly altered the allelopathic potency of cultured *Karenia brevis* (Fig. 3). Three species, *Akashiwo* cf. *sanguinea*, *Amphora* sp., and *Prorocentrum minimum* were significantly inhibited by extracts of *K. brevis* cultures and *K. brevis* + competitor cultures, indicating susceptibility to *K. brevis* allelopathy ($p < 0.01$ for all, using contrasts of maximum cell concentration). Five species, *Peridinium* sp., *Prorocentrum mexicanum*, *Rhizosolenia* cf. *setigera*, *Rhodomonas lens*, and *Thalassiosira* sp., were not inhibited by *K. brevis* extracts or *K. brevis* + competitor extracts, suggesting insensitivity to *K. brevis* allelopathy ($p = 0.097$-0.87). *A. glacialis*, in contrast, was significantly inhibited by *K. brevis* extracts ($p < 0.001$), but not by *K. brevis* + *A. glacialis* extracts ($p = 0.70$), indicating loss of *K. brevis* allelopathy caused by exposure to *A. glacialis*. No species was significantly inhibited by their own exudates

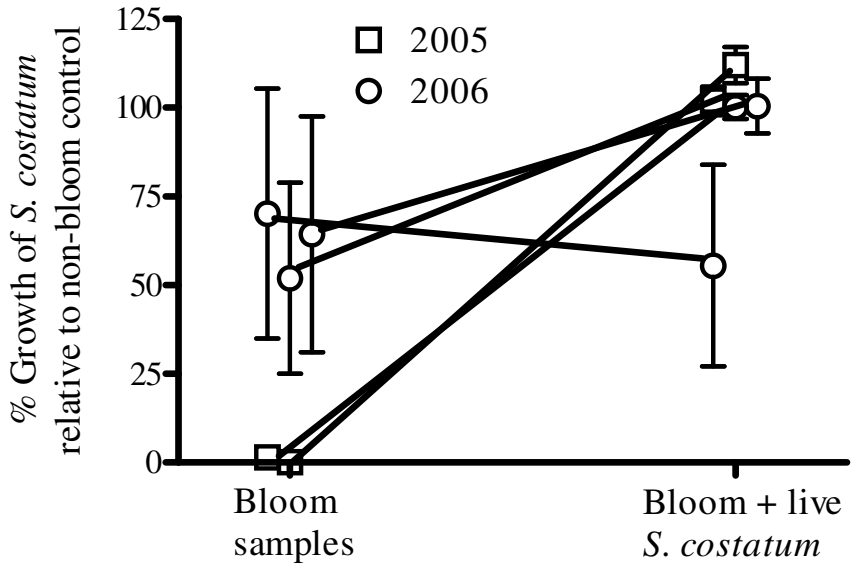

Figure 5.2. Effects of live *Skeletonema costatum* cells on the allelopathic effects of *Karenia brevis* bloom extracts (Experiment 2). Paired samples are connected by a line. Error bars indicate standard error. The mean is significantly different between treatments ($p < 0.001$; $n = 5$ bloom samples; $n = 3$-10 allelopathy assays per sample).

(p = 0.083-0.83), although *R.* cf. *setigera* and *P. mexicanum* were stimulated by their own exudates (data not shown).

The effects of *Skeletonema costatum* on cultured *Karenia brevis* allelopathy were extremely variable. In two separate experiments, *S. costatum* strongly undermined the allelopathic potency of *K. brevis* cultures ($p < 0.001$ for both; Fig. 3B-2, 3B-4). However, in two other experiments *S. costatum* strongly induced allelopathic potency in *K. brevis* cultures ($p < 0.001$ for both; Fig. 3B-1, 3B-3), and in one case weakly induced *K. brevis* allelopathy ($p = 0.038$; Fig. 3B-5). In one case, *K. brevis* cultures were not allelopathic to *S. costatum* whether or not *K. brevis* was exposed to *S. costatum* ($p = 0.30$; Fig. 3B-6).

Correlation of Allelopathy with *Karenia brevis* Cell Density and with Brevetoxin Concentration:

We detected no quantitative relationship between the magnitude of inhibition of *Skeletonema costatum* by *Karenia brevis* and the concentration of *K. brevis* cells present in cultures ($r^2 = 0.26$; $p = 0.13$) or 2005-2006 blooms ($r^2 = 0.053$; $p = 0.50$) over the range of *K. brevis* cell concentrations present in these samples, (i.e., (8.5-17) x 10^3 cells ml^{-1} present in cultures and (2.1-4.4) x 10^2 cells ml^{-1} present in blooms). When non-bloom samples were included in analysis of field data (consisting of 0 cells ml^{-1} *K. brevis*), we detected a weak but significant positive correlation between bloom density and allelopathy ($r^2 = 0.19$; $p = 0.046$). We detected no relationship between the inhibition of *S. costatum* by *K. brevis* blooms and concentration of waterborne brevetoxin B (PbTx-2) in the 2005-2006 samples ($r^2 = 0.19$; $p = 0.18$) or PbTx-3 in the 2006 samples ($r^2 = 0.24$; $p = 0.26$). PbTx-3 concentrations were not measured in 2005. Allelopathic potency

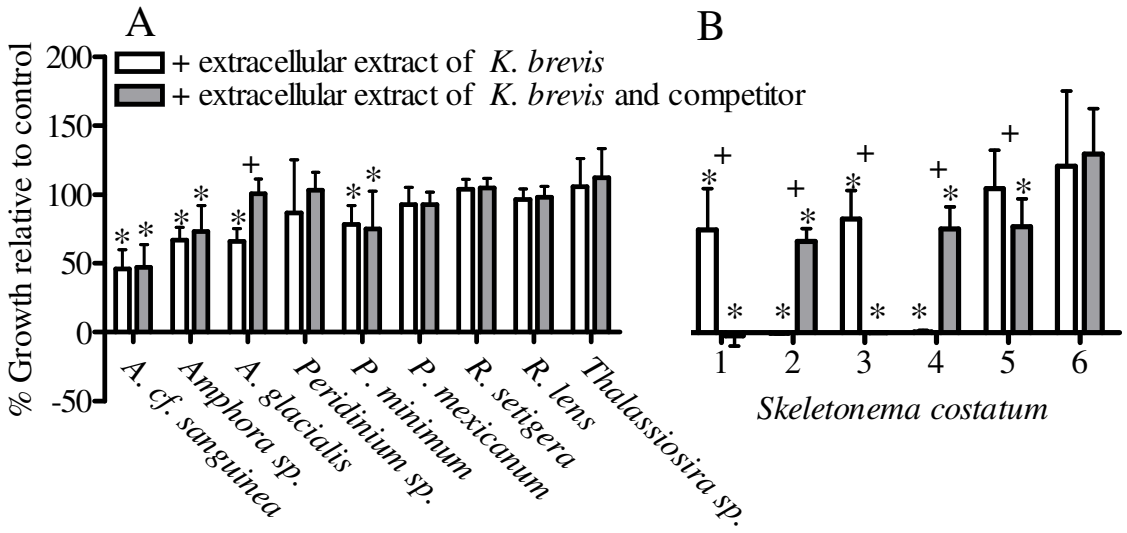

Figure 5.3. (A) The effects of extracellular extracts of *Karenia brevis* cultures exposed to seawater or competitor culture, on the growth of nine competitor species. Each bar represents the maximum cell density of a phytoplankton species normalized to growth of the same species exposed to a seawater control. (B) Six independent tests of the growth of *Skeletonema costatum* exposed to the same treatments as A. Asterisks (*) indicate a significant allelopathic effect relative to seawater control and plus signs (+) indicate a significant difference between treatments for a given species (n = 5-8).

and the concentration of all waterborne type-II brevetoxins (which include PbTx-2, -3, -9) were marginally inversely related over the range of brevetoxins present in *K. brevis* cultures, 9.0-35.5 ng ml^{-1} ($r^2 = 0.32$; $p = 0.054$)

Discussion

Skeletonema costatum undermines allelopathy of *Karenia brevis* blooms

In this study, we provide the first example of allelopathy undermined by a phytoplankton competitor. *Karenia brevis* allelopathic effects decreased significantly when bloom samples were exposed to live *Skeletonema costatum* cells (Fig. 2), indicating that *S. costatum* possesses a mechanism to undermine the allelopathic potency of *K. brevis* blooms. *S. costatum* also undermined allelopathy in two independent experiments with cultured *K. brevis* (Fig. 3B), but there was substantial variability in this process for both natural bloom and cultured *K. brevis* (Figs. 3-4).

Although rare, other studies have proposed mechanisms by which phytoplankton resist or lessen the effects of allelopathy. Reports of co-occurring phytoplankton reciprocally inhibited by each other's exudates suggest that some phytoplankton may counter allelopathic effects by producing their own allelopathic compounds (e.g., Vardi et al. 2002; Yamasaki et al. 2007a). If *Skeletonema costatum* was allelopathic to *Karenia brevis* we would have expected that live *S. costatum* added to blooms would affect the number, morphology, or behavior of *K. brevis* cells. However, we observed no such effects in bloom samples over 36 h. Using a different mechanism to resist allelopathy, the dinoflagellate *Scrippsiella trochoidea* formed temporary cysts in response to exudates from potentially allelopathic phytoplankton (including dinoflagellates *Alexandrium* spp.

and *Karenia mikimotoi*) which may have enabled *S. trochoidea* to escape mortality from allelopathic compounds (Fistarol et al. 2004a; Tillmann et al. 2007). However, the direct interference of *K. brevis* allelopathy by *S. costatum* (Fig. 2) appears to be markedly different from these previously observed instances of allelopathy resistance.

Skeletonema costatum is known to exhibit higher growth rates and lower nutrient requirements than *Karenia brevis* (Furnas 1990; Steidinger et al. 1998), suggesting that it may out-compete *K. brevis* for resources, and indirectly undermine allelopathy by depriving *K. brevis* of the resources needed for the production of allelopathic compounds. Alternatively, *S. costatum* may stop biosynthesis or exudation of *K. brevis* allelopathic compounds, a mechanism used by pathogenic bacteria to avoid defenses of terrestrial plants (Abramovitch & Martin 2004; Bais et al. 2005). Third, *S. costatum* may metabolize or degrade allelopathic compounds released by *K. brevis*, a strategy used by the fungal pathogen *Septoria lycopersici* in response to the defensive compounds of the tomato plant (Bouarab et al. 2002). Finally, *S. costatum* may produce compounds counteracting the physiological damage of allelopathy, observed for two plants responding to the allelopathic plant *Centaurea maculosa* (Weir et al. 2006).

The ability of competitors to influence *Karenia brevis* allelopathy is rare

When we tested ten species of phytoplankton common to the Gulf of Mexico for their ability to influence the allelopathy of cultured *Karenia brevis*, we found that competitor-mediated changes in allelopathic potency were rare. Two dinoflagellates and a diatom had no effect on *K. brevis* allelopathic potency, and two dinoflagellates, two diatoms, and a cryptophyte were not susceptible to *K. brevis* allelopathy at all (Fig. 3).

Only two diatoms, *Asterionellopsis glacialis* and *Skeletonema costatum*, affected the allelopathic potency of *K. brevis* cultures (Fig. 3), suggesting that like *S. costatum*, *A. glacialis* possesses a mechanism for undermining *K. brevis* allelopathy. Although the concentration and biovolume of competitor cells added to *K. brevis* cultures varied by several orders of magnitude among the ten competitor species tested, concentrations and biovolumes of *A. glacialis* and *S. costatum* represented neither the highest nor the lowest values, indicating the ability to undermine *K. brevis* allelopathy was not dependent on cell abundance alone.

Exposure to *S. costatum* affected the allelopathic potency of *K. brevis* cultures in a manner that was dramatic, but highly variable. The factors that influence allelopathy may differ between cultured vs. field phytoplankton, since we occasionally observed induction of allelopathic potency of cultured *K. brevis* by exposure to *S. costatum* (Fig. 3B-1, 3B-3) but allelopathic induction was not observed with natural bloom samples (Fig. 2). However, the variability in the allelopathic potency of *K. brevis* blooms and cultures indicates that although competitors significantly influence *K. brevis* allelopathy, other factors are likely at play.

Allelopathic potency of *Karenia brevis* blooms and cultures is variable

As previously established, compounds exuded during natural blooms of *Karenia brevis* and from cultures of *K. brevis* were significantly allelopathic to the diatom *Skeletonema costatum* (Prince et al. 2008), but the magnitude of allelopathic effects varied widely among field samples and cultures of *K. brevis* ($p < 0.001$; Fig. 1 and 3B). We tested a total of 11 samples from *Karenia brevis* blooms over two years, of which

three suppressed growth of *S. costatum* by more than 95%, three suppressed growth of *S. costatum* by at least 30%, and five did not suppress *S. costatum* growth (Fig. 1). Similarly, of six extracts of *K. brevis* cultures, two suppressed the growth of *S. costatum* by more than 95%, two modestly but significantly suppressed *S. costatum*, and two had no effect (Fig. 3B). However, the variability of allelopathic potency in *K. brevis* blooms and cultures was not related to concentration of water-borne brevetoxins (see Results), indicating that other *K. brevis* compounds were responsible for the majority of observed allelopathic effects

While published reports indicate that allelopathy varies with cell concentration (e.g., Vardi et al. 2002; Yamasaki et al. 2007a), we found no direct relationship between cell density and allelopathic potency for either bloom or cultures of *K. brevis* (see Results). When non-bloom samples (none of which contained *K. brevis*) were included in our analysis, a weak but significant correlation between *K. brevis* cell concentration and allelopathy was detected (see Results) in bloom samples, confirming that some *K. brevis* cells are necessary for allelopathy. All of the bloom samples contained hundreds of cells ml^{-1}, which is considered the low end of a medium-density bloom (Florida Fish and Wildlife Research Institute; http://research.myfwc.com/). Cell concentrations of *K. brevis* cultures were near 10,000 cells ml^{-1}, corresponding with high density blooms. However, *K. brevis* concentrations in the Gulf of Mexico range from undetectable to thousands of cells/mL (Steidinger & Haddad 1981). It is possible that *K. brevis* blooms with concentrations of thousands of cells ml^{-1} are more consistently allelopathic than the less concentrated blooms used in this study, or that there is an approximate cell density threshold, above which blooms are more likely to be allelopathic.

In a previous study of *Karenia brevis*, Kubanek et al. (2005) found that allelopathy of cultured *K. brevis* did vary with growth stage, as previously observed in other systems (e.g., Schmidt & Hansen 2001; Wang et al. 2006). However, in the current study, all cultures of *K. brevis* were extracted during exponential growth stage yet had widely variable allelopathic effects (Fig. 3B), indicating that *K. brevis* growth stage alone cannot explain variance in allelopathic potency among cultures. Because allelopathic potency of field samples has not been previously assessed, it is difficult to determine if allelopathy varies with bloom development stage. In this study, all samples of *K. brevis* blooms were taken from established blooms during "maintenance" stage, a time characterized by low growth rates and probable nutrient limitation (Steidinger et al. 1998). Bloom samples from the same year were collected within 1 h of each other, no more than 70 m apart, suggesting samples of a given year contained *K. brevis* of approximately the same growth stage. However, allelopathy varied dramatically among the field samples (Fig. 1), indicating that factors other than *K. brevis* bloom stage must play a role in allelopathy.

The observed variability in allelopathic effects may have been caused by a variety of factors. Plankton community interactions are known for their variable and even chaotic outcomes (Beninca et al. 2008). It has previously been noted that competitor physiological status affects susceptibility to allelopathy (Fistarol et al. 2005). *S. costatum* used in Experiment 2 was in stationary phase but *S. costatum* was growing exponentially at the time of Experiment 3, indicating that the physiological status of *S. costatum* was different in these two experiments. However, these differences cannot explain the high variance within Experiments 2 and 3. Although the production of secondary metabolites

has also been shown to vary with time of day for some phytoplankton (Taroncher-Oldenburg et al. 1997), this factor could not have been responsible because we started and terminated lab experiments at the same time of day for all tests. In contrast, *S. costatum* was added to field samples of *K. brevis* blooms at varying times of day in 2005 versus 2006, which may have been partially responsible for differences in allelopathy (Fig. 2). However, because allelopathy of bloom samples also varied within years, factors other than time of day must also influence allelopathic potency of *K. brevis* blooms. The variability of allelopathic potency among cultures of *K. brevis* may have also been influenced by factors that we did not measure in this experiment, including bacteria present in cultures. Likewise, the variability of allelopathic potency among *K. brevis* bloom samples may have been caused by localized differences in field conditions including nutrient concentrations, light levels, mixing intensity, as well as the presence of other community members.

Chemically-mediated interactions among competitors may have community-wide consequences

Our results suggest that the dynamics of *Karenia brevis* blooms may be influenced by the presence of a few specific competitors, such as *Skeletonema costatum* and *Asterionellopsis glacialis*, which occasionally undermine bloom allelopathy (Figs. 2-3). We focused on the cosmopolitan diatom, *S. costatum*, because it is one of the dominant phytoplankton species present in the Gulf of Mexico (Saunders & Glenn 1969) and routinely found at densities greater than a million cells per liter during the fall (Turner 1974; Badylak et al. 2007) when *K. brevis* blooms frequently occur (Tester &

Steidinger 1997). We found that while *S. costatum* was not present in any 2005 samples nor in 2006 non-bloom samples, the concentration of *S. costatum* was 67 $\pm$ 25 cells ml^{-1} in 2006 bloom samples, making it one of the most abundant phytoplankton species other that *K. brevis*. Although a number of factors including levels of light, nutrients, and turbulence, may have influenced the allelopathic potency of *K. brevis* bloom samples, the presence of *S. costatum* in field samples may explain differences in allelopathic potency between 2005 and 2006 samples. If *S. costatum* undermines *K. brevis* allelopathy in the field, then its presence in 2006 may help explain why these bloom samples were only marginally allelopathic (Fig. 1) and why the addition of *S. costatum* cells to *K. brevis* blooms did not significantly decrease the allelopathic potency of 2006 samples (Fig. 2), since live *K. brevis* cells in bloom samples had already been exposed to *S. costatum* in the field. The fact that *S. costatum* was relatively common in the 2006 blooms compared to other, non-*Karenia* species, also suggests that the undermining of *K. brevis* allelopathy by *S. costatum* is ecologically meaningful: the ability of *S. costatum* to co-exist with *K. brevis* may provide it with a competitive advantage over other phytoplankton species, potentially altering the composition of the bloom community.

In conclusion, we found *Karenia brevis* blooms to be allelopathic, but the magnitude of allelopathy varied dramatically between collections and years (Fig. 1). Neither brevetoxin concentration nor *K. brevis* cell density explained the variance in allelopathy. However, when we directly tested whether competitive interactions influenced allelopathy, we found that the diatom *Skeletonema costatum* was able to substantially undermine the allelopathic potency of *K. brevis* blooms (Fig. 2). This ability is not common to most Gulf of Mexico phytoplankton (Fig. 3), suggesting that *S.*

costatum may be able to occur during *K. brevis* blooms because of an effective method to overcome allelopathy. Our results indicate that phytoplankton competitors may play an important role in *K. brevis* bloom dynamics, and underscore the importance of reciprocal competitive interactions and the complexity of species interactions in planktonic communities.

CHAPTER 6

CHARACTERIZATION OF ALLELOPATHIC COMPOUNDS FROM THE RED TIDE DINOFLAGELLATE *KARENIA BREVIS*

Abstract

Blooms and cultures of the red tide dinoflagellate *Karenia brevis* exude compounds that inhibit the growth of competing phytoplankton, but the identity of these compounds is unknown. We partially purified and characterized allelopathic compounds from *K. brevis* using a variety of chromatographic and spectroscopic techniques. *K. brevis* produced multiple compounds which inhibited the growth of the competing diatom, *Asterionellopsis glacialis*. However, brevetoxins, potent neurotoxins responsible for massive fish kills and neurotoxic shellfish poisoning in humans, had no effect on *A. glacialis* growth. We determined that allelopathic compounds produced by *K. brevis* are unstable polar organic molecules produced at low concentrations, which are either neutral or positively charged. The two allelopathic compounds characterized in more detail had molecular weights between 500 and 1000 Da and possessed aromatic functional groups. Our results indicate that the ability of *K. brevis* to out-compete co-occurring phytoplankton may be facilitated by the production of multiple allelopathic compounds, but that red tide neurotoxins are not allelopathic.

Introduction

A number of phytoplankton taxa, including cyanobacteria (Suikkanen et al. 2004), dinoflagellates (Tillmann et al. 2007), diatoms (Yamasaki et al. 2007a), and haptophytes (Fistarol et al. 2003) are known to exude compounds which inhibit the growth of competitors, potentially with community-wide effects. Because competitors exhibit different levels of susceptibility to allelopathic compounds, allelopathy can influence the species composition of phytoplankton assemblages (Fistarol et al. 2003; Fistarol et al. 2004b). Allelopathic interactions may also influence or drive patterns of succession. For example, in a series of experiments Keating (1977; 1978) found that phytoplankton isolated from a temperate pond exuded compounds that inhibited the growth of species that occurred before them in succession but either stimulated or had no effect on species succeeding them. Allelopathic interactions may also facilitate invasion. The range of the cyanobacterium *Cylindrospermopsis raciborskii* is expanding potentially in part because *C. raciborskii* exudes compounds that inhibits photosynthesis of native phytoplankton in lakes that it invades (Figueredo et al. 2007).

Allelopathy has been hypothesized to facilitate the formation of harmful algal blooms (reviewed in Legrand et al. 2003). Some bloom-forming species produce potent toxins, which can kill fish, birds, and marine mammals and accumulate in shellfish, rendering them unsafe for human consumption (Landsberg 2002); however, these toxins are rarely linked to allelopathic effects. For example, the allelopathic dinoflagellates *Alexandrium* spp. produce a group of sodium channel blockers which cause paralytic shellfish poisoning in humans (Baden et al. 1998), but these toxins do not account for the negative effects (growth inhibition, paralysis, and cell lysis) of *Alexandrium* spp. filtrates

towards cultures and natural assemblages of phytoplankton competitors (Tillmann and John 2002; Fistarol et al. 2004b). Similarly, while the allelopathic dinoflagellate *Prorocentrum lima* produces the toxin okadaic acid which causes diarrhetic shellfish poisoning in humans, bioassay-guided fractionation of *P. lima* exudates indicated that okadaic acid was not present within the allelopathic fraction (Sugg and Van Dolah 1999).

Considering the attention that allelopathy has recently received as an important mediator of competition in the plankton (Gross 2003; Legrand et al. 2003), very few studies have identified compounds that are allelopathic to competitors at natural, waterborne concentrations. In one case, methyl esters of fatty acids isolated from the green alga *Nannochloris oculata* were shown to inhibit the growth of a competing dinoflagellate (Perez et al. 1997), and in another, a sesquiterpene alcohol isolated from the dinoflagellate *Gymnodinium nagasakiense* caused cell lysis of several competitor species (Kajiwara et al. 1992). However, neither study demonstrated that these compounds were allelopathic at naturally exuded concentrations. Similarly, fischerellins which were found to be exuded by the cyanobacterium *Fischerella mucosa* (Gross et al. 1991) and whose structures were later determined (Hagmann and Juttner 1996; Papke et al. 1997), inhibited the growth of photosynthetic competitors, but whether waterborne concentrations of fischerellins are high enough to inhibit competitors is unknown. In some cases, compounds that are allelopathic at natural, waterborne concentrations have been isolated, but their structures remain unknown. For example, karlotoxins exuded by the dinoflagellate *Karlodinium micrum* were found to inhibit the growth of competing phytoplankton (Adolf et al. 2006), but the complete structures of these molecules have not been reported.

Because allelopathic compounds have so rarely been identified, most studies testing allelopathy among planktonic organisms have used mixtures of exuded compounds, either in the form of filtrates (Schmidt and Hansen 2001; Tillmann et al. 2007) or extracellular extracts of phytoplankton (Prince et al. in press; Prince et al. submitted). Although the occurrence of allelopathy can be established using filtrates or extracellular extracts, the identification of specific allelopathic compounds is expected to enable researchers to explore the nature of allelopathic interactions in more depth. With pure compounds of known molecular structure in hand, the biosynthesis, cellular release, and waterborne transport of allelopathic compounds could be experimentally studied. Knowledge of the molecular structures of allelopathic compounds could help researchers determine whether their effects are general or species-specific, as well as the molecular mechanisms of action involved.

Karenia brevis is an ecologically and economically important dinoflagellate that blooms seasonally in the Gulf of Mexico, altering the phytoplankton community (West et al. 1996), perhaps in part through the production of allelopathic compounds (Kubanek et al. 2005). Compounds exuded by *K. brevis* cultures and natural blooms have been shown to inhibit the growth of several co-occurring phytoplankton species (Kubanek et al. 2005; Prince et al. in press; Prince et al. submitted). Two mechanisms of action have been identified in this system: allelopathic extracellular extracts of *K. brevis* lowered the photosynthetic efficiency of five competitors and damaged cell membranes of three of these five species (Prince et al. in press). *K. brevis* produces a group of neurotoxic compounds called brevetoxins which cause massive fish kills and neurotoxic shellfish poisoning in humans (Baden et al. 1998), but in laboratory experiments brevetoxins were

allelopathic to only one competitor, the diatom *Skeletonema costatum* (Kubanek et al. 2005). However, in subsequent experiments, concentrations of brevetoxins in cultures and field samples of *K. brevis* did not correlate with allelopathic effects of *K. brevis* on *S. costatum*, suggesting that brevetoxins may play, at most, a minor role in allelopathy (Prince et al. submitted). In addition, seven other species of competing phytoplankton, including the diatom *Asterionellopsis glacialis*, were found to be susceptible to unidentified allelopathic compounds produced by *K. brevis* (Kubanek et al. 2005; Prince et al. in press) but were not inhibited by pure brevetoxins at realistic waterborne concentrations (Kubanek et al. 2005).

Here, we report the partial characterization of allelopathic compounds exuded by *Karenia brevis*, using a variety of chromatographic and spectroscopic techniques. We assess the polarity, charge, molecular weight, and functional group composition of *K. brevis* compounds found to be allelopathic to the diatom *Asterionellopsis glacialis*. *A. glacialis* was chosen as a model competitor because it was consistently inhibited by extracellular compounds extracted from both *K. brevis* cultures and *K. brevis* blooms (Kubanek et al. 2005; Prince et al. in press; Prince et al. submitted). In addition, the strain of *A. glacialis* used in this experiment was originally isolated from the Gulf of Mexico, and *A. glacialis* has been found in the Tampa Bay estuary (Badylak et al. 2007) and in along the Florida panhandle (T. L. Myers, personal observation), areas that experience frequent *K. brevis* blooms.

Materials and Methods

Phytoplankton culturing

Non-axenic clones of the diatom *Asterionellopsis glacialis* (CCMP 137) and the red tide dinoflagellate *Karenia brevis* (CCMP 2228) were obtained from the Provasoli-Guillard National Center for Culture of Marine Phytoplankton (CCMP). Both species were originally isolated from the Gulf of Mexico. Cultures were maintained at 22 °C with a 12 h light:dark cycle in a Percival incubator with Philips F32T8/TL741 Universal/Hi-Vision fluorescent bulbs mounted vertically, producing irradiance of 100-145 μmol m^{-2} s^{-1} throughout the incubator (measurement using Biospherical Instruments Inc light meter model QSL2100). Cultures of *K. brevis* used to generate extracellular extracts were grown in 2.5 L Fernbach flasks in L1 + silicate media (CCMP) made with filtered Maine seawater. Experiments with *A. glacialis* were also conducted in L1 + silicate media, but *A. glacialis* stock cultures were maintained in F/2 + silicate media (Sigma-Aldrich Co). Initial growth curves for all species were generated by visual cell count data using an Olympus IX-50 inverted microscope with a Palmer-Maloney settling chamber on culture samples preserved with Lugol's solution.

Generation of extracellular extracts

When *Karenia brevis* cultures were in exponential growth stage, extracellular organic materials were extracted from each culture using a mixture of three adsorbent resins optimized for the extraction of lipophilic materials from aqueous media (Prince et al. 2006). The advantages of this method over traditional methods of generating cell-free filtrates is described in Prince et al. (in press). The resin was incubated within *K. brevis*

cultures for 12-15 h, and then recovered by slow filtration through Nitex nylon mesh. The recovered resin was rinsed with deionized water, and organic compounds were eluted from the resin using methanol followed by acetone, and the solvents were removed by rotary evaporation. The extracellular extract used for high performance liquid chromatography (HPLC) fractionation (described below) was generated from cultures containing 1.23 x 10^4 *K. brevis* cells mL^{-1} and the extract used for size-exclusion chromatography was generated from cultures containing 3.02 x 10^4 cells mL^{-1}. The extract used for all other experiments was generated from cultures of *K. brevis* containing 7.57 x 10^3 cells mL^{-1}.

Partial purification of allelopathic compound(s) exuded by *Karenia brevis*

HPLC fractionation of extracellular Karenia brevis *extracts*

We fractionated extracellular extracts generated from 500 mL of *Karenia brevis* culture using a Waters 1525 binary HPLC pump and a Waters 2487 dual wavelength detector set at 215 and 254 nm, coupled to Waters Breeze software. We used a C-18 reversed-phase silica column (4.6 x 250 mm with a 5 μm particle size Zorbax SB-C18) and a flow rate of 1 mL min^{-1}. A 1:1 ratio of methanol/water was used as the mobile phase for the first 10 min, after which the proportion of methanol linearly increased to 100% over a period of 20 min. The mobile phase remained at 100% methanol for 10 min, and then returned to starting conditions over the next 3 min. Thus, more polar compounds were eluted from the column before less polar compounds. We collected 23 fractions based on peak shape. In order to assess the overall effects of *K. brevis* extracellular extracts and to test whether allelopathic compounds were decomposed by HPLC, we

recombined half of each fraction into a single treatment. The effects of each individual HPLC fraction and the recombined treatment on the growth of *Asterionellopis glacialis* were tested using the assay described below.

Allelopathy assay of HPLC fractions

In order to balance the need for statistical power with keeping the number of experimental units manageable, six individual treatments were blocked with each control. For example, fractions A-F were grouped with control 1, fractions G-L with control 2, etc. The recombined fraction ("ALL") was tested with fraction S, T, U, V, and W. On the first day of the experiment, ten replicate culture tubes each containing 6.0 mL of L1 + silicate media were inoculated with 250 µL of *Asterionellopsis glacialis* in stationary stage for each treatment and control (280 total culture tubes, with an initial concentration of 2.1 x 10^3 cells mL^{-1}). Prior to addition of HPLC fractions, the initial cell concentration of each culture was approximated using a Turner Designs TD-700 fluorometer calibrated with chlorophyll a (the relationship between fluorescence and visual cell counts was linear for *A. glacialis*; $r^2 = 0.95$). In order to counteract the probable partial decomposition of the allelopathic compounds during fractionation, twice the approximate natural concentration of compounds exuded by *K. brevis* cultures was added to each experimental tube (i.e., the amount of each fraction equivalent to that generated from 12.5 mL of *K. brevis* culture was tested in a total volume of 6.25 mL). Each experimental tube received a given fraction dissolved in 10 µL DMSO (dimethyl sulfoxide); solvent control tubes also received 10 µL DMSO. Next, treatments for each group (i.e., A – F, G – L, M – R, and S – ALL) were blocked by replicates and randomly placed in the

incubator at 22 °C with a 12 h light:dark cycle. Tubes were inverted every 1-2 d and their position in the incubator randomly reassigned. Fluorescence of each culture was measured again after 1, 3, 5, 7, 10, 14, 18, and 21 d. Subsequent additions of extracts were made 7 and 14 d after the experiment was begun, in order to counteract probable decomposition of allelopathic compounds in experimental tubes. The maximum fluorescence of each treatment (measured after 21 d) was converted using equation 1:

$$Max.\ density\ of\ \ A.glacialis\ relative\ to\ solvent\ control = \frac{treatment\ \ fluorescence}{control\ \ fluorescence} \times 100\%$$

Characterization of *Karenia brevis* allelopathic compound(s)

HP20ss column separation of Karenia brevis *extracellular extract*

We used an HP20ss column to assess the polarity of allelopathic compound(s) exuded by *K. brevis*. First, we packed a column of HP20ss (Supleco) 2.5 cm high and 1 cm in diameter in methanol, and then rinsed with deionized water. Extracellular extract of *K. brevis* equivalent to the amount exuded by 100 mL of culture was dissolved in 500 µL of deionized water and loaded onto the column. The column was flushed with 35 mL of deionized water. The eluate was collected and the solvent removed by rotary evaporation. Next, the column was rinsed with 20 mL of methanol followed by 15 mL of acetone. The combined methanol-acetone eluate was collected and dried under rotary evaporation. Half of the material from each fraction was used to make a recombined treatment to test for possible decomposition of allelopathic compounds. The effects of the fraction that was eluted from the column with water ("WATER"), the fraction that eluted from the column with organic solvents ("ORGANIC"), and the recombined treatment

("ALL") were each tested on the growth of *A. glacialis* using the allelopathy assay described below.

SAX column separation of Karenia brevis *extracellular extract*

We tested whether the allelopathic compounds produced by *Karenia brevis* are negatively charged using strong anion exchange (SAX) chromatography. First, a column of 1.2 g of LC-SAX (Supelclean) was packed and rinsed with 1:1 methanol/water mixture. Extracellular extract of *K. brevis* equivalent to the amount exuded by 100 mL of culture was dissolved in a basic solution of 2 mL of a 1:1 mixture of 5 mM aqueous ammonium hydroxide and methanol, and loaded onto the column. Compounds were eluted from the column in three steps: first with a mobile phase of 15 mL of 75:25 methanol/water ("SAX1"), second with 15 mL of 75:23:2 methanol/water/1M aqueous formic acid ("SAX2"), and finally with 15 mL of 75:25 methanol/1M aqueous formic acid ("SAX3"). Each eluate was dried by rotary evaporation. Half of the material from each fraction was recombined. The allelopathic effects of SAX1, SAX2, SAX3, as well as the recombined treatment ("ALL") were each tested using the 48 h assay described below.

Dialysis membrane separation of Karenia brevis *extracellular extract*

This method was used to test whether allelopathic compounds exuded by *K. brevis* were larger or smaller than 1000 daltons (Da). Extracellular extract of *K. brevis* equivalent to the amount exuded by 100 mL of culture was dissolved in 5.0 mL of deionized water and placed inside a 1000 Da molecular weight cut-off dialysis membrane

tube (Spectrum Laboratories). The membrane was clamped at both ends, placed in a beaker containing 500 mL of deionized water, and gently stirred at 4 °C. After 7 h, the material inside (i.e., the > 1000 Da fraction) and outside (i.e., the < 1000 Da fraction) the membrane was collected and the deionized water removed by rotary evaporation. In order to assess the overall effects of *K. brevis* extracellular extracts and to test whether allelopathic compounds were decomposed by dialysis, we recombined half of each fraction into a single treatment ("ALL"). The effects of the > 1000 Da, the < 1000 Da, the recombined fraction, and a portion of the original unfractionated *K. brevis* extract ("WHOLE EXTRACT") were tested on the growth of *A. glacialis* using the 48 h allelopathy assay described below.

Size-exclusion chromatographic separation of Karenia brevis *extracellular extract*

Approximate molecular weight ranges of the allelopathic compound(s) from *Karenia brevis* were determined using size-exclusion chromatography. A P-2 Biogel column (Bio-Rad Laboratories), 31 cm tall and 0.5 cm in diameter, was flushed with 30 mM aqueous ammonium formate. Extracellular extract of *K. brevis* equivalent to the amount exuded by 500 mL of culture was dissolved in 250 µL of 30 mM aqueous ammonium formate, loaded onto the column, and substances were eluted with a mobile phase of 30 mM aqueous ammonium formate (high-molecular weight materials eluted before lower-molecular weight materials). Fraction 1 contained the first 2 mL eluting from the column; subsequently fractions (2-7) contained between 0.5 and 1 mL; and fraction 8 contained all remaining materials washed from the column in 20 mL. Fractions 1-8 and a recombined fraction ("ALL") were tested using the allelopathy assay described

below. The allelopathic size-exclusion fraction (fraction 8) was further fractionated using the same size-exclusion column and mobile phase. Fraction 8'1 contained first 5 mL eluted from the column; fractions 8'2 to 8'9 each contained 2 mL; and fraction 8'10 consisted of a final 20 mL collected from the column. The allelopathic effects of each of these fractions, and a recombined fraction ("ALL"), were assessed using the assay described below. Two groups of fractions, 8'2-4 and 8'5-7, which were adjacent to one another and similar in allelopathic potency, were combined, tested again, and subjected to NMR and mass spectral analysis (see below). Allelopathic effects of the combined fractions were assessed using the same assay eight months after initial tests, and the combined fractions were again subjected to NMR and mass spectral analysis.

The molecular weight of each fraction was approximated by comparison of elution patterns with molecular weight standards. A mixed solution of cyclodextrin (Sigma, MW 1135), napthol blue-black (Sigma, MW 570 Da), rhodamine (Aldrich, MW 443 Da), penicillin (FisherBiotech, MW 333 Da), and glucose (Aldrich, MW 180 Da) was prepared (approximately 4 μg mL^{-1} of each compound in 30mM ammonium formate). We loaded 200 μL of the solution onto the size-exclusion column, and collected fractions of the same volume described for the second size-exclusion fractionation of *K. brevis* extract. Three compounds, cyclodextrin, napthol blue-black, and rhodamine eluted from the column within the elution volumes reported earlier. The relative concentration of these compounds in each fraction was determined by LC-MS analysis (see below).

48 h Allelopathy assay

We determined the allelopathic activity of each fraction generated from dialysis, HP20ss chromatography, and SAX chromatography using a 48 h assay with the model competitor *Asterionellopsis glacialis*. Culture tubes containing 2.8 mL of L1+silicate media made with filtered Maine seawater (36 ppt) were inoculated with 200 µL of *A. glacialis*. When *A. glacialis* reached exponential growth stage (approximately 3 to 7 d), initial fluorescence measures were taken and replicates were created by grouping cultures with similar fluorescence (n = 7 for all experiments). Next, to each treatment tube we added the amount of extract or fraction generated from 3.0 mL of *K. brevis* culture (i.e., approximately natural concentration) dissolved in 20 µL deionized water, and 20 µL deionized water was added to each control. Groups of experimental tubes were randomly placed in the incubator at 22 °C with a 12 h light:dark cycle. After 48 h, the approximate cell concentration within each experimental tube was assessed by measuring its fluorescence, and the percent growth of *A. glacialis* within each tube was calculated using the equation 2:

$$\%\ Growth\ of\ A.glacialis\ over\ 48\ hours = \frac{final\ \ fluorescence - initial\ \ fluorescence}{initial\ \ fluorescence} \times 100\%$$

We also used a 48 h assay to determine the allelopathic activity of each fraction generated from size-exclusion chromatography. However, in order to assure adequate statistical power, we grouped size exclusion fractions as described for HPLC fractions. Three treatments were tested with one control following the first column separation (n = 5). Three or four treatments were tested with one control for the fractions generated from the second size-exclusion column (n = 7). These assays were conducted in the same way

as assays of fractions generated from dialysis, HP20ss, and SAX. The growth of *A. glacialis* in each experimental tube was also calculated using equation 2. However, in order to enable direct comparisons among treatments, we also normalized the growth of *A. glacialis* for treatments vs. controls using equation 3:

$$\%\ Growth\ of\ \ A.\ glacialis\ relative\ to\ solvent\ control = \frac{\%\ Growth\ of\ treatment\ (48\,h)}{\%\ Growth\ of\ control\ (48\,h)} \times 100\%$$

Statistical analyses

For the results of the allelopathy assay for HPLC fractions of *K. brevis* extracellular extracts, the maximum fluorescence of each treatment after 21 d was compared within groups using a 1 way ANOVA with a Tukey post-hoc test using the software program SYSTAT 9 (Zar 1999). Differences between treatments were accepted as significant when $p \leq 0.05$. For the results of the other assays, the percent growth of *A. glacialis* over 48 h was compared within groups using the same statistical test.

Characterization of allelopathic fractions

The combined size-exclusion fractions 8'2-4 and 8'5-7 were characterized by ^{1}H nuclear magnetic resonance (NMR) spectroscopy, with data acquired in D_2O at 25 °C using a Bruker 500 MHz spectrometer. The same fractions were analyzed by the Georgia Institute of Technology mass spectrometry lab by high resolution electrospray ionization (ESI) mass spectrometry (MS) in positive mode using an Applied Biosystems QSTAR XL spectrometer. Identical analyses for all samples were conducted again after eight months storage at -20 °C.

LC-MS analysis of brevetoxins and molecular weight standards

We analyzed by LC-MS all HPLC fractions for the presence of brevetoxin B and brevetoxin 3 (PbTx-2 and PbTx-3, respectively). We determined PbTx-2 concentrations by comparison to a PbTx-2 standard curve (5-7 standard concentrations of 0–2 μg mL^{-1}). Because PbTx-2 and PbTx-3 shared almost identical standard curves below 1 ug mL^{-1}, we also quantified PbTx-3 concentrations by comparison to a PbTx-2 standard curve (PbTx-3 concentrations in all samples were < 1 ug mL^{-1}) (Kubanek et al. 2007; Myers et al. submitted). We also used LC-MS to assess relative concentrations of molecular weight standards by comparing ion profiles of size-exclusion fractions to the ion profiles of pure molecular weight standard samples.

Results

Polarity of allelopathic compounds from *Karenia brevis*

HPLC fractionation of *Karenia brevis* extracellular extract yielded 23 fractions (Figure 5.1A), of which five (fractions B, G, H, I, and S) significantly inhibited the growth of *Asterionellopsis glacialis* (Figure 5.1B), and four (fractions C, D, E, and M) significantly stimulated the growth of *A. glacialis*. When all HPLC fractions were combined and tested at natural concentration, the recombined extract completely suppressed *A. glacialis* growth ($p < 0.001$), with cultures of *A. glacialis* never growing beyond starting concentrations. Thus, although *K. brevis* exudates contained multiple allelopathic and stimulatory compounds, the overall effect of *K. brevis* on its competitor *A. glacialis* was allelopathic.

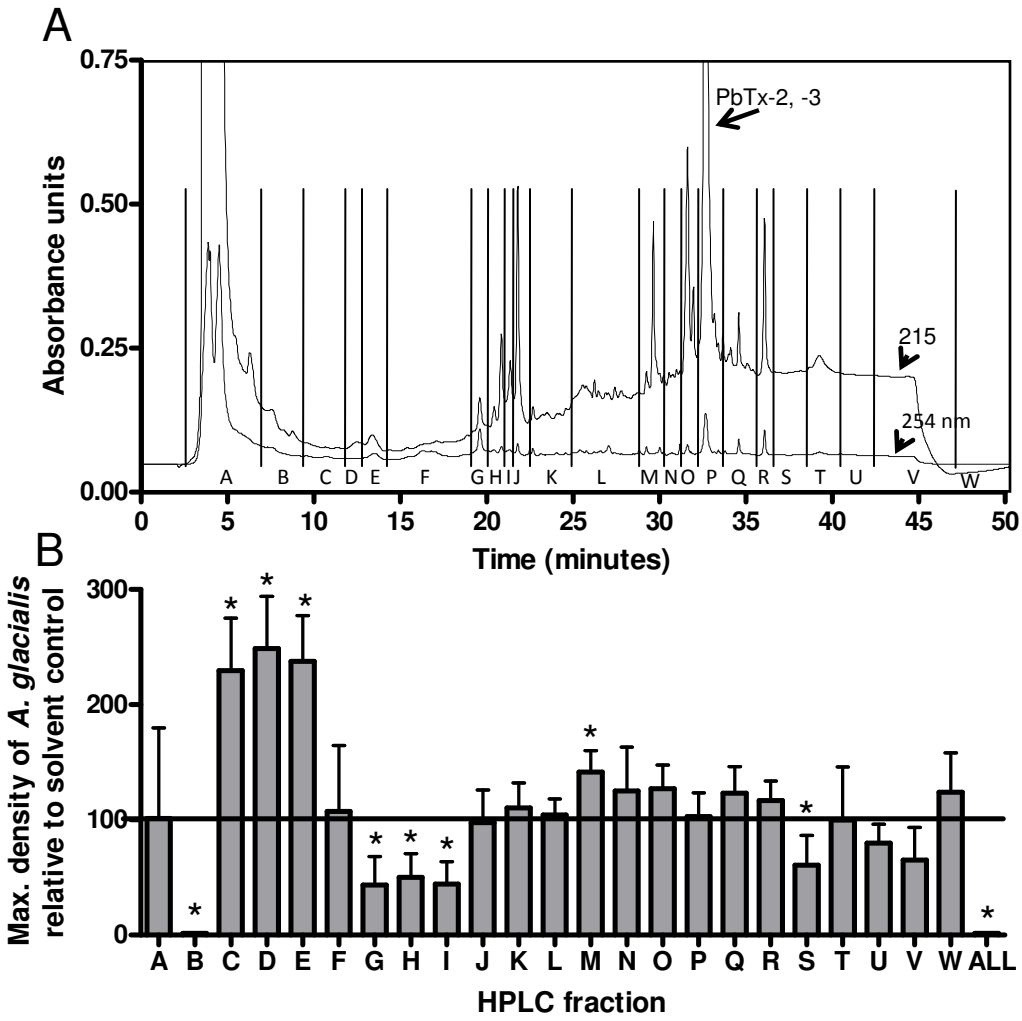

Figure 6.1. HPLC fractionation of *Karenia brevis* extracellular extracts. (A) C-18 reversed-phase HPLC chromatogram of *K. brevis* extracellular extracts in a gradient of aqueous methanol, with absorbance measured at 215 and 254 nm. Vertical lines indicate fractions collected. (B) Growth of the diatom *Asterionellopsis glacialis* exposed to HPLC fractions normalized to a solvent control. "ALL" represents a treatment containing recombined HPLC fractions. Bars above the reference line indicate increased *A. glacialis* growth relative to solvent controls, while bars below the reference line indicate suppressed *A. glacialis* growth relative to solvent controls. Asterisks indicate *A. glacialis* growth significantly different from solvent controls ($p \leq 0.05$; $n = 10$ for all).

Only one *K. brevis* HPLC fraction (B) completely suppressed the growth of *A. glacialis* ($p = 0.001$), explaining most of the allelopathic effect of the *K. brevis* extracellular extract (Figure 5.1B). Fraction B eluted from the reversed-phase HPLC column with 50% aqueous methanol very shortly after the solvent front, indicating that allelopathic compound(s) within this fraction were polar relative to other compounds exuded by *K. brevis* (Figure 5.1A). Three other allelopathic fractions (G, H, and I), which also eluted relatively early in the fractionation, suppressed *A. glacialis* growth by 50-57% relative to solvent controls ($p < 0.001$ for all; Figure 5.1). The most lipophilic allelopathic fraction (S), corresponding with no obvious UV absorption, suppressed *A. glacialis* growth by 39% relative to controls ($p = 0.040$). Some allelopathic fractions were characterized by a single UV peak (i.e., fractions G and I; Figure 5.1A), suggesting that these fractions contained only one major UV-absorbing compound. Others (i.e., fractions B and H; Figure 5.1A) were each associated with multiple UV peaks, indicating that they contained several compounds. However, allelopathic compounds in all fractions decomposed with further attempts at purification and characterization (data not shown).

Brevetoxin B (PbTx-2) and brevetoxin 3 (PbTx-3) co-eluted with HPLC fraction P (Figure 5.1A). As determined by LC-MS analysis, PbTx-2 was present at 2.50 ng mL^{-1} and PbTx-3 at 7.61 ng mL^{-1} in the extracellular portion of *Karenia brevis* culture. Other brevetoxins were not observed. Since extracellular extracts were generated from *K. brevis* culture containing 1.23×10^4 cells mL^{-1}, this represents 0.20 pg PbTx-2 and 0.62 pg PbTx-3 exuded per *K. brevis* cell. The growth of *Asterionellopsis glacialis* exposed to fraction P was not significantly different from *A. glacialis* exposed to a DMSO control ($p = 0.84$; Figure 5.1B). No brevetoxins were detected in any of the other HPLC fractions,

including the allelopathic fractions, indicating that brevetoxins appear to play no role in the allelopathy of *K. brevis* towards *A. glacialis*.

Allelopathic extracts of *Karenia brevis* were retained on an HP20ss column (Figure 5.2A). Compounds that eluted from the column with water did not inhibit *Asterionellopsis glacialis* growth ($p = 1.00$). However, compounds present in the fraction that eluted from the column with organic solvents did inhibit *A. glacialis* ($p = 0.014$). When fractions were combined, the resulting treatment also significantly inhibited *A. glacialis* growth ($p = 0.003$) but not significantly more than the organic fraction ($p = 0.92$), indicating that the allelopathic compounds were not extremely water-soluble.

Charge status from ion exchange chromatography

Anion exchange chromatography failed to retain allelopathic compounds (Figure 5.2B), indicating they were not negatively charged. Allelopathic compounds eluted from the column with a basic-to-neutral eluant as part of fraction SAX1 ($p < 0.001$). Compounds collected with increasing concentrations of formic acid (fractions SAX2 and SAX3), did not inhibit *A. glacialis* growth ($p > 0.84$ for both). The recombined treatment did inhibit the growth of *A. glacialis* ($p < 0.001$), similarly to fraction SAX1 ($p = 0.84$). Cation exchange chromatography lead to inadequate separation of allelopathic compounds with positively charged functional groups (data not shown).

Molecular weights of allelopathic compounds

Dialysis membrane separation of *Karenia brevis* extracellular extracts yielded two fractions: one consisting of all compounds with molecular weights higher than 1000 Da,

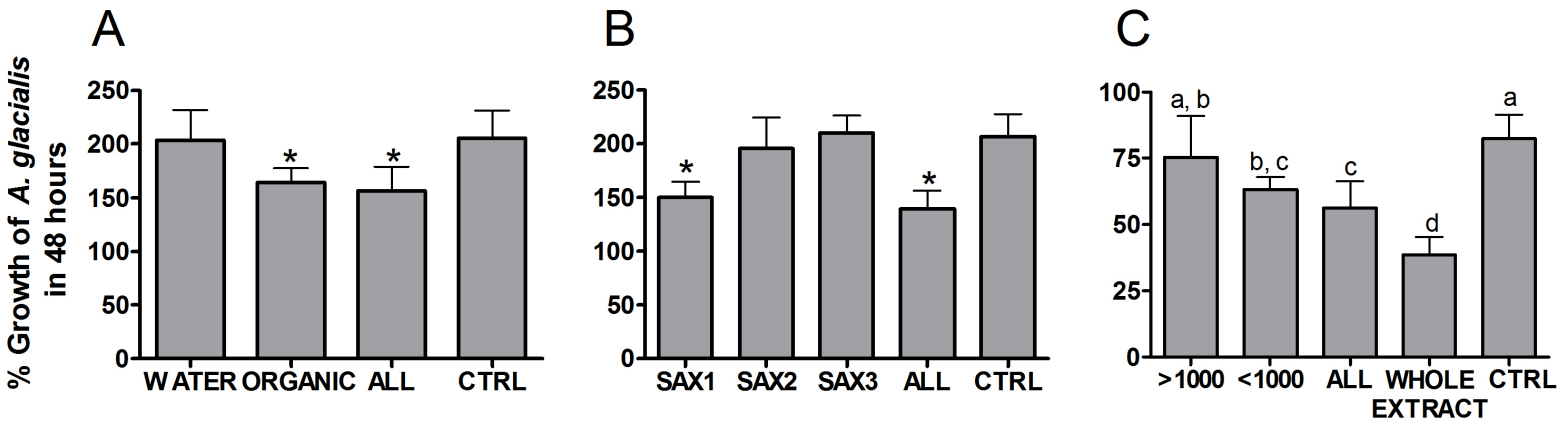

Figure 6.2. Growth of *A. glacialis* exposed to *Karenia brevis* extracellular extracts fractionated by three methods. (A) HP20ss fractionation. Fractions represent substances eluted from the column with water ("WATER"), with methanol/acetone ("ORGANIC"), and recombined fractions ("ALL"). (B) Strong anion exchange chromatography. Fractions represent those that were not negatively charged ("SAX1"), to those that were charged ("SAX2", "SAX3"), and a recombined fraction ("ALL"). For panels (A) and (B) asterisks represent treatments significantly different from controls ($n = 7$; $p \leq 0.05$) (C) Dialysis membrane separation. *A. glacialis* was exposed *K. brevis* materials greater than 1000 Daltons (>1000), less than 1000 Daltons (< 1000), the recombined fraction ("ALL"), unfractionated extract ("WHOLE EXTRACT"), and solvent control ("CTRL"). Letters represent treatments significantly different from one another ($n = 7$; $p \leq 0.05$).

and another with compounds of molecular weight less than 1000 Da. Of these, only compounds < 1000 Da inhibited the growth of *Asterionellopsis glacialis* relative to controls ($p = 0.008$; Figure 5.2C). When the two fractions were recombined, the resulting treatment also inhibited the growth of *A. glacialis* ($p < 0.001$), but not significantly more than the low molecular weight fraction ($p = 0.67$). The recombined treatment did not inhibit the growth of *A. glacialis* as strongly as *K. brevis* extract prior to dialysis ($p = 0.017$), indicating that some of the allelopathic effect was lost by decomposition during dialysis.

When exposed to fraction 8 from the size-exclusion column, *Asterionellopsis glacialis* growth was inhibited by 71% relative to solvent controls ($p < 0.001$; Figure 5.3A). The growth of *A. glacialis* exposed to fractions 1-7 was not significantly different from controls ($p = 0.70$-0.94 for all), indicating that all significantly allelopathic compounds eluted with fraction 8. The recombined fraction from the size-exclusion column also inhibited the growth of *A. glacilalis* relative to controls, and actually caused the cell density of *A. glacialis* culture to decrease by approximately 6% over 48 h ($p < 0.001$).

When fraction 8 was further separated by size-exclusion chromatography, the allelopathic effects were spread among several fractions (Figure 5.3C), supporting the hypothesis that multiple allelopathic compounds are exuded by *K. brevis*. While fractions 8'1 and 8'8 modestly inhibited the growth of *A. glacialis*, by 37% ($p = 0.003$) and 73% ($p < 0.001$) respectively, fractions 8'2, 8'3, and 8'4 inhibited the growth of *A. glacialis* more dramatically, by 81-85% ($p < 0.001$ for all). However, the most allelopathic fractions (8'5, 8'6, 8'7) inhibited the growth of *A. glacialis* by between 109 and 112% ($p < 0.001$

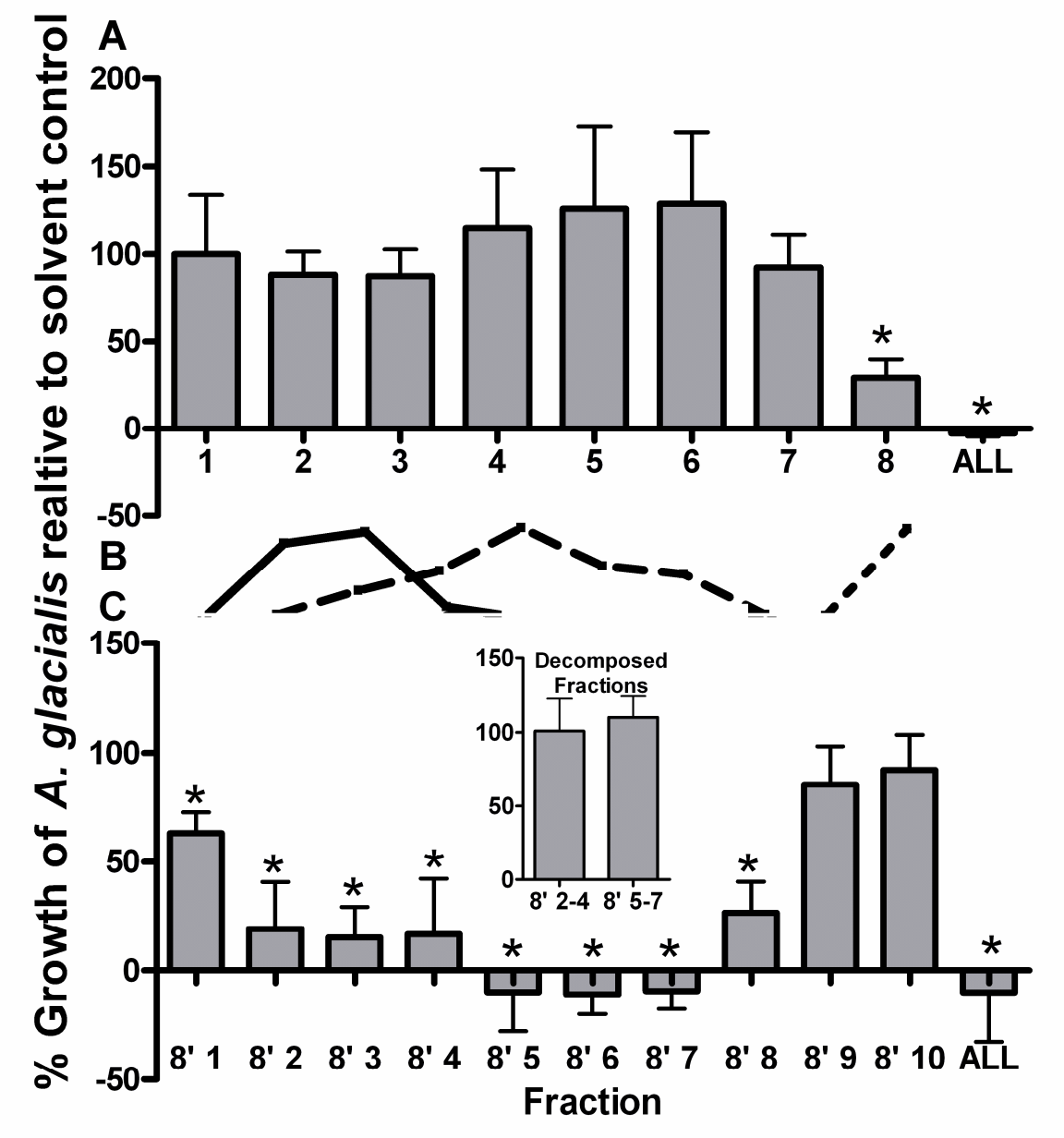

Figure 6.3. Effects of fractions from size exclusion column on growth of *A. glacialis*, normalized to solvent controls. (A) Initial separation (n = 5). (B) The relative concentrations of molecular weight standards in each fraction (in panel C). The solid line represents the relative abundance of cyclodextrin (1135 Da), the dashed line napthol blue black (570 Da), and the dotted line rhodamine (433 Da). (C) Further separation of fraction 8 (n = 7). The inset shows effects of combined fractions 8'2-4 and 8'5-7 on the growth of *A. glacialis* after eight months storage at -20 °C (n = 7). For all panels, asterisks indicate significant differences relative to controls ($p \leq 0.05$).

for all), indicating substantial *A. glacialis* cell death, not just inhibition of cell growth. Two fractions, 8'9 and 8'10, did not significantly inhibit the growth of *A. glacialis* (p = 0.39-0.82). When all of the fractions were recombined and tested for allelopathic effects, *A. glacialis* growth was inhibited by 110% ($p < 0.001$).

Three standards with molecular weights of 433-1135 Da eluted from the column within the retention window of fractions 8'1-10 (Figure 5.3B). These results indicate that the molecular weights of allelopathic compounds from *K. brevis* extracellular extracts (eluting in fractions 8'1-8 but peaking in fractions 8'5-7) were likely between 500 and 1000 Da, and that the compounds in fractions 8'2-4 were of higher molecular weight than the compounds in fraction 8'5-7. When the allelopathic effects of combined fractions 8'2-4 and 8'5-7 were tested again after eight months storage at -20 °C, neither combined fraction inhibited the growth of *A. glacialis* (p = 0.60-1.00; inset Figure 5.3C), indicating decomposition of allelopathic compounds over time.

The initial mass spectral analyses of two allelopathic size-exclusion fractions showed several peaks in the molecular weight range of 500-1000 Da. In combined size-exclusion fraction 8'2-4, peaks with a mass-to-charge ratios of 663, 963, and 991 (which each likely represent the molecular weight of a compound plus proton) were visible (Figure 5.4A). However, when the sample was analyzed after decomposition, the only peaks visible were from a polyethylene glycol (PEG) impurity (Figure 5.4B). This PEG impurity, likely from laboratory plastic materials (e.g., pipet tips), was probably also present in the initial sample, but because peaks from the candidate molecular ions of allelopathic compounds were much larger, the PEG-associated peaks were hidden in baseline noise. A similar pattern was observed in the mass spectrum of the combined

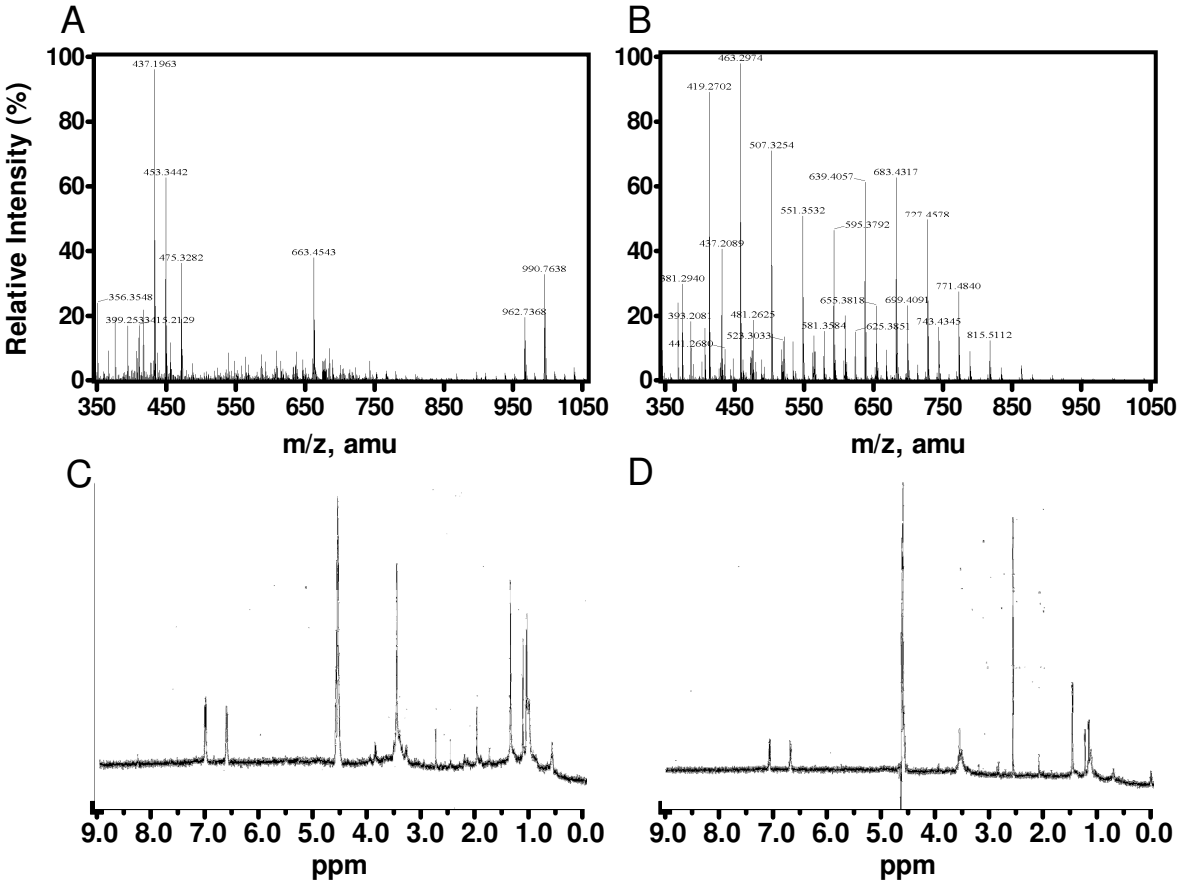

Figure 6.4. Spectroscopic analysis of combined size exclusion fraction 8'2-4. High resolution electrospray positive ion mass spectrum (A) before, and (B) after decompostion. ^{1}H NMR spectrum (C) before, and (D) after decomposition.

fraction 8'5-7. Initially, peaks at mass-to-charge ratios of 663, 685, 907, and 991 were visible (Figure 5.5A) but after decomposition only peaks from the PEG impurity were obvious (Figure 5.5B).

Functional group composition from NMR spectral data

^{1}H NMR spectral analyses of combined size-exclusion fractions 8'2-4 and 8'5-7 acquired prior to decomposition suggested mixtures of substituted aromatic compounds with relatively few hydrogen atoms attached to carbons (Figures 5.4C, 5.5C). However, the poor signal-to-noise ratio for each NMR spectrum indicated that compounds were present in low abundance, and thus full molecular structures could not be determined. Because the peak shapes corresponding to the aromatic protons indicated a different substitution pattern around putative benzene rings for each of the two combined size-exclusion fractions, major compound(s) associated with fractions 8'2-4 (Figure 5.4C) were likely different from major compound(s) present in fractions 8'5-7 (Figure 5.5C). When the same samples were analyzed by ^{1}H NMR spectroscopy after they had lost allelopathic activity, no aromatic protons were detected in combined fraction 8'5-7, and other proton signals decreased in intensity (Figure 5.5D). In contrast, the aromatic protons were still obvious in combined fraction 8'2-4, although all proton signals decreased somewhat in intensity, relative to background noise (Figure 5.4D).

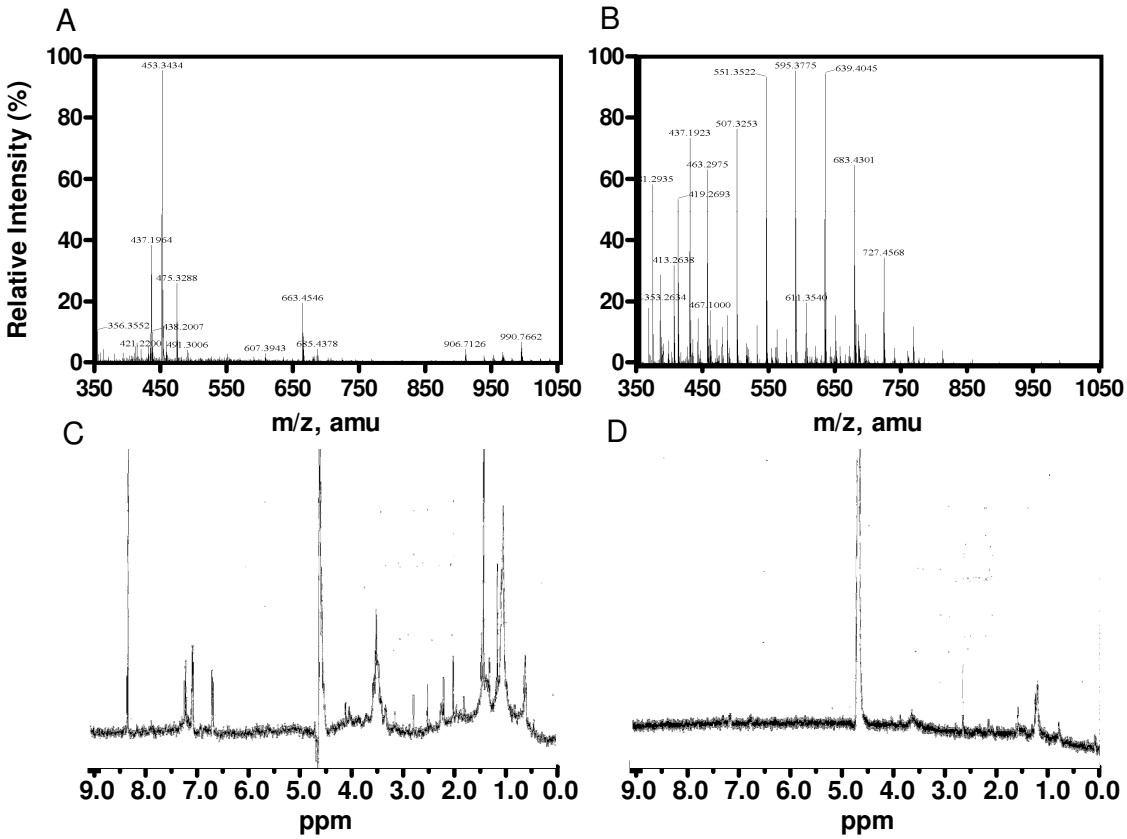

Figure 6.5. Spectroscopic analysis of combined size exclusion fraction 8'5-7. High resolution electrospray positive ion mass spectrum (A) before, and (B) after decompostion. ^{1}H NMR spectrum (C) before, and (D) after decomposition.

Discussion

Characterization of allelopathic *Karenia brevis* compounds

Karenia brevis exuded multiple compounds that inhibited the growth of a model competitor, the diatom *Asterionellopsis glacialis* (Figure 5.1B, Figures 5.4-5.5). Allelopathic compounds were not negatively charged (Figure 5.2B), and possessed molecular weights in the range of 500-1000 Da (Figures 5.2-5.5). Whereas allelopathic compounds were lipophilic enough to be retained on a hydrophobic resin column (Figure 5.2A), the most potent compounds eluted by reversed-phase HPLC with 50% aqueous methanol shortly after the solvent front, indicating greater polarity than most other compounds exuded by *K. brevis* (Figure 5.1B). The ^{1}H NMR spectral data indicated the presence of aromatic moieties on at least two allelopathic compounds, with relatively few (< 20) hydrogen atoms bound to carbons (Figures 5.4-5.5). These allelopathic compounds were unstable and present in low abundance (Figures 5.4-5.5).

Brevetoxins are not allelopathic to *Asterionellopsis glacialis*

Brevetoxin-B and brevetoxin-3 (PbTx-2 and PbTx-3, respectively), the two most abundant brevetoxins in water samples during *Karenia brevis* blooms (Pierce et al. 2005), eluted by HPLC as part of fraction P (Figure 5.1A), but the growth of *Asterionellopsis glacialis* was not affected by this fraction (Figure 5.1B). Other brevetoxins were not evident in *K. brevis* extracellular extracts when analyzed by LC-MS. Thus, although brevetoxins act as potent neurotoxins to mammals (Baden et al. 1998), our results indicate that brevetoxins at naturally exuded concentrations do not inhibit the growth of *A. glacialis*. This is consistent with previous studies (Kubanek et al. 2005; Prince et al.

submitted), which reported that allelopathic effects of *K. brevis* can rarely be attributed to brevetoxins. Kubanek et al. (2005) found that a mixture of three common brevetoxins (PbTx-2, -3, -9) totaling 8.8 ng mL^{-1} did not inhibit the growth of *A. glacialis*. Indeed, brevetoxins consistently inhibited the growth of only one competitor, the diatom *Skeletonema costatum*. However, Prince et al. (submitted) found no correlation between waterborne concentrations of brevetoxins during *K. brevis* blooms and the allelopathic potency of these blooms to *S. costatum*, suggesting that compounds other than brevetoxins were responsible for allelopathic effects of natural *K. brevis* blooms.

Smayda (1997) suggested that some microalgae may be able to form blooms in part by the production and exudation of allelopathic compounds; however, he noted more experiments must be conducted in order to determine whether compounds previously identified as neurotoxins and hepatotoxins act as allelopathic compounds. Since that time, the allelopathic effects of many microalgal metabolites toxic to vertebrates have been tested. Neither saxitoxin nor its derivatives, nor the spirolides produced by *Alexandrium* spp. were allelopathic (Tillmann and John 2002; Fistarol et al. 2004b; Tillmann et al. 2007). Similarly, okadaic acid and derivatives produced by *Prorocentrum lima*, did not inhibit the growth of competitors at naturally exuded concentrations (Sugg and Van Dolah 1999). Domoic acid, a neurotoxin produced by the diatoms *Pseudo-nitzschia* spp., also caused no allelopathic effects towards competitors (Lundholm et al. 2005). Neither have cyanobacterial toxins, including microcystins and nodularin, been found to be allelopathic (Babica et al. 2006; Suikkanen et al. 2006). While exceptions are likely to exist, based on the current evidence the generalization that algal compounds toxic to vertebrate animals function as allelopathic compounds should be rejected.

Karenia brevis produces multiple allelopathic compounds

Karenia brevis produces multiple allelopathic compounds that differ in polarity and allelopathic potency. When *K. brevis* extracellular extract was separated into 23 fractions by reversed-phase HPLC, five fractions (most containing distinct UV peaks) significantly inhibited the growth of *Asterionellopsis glacialis* (Figure 5.1). Additionally, allelopathic compounds fractionated by size-exclusion chromatography differed in potency (Figure 5.3B), molecular weight (Figure 5.3B), and functional group composition (Figures 5.4–5.5). Four HPLC fractions of *Karenia brevis* extracellular extracts stimulated the growth of *A.glacialis* (Figure 5.1B). However, the recombined HPLC fraction totally suppressed the growth of *A. glacilalis* indicating that the overall effect of compounds exuded by *K. brevis* is inhibition of competitors.

Previous studies that have attempted to identify allelopathic compounds have also suggested the involvement of multiple compounds. For example, fractionation of filtrates from cultures of the allelopathic cyanobacterium *Trichormus doliolum* yielded three fractions that inhibited growth of a cyanobacterial competitor, *Anabaena variabilis* (Von Elert and Juttner 1997). Multiple compounds inhibitory to competitor growth have been isolated from whole cell extracts of other cyanobacteria, including *Fischerella mucosa* (Gross et al. 1991; Hagmann and Juttner 1996; Papke et al. 1997) and *Nostoc* 31 (Todorova and Juttner 1995; Juttner et al. 2001). However, since the natural waterborne concentrations of these compounds are unknown, it is difficult to determine whether they are allelopathic in natural systems. In contrast to these studies, Sugg and Van Dolah (1999) found that allelopathic effects of filtrates of *Prorocentrum lima* were confined to

two adjacent HPLC fractions, suggesting that one or a few structurally similar compounds may be responsible for *P. lima* allelopathy.

Production of multiple allelopathic compounds by *Karenia brevis* (Figures 5.1, 5.3B) seems unnecessary given that the compound(s) contained in only one HPLC fraction (B) were sufficient to completely suppress the growth of *Asterionellopsis glacialis* (Figure 5.1). However, because *K. brevis* blooms occur within a complex ecological community, multiple allelopathic compounds may be advantageous to *K. brevis*. First, in some cases multiple allelopathic compounds may be necessary to inhibit the growth of *A. glacialis* if *A. glacialis* is more or less susceptible to allelopathic compounds depending upon its own physiological state. As a precedent for this concept, the diatom *Thalassiosira weissflogii* was more resistant to allelopathic compounds from the haptophyte *Prymnesium parvum* (Fistarol et al. 2005) when grown under nutrient replete, rather than nutrient limited conditions. Because in the current study *A. glacialis* was grown in nutrient replete culture media, it may have been more resistant to allelopathic compounds that it would typically be in the field. Second, *K. brevis* competes with a complex assemblage of phytoplankton species in the field, rather than a single species. *K. brevis* may produce multiple allelopathic compounds because not all competitors are equally susceptible to the same compounds.

Because allelopathic compounds produced by phytoplankton have generally not been identified, determining whether a single compound is allelopathic to multiple species or whether multiple allelopathic compounds are produced with species-specific effects has been difficult. However, different competitors clearly have differing susceptibilities to allelopathic compounds. For example, yet-uncharacterized allelopathic

compounds (karlotoxins) of the mixotrophic dinoflagellate *Karlodinium micrum* inhibited the growth of some competitors, including the chryptophyte *Storeatula major* and the raphidophyte *Heterosigma akashiwo*, but did not inhibit chryptophyte *Pyrenomonas salina* or the dinoflagellate *Amphidinium carterae*. Similar patterns have been found for other allelopathic phytoplankton, including dinoflagellates *Alexandrium* spp. (Fistarol et al. 2004a; Tillmann et al. 2007), the haptophyte *Prymnesium parvum* (Fistarol et al. 2003), and *Karenia brevis* (Kubanek et al. 2005; Prince et al. in press). Kubanek et al. (2005) found that some filtrates of *K. brevis* were allelopathic to some species, and other filtrates were allelopathic to other species, suggesting allelopathy may be mediated by several species-specific compounds.

Most of the allelopathic compounds produced by *Karenia brevis* are polar and either neutral or positively charged

Allelopathic compounds produced by *Karenia brevis* are organic, polar molecules with no negative charge. All allelopathic activity from *K. brevis* extracellular extracts was retained on a hydrophobic resin column, eluting when the column was rinsed with methanol and acetone (Figure 5.2A), indicating that allelopathic effects were attributable to organic compounds. Yet, when *K. brevis* extracts were fractionated by reversed-phase HPLC, the most potent fraction eluted with 50% aqueous methanol, and three other allelopathic fractions eluted from the column in 50-70% aqueous methanol, indicating that these compounds were also relatively polar compared to the majority of compounds exuded by *K. brevis* (Figure 5.1). Using anion exchange chromatography we determined that allelopathic compounds exuded by *Karenia brevis* are not negatively charged (Figure

5.2B). These results indicate that no *K. brevis* allelopathic compounds contain carboxylic acid functional groups. Our method to determine whether allelopathic compounds contain positively charged functional groups using cation exchange chromatography yielded inadequate separation of the active compound (data not shown). Therefore, it is unclear whether *K. brevis* allelopathic compounds contain ammonium or some other positively charged group.

In contrast to our results, many previous studies of other planktonic systems reported lipophilic allelopathic compounds. For example, several studies have claimed that long-chain fatty acids or their methyl esters are allelopathic (Perez et al. 1997; Wu et al. 2006). However, the concentrations of methyl palmitate and methyl stearate necessary to cause allelopathic effects (Perez et al. 1997) probably do not occur in the field. Similarly, several fatty acids including linoleic and oleic acids caused the chlorophyte *Monoraphidium contortum* to leak K^+ ions, suggesting physiological stress (Wu et al. 2006). Although some microalgae have been documented to contain these fatty acids (Chiang et al. 2004), it is not clear than they are exuded at concentrations high enough to be allelopathic. In addition, fischerellins A and B isolated from the cyanobacteria *Fischerella muscicosa* are quite lipophilic (Hagmann and Juttner 1996; Papke et al. 1997), but these compounds have not been shown to be allelopathic at naturally exuded concentrations.

Several studies have partially characterized allelopathic compounds from extracellular filtrates of phytoplankton. The allelopathic compounds present in *Trichormus doliolum* filtrates eluted from a reversed-phase HPLC column with 90-100% aqueous methanol (Von Elert and Juttner 1997), indicating that they were less polar that

the allelopathic compounds exuded by *K. brevis*. In contrast, allelopathic compound(s) present in *Prorocentrum lima* filtrates eluted from a reversed-phase HPLC column with 50% aqueous acetonitrile (Sugg and Van Dolah 1999), indicating that allelopathic compounds contained in this fraction may have been similar in polarity to those reported in this study. Although only a few naturally exuded allelopathic compounds have been partially characterized, the results of these studies indicate that allelopathic compounds may possess a wide range of polarities.

The polarity of allelopathic compounds has implications for their mechanisms of action and transport through the environment. Von Elert and Juttner (1997) suggested that hydrophobic compounds are more likely to be allelopathic than hydrophilic compounds, because they are better able to pass through cell membranes of competitors and to attack intracellular targets. However, transport across cell membranes may not be passive, or the site of action for allelopathy may be outside the cell. Previously we found that allelopathic compounds exuded by *Karenia brevis* increased the membrane permeability of competitor species resulting in cell membrane damage (Prince et al. in press), although the mechanism by which this occurred is unknown. Alternatively, exuded compounds may be chemically modified to become more allelopathic once they enter target cells. This "trojan horse" hypothesis has been proposed for okadaic acid and derivatives, but whether this mechanism is effective in natural systems is unclear (Hu et al. 1995; Sugg and Van Dolah 1999).

In order for allelopathic molecules to be effective, they must be transported through the environment to the target organism. The polarity of a compound influences its solubility in an aqueous medium, and polar and charged molecules can be hydrated

with water, changing their diffusion rate (Cussler 1997). Extremely lipophilic compounds may remain associated with producing cells or adsorb onto abiotic particles instead of diffusing freely through the surrounding aqueous environment. Additional allelopathic compounds will need to be characterized for scientists to be able to discern a general trend in the chemical characteristics of these compounds, and to determine the mechanisms by which they are transported through the environment and into competitor cells.

Allelopathic compounds produced by *Karenia brevis* have molecular weights of 500-1000 Da

Because allelopathic compounds were not retained by dialysis using a 1000 Da molecular weight cut-off membrane, it appears that they were smaller than 1000 Da (Figure 5.2C). This was confirmed using size-exclusion chromatography (Figure 5.3), whose outcome indicated that most allelopathic compounds eluted between the 1135 and 433 Da standards. Thus, we predict that the molecular weights of allelopathic compounds fall between 500 and 1000 Da, suggesting that *K. brevis* allelopathy is mediated by small organic molecules rather than large proteins or other polymers. Many putative allelopathic compounds have molecular weights in a similar range, including fischerellin A (409 Da) (Hagmann and Juttner 1996) and cyanobacterin (430 Da) (Mason et al. 1982). Molecular weight ranges for naturally exuded allelopathic compounds have not been confirmed; however, the allelopathic dinoflagellate *Alexandrium taylori* was reported to exude a protein-like molecule of molecular weight > 10,000 Da that lyses some

mammalian red blood cells (Emura et al. 2004), although the effects of this compound on competitors is unknown.

When two allelopathic fractions of *K. brevis* extracellular extracts were analyzed by high resolution positive-ion electrospray ionization mass spectrometry (MS), several candidate molecular ions were observed (Figures 5.4A, 5.5A). A unique peak with mass-to-charge ratio (*m/z*) of 963 was observed in allelopathic fraction 8'2-4 (Figure 5.5A) and fraction 8'5-7 contained a unique peak with *m/z* 907. Both samples produced ions with *m/z* of 663 and 991, suggesting that compounds with molecular weights of 662 and 990 Da were present in both fractions. These two fractions eluted from the size exclusion column adjacent to one another, making it likely that some compounds were spread between fractions. Because the peak heights were normalized to the most abundant ion in each sample, the actual concentration of these compounds in each sample cannot be quantified directly from these spectra, but ^{1}H NMR data indicates that the major component of each fraction is not the same. The shared compounds in each fraction may be minor components responsible for allelopathic effects, or the unique major components may be responsible for allelopathy. When these fractions were analyzed by mass spectrometry after decomposition (Figures 5.4B, 5.5B), only impurities were visible. In order to determine which candidate molecular weights can be attributed to the allelopathic compound, the allelopathic fractions must be more pure. However, further attempts to purify allelopathic compounds were unsuccessful.

Allelopathic compounds produced by *Karenia brevis* have aromatic functional groups

Analysis by ^{1}H NMR spectroscopy of two allelopathic fractions generated by size-exclusion chromatography prior to decomposition indicated that both fractions contained an aromatic moiety, but had different substitution patterns around the putative benzene ring (Figures 5.4C, 5.5C). Only a few proton signals were visible upfield of the aromatic proton resonances, indicating that the allelopathic compounds contained fewer than 20 hydrogen atoms bonded to carbon atoms. While these NMR data acquired with poor signal-to-noise ratio were insufficient for determining the complete molecular structures or even the biosynthetic class of allelopathic compounds, it is possible for some molecular characteristics to be confirmed. Because ions observed after mass spectrometry analysis were likely protonated, all of the observed candidate molecular weights were even-numbered (1 Da less than the observed *m/z*), indicating that allelopathic compounds contained either no nitrogen atoms or an even number of nitrogen atoms according to the nitrogen rule (Silverstein et al. 2005). No characteristic m+2 peaks were apparent in the mass spectra of allelopathic fractions, indicating than allelopathic compounds were unlikely to contain chlorine, bromine, or sulfur atoms.

Although the molecular weight range of 500-1000 Da for our compounds rules out the possibility that allelopathy is mediated by proteins, the allelopathic compounds could be peptides containing aromatic amino acids but without positively charged groups. Several putative allelopathic aromatic cyclic peptides produced by the cyanobacterium *Nostoc* 31 have been described (Juttner et al. 2001). Dinoflagellates including *Karenia brevis* are known to produce polyketides (Baden et al. 1998), although these metabolites

are not aromatic. However, aromatic polyketides are commonly produced by bacteria, fungi, and plants (Shen 2000), so it is possible that they might also be produced by a dinoflagellate. Aromatic alkaloids can be biosynthesized through the shikimic acid pathway (Ganem 1978). The allelopathic compounds produced by *K. brevis* may belong solely to one class of these metabolites, or may be products of some mixture of biosynthetic pathways. A small number of natural products have previously been identified that would fit the characteristics of the compounds present in our allelopathic fractions (i.e., organic, polar aromatic compounds containing an even number of nitrogens but no chlorines, bromines, or sulfurs). For example, an aromatic polyester with a molecular weight of approximately 662 which inhibits cell wall biosynthesis in fungi has been identified from a marine fungus (Schlingmann et al. 2002). Additionally, several aromatic peptides with molecular weights of approximately 962 have been identified from other marine organisms (Luesch et al. 2000; Milanowski et al. 2004).

Allelopathic compounds are produced by *Karenia brevis* in low concentrations and are unstable

We were unable to purify and fully characterize the molecular structures of allelopathic compounds exuded by *Karenia brevis*, in part because allelopathic compounds were unstable. Several of the methods that we used to purify compounds from *K. brevis* extracellular extracts resulted in loss of allelopathic effects, including the use of two additional reversed-phase HPLC columns (C-30 and CHP5C) (data not shown). Attempts to purify the allelopathic compounds by C-18 reversed-phase HPLC (Figure 5.1) resulted in eventual decomposition of allelopathic compounds (data not

shown). Although in some cases extracts and fractions remained allelopathic for weeks when stored in the dark at -20 °C under nitrogen gas, after several months allelopathic compounds decomposed even in these conditions (Figures 5.4B, 5.4D, 5.5B, 5.5D). Because allelopathic compounds were unstable, their presence in the environment is likely to be fleeting. The production of allelopathic compounds with a short lifespan may be advantageous to *K. brevis* by reducing the risk that grazers or parasites use these compounds as a cue to locate prey or hosts or that other, resistant but non-allelopathic phytoplankton benefit from allelopathic effects towards susceptible competitors.

Allelopathic compounds exuded by *Karenia brevis* were also present at low concentrations. Extraction of 1 L of *K. brevis* culture of high cell density yielded approximately 200 mg of extracellular organic extract, of which, the allelopathic fractions made up only a small percentage. When *K. brevis* extracellular extracts were separated by HPLC, the most potent allelopathic fraction (B) comprised only 2.5% of mass of the total, and the combined mass of all five allelopathic fractions represented only 13.7% of the total. Although all of the allelopathic material collected from 250 mL of *K. brevis* cultures was analyzed by ^{1}NMR spectroscopy, the low signal-to-noise ratio of the resulting spectra indicated that only microgram quantities of each compound were present in these fractions (Figures 5.4C, 5.5C). Additional efforts to access a greater abundance of allelopathic compounds directly from cultured *K. brevis* cells also met with failure, since compounds with chemical characteristics that matched those described above were not detected within fractions that suppressed *Asterionellopsis glacialis* growth.

Conclusions

We found that *Karenia brevis* produced multiple compounds that inhibited the growth of *Asterionellopsis glacialis* (Figure 5.1). Although one HPLC fraction accounted for most of the observed allelopathic effects, the production of multiple allelopathic compounds may be advantageous in some situations – for example, in the presence of multiple competitors of differing susceptibility. Most allelopathic compounds were relatively polar (Figure 5.1), indicating that they may be soluble in the surrounding water column, but raising questions about how such polar allelopathic compounds can cross competitor cell membranes. Partial characterization of two allelopathic compounds revealed molecular weights between 500 and 1000 Da (Figure 5.3), neutral or positive charge (Figure 5.2B) and aromatic moieties (Figures 5.4C, 5.5C) for both compounds. Compounds were produced at low concentrations and were unstable, suggesting that their presence in the environment is fleeting. The short lifespan of allelopathic compounds suggests that allelopathic effects of *K. brevis* on the plankton community dissipate with blooms. Future studies should attempt to elucidate the structures of *K. brevis* allelopathic compounds and to determine whether multiple compounds have species-specific effects on competitors.

CHAPTER 7

ALLELOPATHY IN MARINE SYSTEMS: PERSPECTIVES AND FUTURE DIRECTIONS

Recent experimental evidence supports the hypothesis that allelopathy plays an important role in competitive interactions between pairs of species in planktonic and benthic marine communities. However, in order for research on allelopathy to continue to contribute to the understanding of marine ecology, future studies must first address current methodological concerns and constraints, focusing on the production and release of allelopathic compounds, the transport of these compounds through the environment, and the physiological effects of allelopathy on competitors. More importantly, in order to understand the consequences of allelopathic interactions, future research must investigate the effects of allelopathy on marine communities and must place allelopathic interactions in an evolutionary context.

Current Methodological Concerns and Constraints

Convincing evidence of allelopathy in marine systems will require improvements in methodology at all levels. First, researchers should focus on identifying allelopathic compounds that are naturally released or found at the surface of organisms. Second, researchers must consider the waterborne transport of allelopathic compounds, designing experiments that address the fluid dynamics of the specific system. Finally, researchers should consider the physiological effects of allelopathic compounds on competing species. Here, we discuss limitations of current methods used for researching allelopathic

interactions and suggest alternative methods that may provide more ecologically relevant results and their potential benefits.

Production and release of allelopathic compounds

Identification of allelopathic compounds

Few studies have successfully led to identification of allelopathic compound(s). Instead, researchers have typically utilized mixtures of compounds exuded or extracted from allelopathic organisms to answer ecological questions. Both approaches have advantages. Because organisms may produce multiple compounds that both inhibit and stimulate competitors (e.g., Chapter 5 of this thesis) researchers must test the overall effects of one organism on another using whole extracts or exudates of a potentially allelopathic organism. Researchers can also assess whether total allelopathic potency changes with changing situations (e.g., in nutrient limiting conditions or the presence of specific community members). In contrast, conducting ecological tests with isolated, pure allelopathic compounds does not give researchers a complete picture of the allelopathic interactions between organisms.

The isolation and identification of allelopathic compounds also had advantages, however. For example, if allelopathic compounds were successfully isolated and their structures elucidated, one could also test factors that influence the biosynthesis and exudation of allelopathic substances. Also, as with chemical defenses against consumers (Koricheva 2002), the use of allelopathic compounds may be costly. Because the cost of using chemical cues may be affected by the availability of specific nutrients (Bryant et al. 1983), knowing the molecular structures of allelopathic compounds would enable

scientists to form and test hypotheses concerning the cost of producing compounds under specific nutrient regimes. In addition, many questions about the transport of waterborne allelopathic compounds through the environment could only be asked once molecular structures are known. The exudation rate, the solubility constant, and the diffusion rate of a pure compound can be measured or calculated (Cussler 1997), allowing researchers to assess natural localized concentrations of allelopathic compounds and thus to determine the active space of allelopathy. Working with individual compounds, one could also determine whether allelopathic effects are general (i.e., inhibit all species) or if some organisms produce multiple compounds, each with species-specific allelopathic effects. In addition, if the molecular structure of an allelopathic compound is similar to that of other biologically active compounds with known modes of action, knowledge of its structure may provide insight into allelopathic mechanisms of action.

To date, studies that aim to track allelopathy to its molecular source in a given benthic or pelagic marine system have primarily investigated the allelopathic effects of previously identified compounds. In pelagic systems, several species of marine dinoflagellates and haptophytes are known to produce compounds that are neurotoxic to vertebrates, but which have largely unknown ecological functions within plankton communities. Although these compounds can be responsible for mass mortalities of fish, birds, and marine mammals (Landsberg 2002), they have rarely been shown to be allelopathic to phytoplankton competitors. For example, *Alexandrium* spp. dinoflagellates produce saxitoxin and structurally related compounds whose neurotoxicity stems from their blockage of sodium channels (Baden et al. 1998). While filtrates of *Alexandrium* spp. are allelopathic to other dinoflagellates, diatoms, and chryptophytes, the effects seem

to be unrelated to saxitoxins (Tillmann & John 2002; Fistarol et al. 2004b). Similarly, the dinoflagellate *Karenia brevis* produces a group of neurotoxins called brevetoxins, which are largely not responsible for the allelopathic effects of *K. brevis* on competitors (Kubanek et al. 2005; Chapters 4 and 5 of this thesis). However, unidentified compounds isolated from exudates of the dinoflagellate *Karlodinium micrum* are icthyotoxic and hemolytic (Deeds et al. 2002), and were later also found to be allelopathic (Adolf et al. 2006). In a few cases, allelopathy among phytoplankton has been linked to specific compounds; for example, several sequiterpene alcohols from the dinoflagellate *Gymnodinium nagasakiense* (Kajiwara et al. 1992) and fatty acid methyl esters produced by *Nanochloris oculata* were shown to inhibit the growth of competitors. However, in neither study were these compounds shown to be effective at natural waterborne concentrations. In contrast, allelopathic effects of benthic organisms have more often been explained by fully characterized compounds. Several sponges and macroalgae use compounds previously identified as feeding deterrents to overgrow competitors or avoid being overgrown by competitors (de Nys et al. 1991; Thacker et al. 1998; Kubanek et al. 2002).

Although sometimes successful, currently available methods for testing allelopathic effects of known compounds have drawbacks. For example, (Windust et al. 1996; Windust et al. 1997) found that okadaic acid, produced by the dinoflagellate *Prorocentrum lima,* suppressed the growth of several species of phytoplankton. However, when filtrates of *P. lima* were fractionated by high performance liquid chromatography (HPLC) and fractions tested at realistic waterborne concentrations (much lower than the concentrations used by Windust et al.), unidentified compound(s) were found to be

responsible for allelopathy rather than okadaic acid (Sugg & Van Dolah 1999). The results of this study suggest that bioassays must be carefully designed to mimic natural conditions, using realistic compound concentrations and ecologically relevant competitors. In addition, focusing only on previously identified compounds biases against the discovery of new allelopathic compounds and may prevent researchers from observing that multiple compounds (e.g., one previously identified and one unknown compound) act together to create allelopathic effects.

Researchers may employ a variety of techniques to isolate allelopathic compounds, including bioassay-guided fractionation to narrow down the number of possible allelopathic compound from hundreds to only a few, within a manageable number of experimental steps. While bioassay-guided fractionation is ideal for exploring interactions involving a single, relatively stable compound, multiple fractionation steps can lead to loss of allelopathic compounds, and additive or synergistic effects of compounds can be difficult to test. While scientists have partially purified and characterized several novel allelopathic compounds by bioassay-guided fractionation (Suzuki et al. 1998; Sugg & Van Dolah 1999; Koh & Sweatman 2000; Deeds et al. 2002; Chapter 6), their molecular structures have not been fully determined.

Alternatively, researchers can employ comparative approaches to discovering novel allelopathic compounds. In cases in which production of allelopathic compounds is variable (e.g., Prince et al. 2008; Prince et al. submitted), chemical profiles can be compared between allelopathic and non-allelopathic exudates or extracts. A metabolomics approach, allowing simultaneous comparison of the concentrations of hundreds of metabolites (Viant 2007), could be used to determine whether the presence

of certain metabolites correlates with allelopathic activity. However, a compound identified by this type of correlative approach may not be responsible for the overall allelopathic effect. In addition, chemical variability inherent in complex extracts and exudates may make the signal of allelopathy difficult to detect against background noise.

Researchers may also investigate changes in gene expression when the production of allelopathic compounds is variable. Differential expression of genes related to the production of allelopathic compounds may be determined using expressed sequence tags (ESTs) from a cDNA library of an allelopathic organism or transcriptional changes of mRNA using microarrays (Dupont et al. 2007). If the differentially expressed genes code for enzymes known to be involved in the production of secondary metabolites (e.g., polyketide synthase genes Staunton & Weissman 2001), researchers might gain some insight into the class of molecules to which allelopathic compounds belong. This approach is complicated by the lack of well annotated genomes (Johnson & Browman 2007) for most allelopathic organisms, making it possible that these methods will provide a sequence that does not correspond to a known gene. In addition, determining the structure of a molecule based on differentially expressed genes is not straightforward, and results may be difficult to interpret.

Allelopathic interactions mediated by cell contact

Experimental demonstration of allelopathy requires the use of ecologically realistic bioassays, in which competitors are exposed only to compounds they would normally encounter in nature. For example, if allelopathic interactions are mediated by contact between competitors, only compounds present at the surface of an organism have

the potential to act as allelopathic agents. Yet, in some cases researchers have reported allelopathic effects using extracts of whole organisms (e.g., Thacker et al. 1998; Engel & Pawlik 2000), which could lead to an over-estimation of allelopathy if compounds extracted from deep within an organism suppress competitors, or an under-estimation if allelopathic surface compounds are tested at reduced, whole organism concentrations. A few studies have acknowledged this problem. For example, after determining that the sponge *Ectyoplasia ferox* produces tritepene glycosides that prevent it from being overgrown by another sponge competitor, Kubanek et al. (2002) found that the concentration of these compounds was significantly higher in the outermost layer of the sponge than in inner portions, suggesting that observed allelopathic effects may have been conservative (however, outer layer concentrations may still have been artificially high compared to actual surface concentrations). One solution to this problem may be the method proposed by de Nys et al. (1998), in which organisms are dipped briefly in hexane to extract lipophilic compounds from the surface without lysing cells. Once collected, allelopathic effects of such compounds can be tested at approximately natural concentrations. In a subsequent test, Nylund et al. (2006) found that results from this method of collecting and testing surface compounds from macroalgae were more predictive of an organism's ability to inhibit fouling than tests that utilized whole organism extracts. However, application of this surface extraction method is likely to be limited to highly lipophilic compounds and organisms whose cells are resistant to brief solvent exposure.

In contrast to benthic systems, no study of planktonic interactions has established that allelopathy occurs through cell contact. However, a few studies have shown that

competition can be mediated by cell contact (e.g., Uchida et al. 1995; Yamasaki et al. 2007b). For example, Uchida et al. (1995) reported that contact with the red tide dinoflagellate *Heterocapsa* sp. caused cells of competitors to lose motility and eventually lyse. Similarly, contact with the dinoflagellate *Cochlodinium polykrikoides* caused the cells of one dinoflagellate competitor to develop an abnormal shape (Yamasaki et al. 2007b). However, neither study demonstrated conclusively that these effects were chemically mediated.

Allelopathic interactions mediated by waterborne compounds

Demonstration of allelopathy via waterborne compounds requires experiments that use only naturally exuded materials, rather than whole cell extracts which contain compounds not normally encountered by competitors (e.g., Freeburg et al. 1979; Wu et al. 1998; Koh & Sweatman 2000). One solution is to expose competitors to filtrates of potentially allelopathic phytoplankton (e.g., Arzul et al. 1999; Kubanek et al. 2005; Tillmann et al. 2007). However, when using filtrates, one must ensure that allelopathic filtrates are not nutrient limited compared to controls. Because cultured organisms deplete nutrients as they grow, filtrates of these cultures have lower nutrient concentrations than fresh media. Thus, in many cases, researchers have added nutrients to filtrates (e.g., Arzul et al. 1999; Yamasaki et al. 2007a), but this approach can lead to experiments in which nutrient concentrations in filtrates are higher than that of control media, potentially stimulating competitor growth and decreasing the likelihood of detecting allelopathic effects. To reduce the problem of nutrient artifacts, Kubanek et al. (2005) diluted filtrates of the dinoflagellate *Karenia brevis* four-fold with culture media,

consequently testing potential allelopathic compounds at artificially low concentrations. Alternatively, the nutrient concentrations of filtrates can be measured directly, and nutrient concentrations of controls adjusted to match treatments (e.g., Fistarol et al. 2004b). Recent studies have reported a method to extract waterborne (extracellular) organic compounds from cultures, in which Prince et al. (2006; 2008) added a mixture of polystyrene resins to live phytoplankton cultures. Adsorbed organic compounds were then eluted using organic solvents. Because only an insignificant fraction of inorganic nutrients were extracted (Prince et al. 2008), this method enables allelopathic compounds to be tested at realistic exuded concentrations without the confounding variable of unequal nutrient concentrations.

In marine benthic systems, waterborne compounds may inhibit settlement and metamorphosis of competitor larvae, but few studies have tested this possibility using ecologically relevant assays. For example, Koh & Sweatman (2000) found that compounds extracted from whole tissues of the scleractinian *Tubastrea faulkneri* inhibited metamorphosis of larvae of coral competitors, but did not test whether these compounds were exuded by *T. faulkneri* in natural environments. Other studies have reported that coral larvae are unlikely to settle near some filamentous cyanobacteria and macroalgae, but this may or may not be explained by the exudation of compounds into the water column (Kuffner & Paul 2004; Kuffner et al. 2006). Maida et al. (1995) found that scleractinian coral settlement was lower around the soft corals *Sinularia flexibilis* and *Sarcophyton glaucum* than around scleractinian coral controls. In subsequent tests to determine whether this pattern was chemically mediated, Maida et al. (1995) observed reduced settlement of scleractinian coral larvae onto ceramic tiles impregnated with

whole soft coral extracts; however, the authors did not determine whether the tiles released compounds at realistic rates. When Kubanek et al. (2002) collected water surrounding the allelopathic sponge *Ectyoplasia ferox*, they found that compounds were not present at high enough waterborne concentrations to significantly inhibit competitors by this mechanism. However, Dobretsov et al. (2006) did find that compounds exuded by the macroalgae *Caulerpa racemosa* inhibited the settlement of bryozoan and polychaete foulers.

Realistic allelopathy assays testing compounds at their natural waterborne concentrations are difficult to conduct because one cannot measure the concentration of an unknown compound and because extracting compounds directly from seawater has historically not been straightforward. However, waterborne extractions are feasible for cultures and even for field samples using techniques such as the polystyrene resin method used by Prince et al. (2006; 2008), by collecting exudates of allelopathic organisms using gas permeable/water impermeable bags, or by pumping water surrounding organisms through a column that collects organic compounds (e.g., Kubanek et al. 2002; Selander et al. in prep).

Transport of allelopathic compounds through the environment

Researchers studying allelopathy in marine environments should also consider the physical environment through which waterborne compounds travel. Within fluids, the relative importance of forces that govern the transport of molecules differs depending upon spatial scale. At small scales (i.e., that of individual phytoplankton cells), viscous forces dominate, and molecules move away from a source primarily by the relatively

slow process of diffusion (Purcell 1977). However, at larger scales (i.e., that of macroscopic benthic organisms), inertial forces dominate and compounds travel by the relatively rapid process of advection (Vogel 1994). Thus, the movement of compounds between single-celled planktonic organisms is fundamentally different than the transport of compounds among larger benthic organisms, which may affect allelopathic outcomes.

Because diffusion is the dominant process moving allelopathic compounds away from phytoplankton cells, cells are expected to be surrounded by a halo of their exuded compounds. Much of the surrounding water and exudate should move with each cell as it swims (Purcell 1977; Dusenbery 1992) since phytoplankton are smaller than the smallest scale of turbulent mixing in the ocean (approximately one millimeter) (Fischer et al. 1979). By working with filtrates or extracellular extracts of cultured phytoplankton, past experiments have been designed to test the average allelopathic effect of exuded phytoplankton compounds. However, on the scale of individual cells, it is likely that there exists a complex chemical landscape, with patches of high concentrations of allelopathic compounds surrounded by areas of comparatively low concentration. The implications of a varied allelopathic landscape are difficult to assess using current methods but could be tackled using a mixture of modeling and innovative experimentation. Although models have been constructed for allelopathy in the plankton (e.g., Sole et al. 2005; Chen et al. 2007; Roy & Chattopadhyay 2007), none have modeled the diffusion of allelopathic compounds away from an individual cell in order to predict this complex landscape. Future models should aim to predict the concentration of compounds surrounding an allelopathic organism, specifically focusing on factors that influence local concentrations of compounds and the gradient and scale over which concentrations of allelopathic

compounds change with increasing distance from the producing organisms. Parameters such as cell size and shape, swimming or sinking speed, and compound exudation and diffusion rates should be included in models of local concentrations of allelopathic compounds. The concentration of compounds necessary to produce allelopathic effects cannot be predicted by modeling, Thus, researchers could also experimentally generate dose-response curves of the allelopathic effects of identified compounds. Experimental results can be paired with models to determine the inter-organism distance over which allelopathy is effective.

Because benthic organisms such as corals, sponges, and seaweeds occupy a scale of centimeters to meters, allelopathic compounds are expected to move between organisms primarily by advection (Vogel 1994). At this scale, understanding current magnitude and direction, turbulent velocity fluctuations, and boundary layer dynamics is central to understanding the fate of exuded allelopathic compounds. The motion of water near the substratum in benthic systems is characterized not only by high turbulence and high instantaneous velocity, but also by slow horizontal movement of water, suggesting that the concentration of exuded compounds may be patchy and rapidly changing (Koehl et al. 2007). Few studies have considered how flow affects allelopathic interactions in benthic systems. Maida et al. (1995) found that coral larvae did not settle evenly around soft corals. Measurements of prevailing currents confirmed that coral larvae recruitment was substantially more inhibited downstream, rather than upstream, of the allelopathic soft coral. The results of this study suggest that understanding the flow in benthic systems is central to determining which organisms will be impacted by allelopathy and how allelopathy will affect species distribution. Future studies should consider experiments

like those conducted by Maida et al. (1995); however, a more detailed map of flow fields would enable scientists to better predict areas where concentrations of allelopathic compounds are sufficient to inhibit competitor settlement or growth. Mixtures of modeling and experiments have been used to answer similar questions in other fields. For example, Koehl et al. (2007) found that compounds exuded by the coral *Porites compressa* are not distributed evenly around corals, but instead form filaments in the turbulent water. Larvae of the sea slug *Phestilla sibogae* may settle onto coral hosts by sinking once larvae have encountered a filament of settlement cues from *P. compressa*. Questions about the fate of allelopathic compounds exuded into the water column could be answered by a similar combination of models to predict the flow field around an organism and experiments designed to visualize flow in the field and to test allelopathic effects of realistic exposure to waterborne compounds present in moving fluids.

Physiological effects of allelopathic compounds on competitors

Although previous research has provided insight into some potential mechanisms of action of allelopathic compounds, additional ecologically relevant experiments are needed to understand their effects in natural systems. Nevertheless, determining how allelopathic compounds affect some model organisms can suggest potential physiological targets of allelopathy. For example, the hemolytic effect of algal toxins and microalgal exudates is often reported. Prymnesins 1 and 2, produced by the haptophyte *Prymnesium parvum*, and maitotoxin, from the dinoflagellate *Gambierdiscus toxicus,* lyse red blood cells of mammals, birds, and fish, a process which may be caused by the formation of cation pores in red blood cell membranes (Igarashi 1998; Igarashi et al. 1999). Although

prymnesins have not been shown to be allelopathic, exudates of *P. parvum* (that may contain prymnesins, among other compounds) have been shown to inhibit the growth of competing phytoplankton (Fistarol et al. 2003). However, cell-free filtrates of *P. parvum* did not possess hemolytic activity (Uronen et al. 2005), suggesting that prymnesins were not exuded from cells at high concentrations. Thus, while the formation of membrane pores and subsequent cell lysis is likely the mode of action of prymnesins on mammalian red blood cells, the presence of prymnesins appears not to account for allelopathy, emphasizing the importance of relying on ecologically relevant experiments to determine the mode of action of allelopathic compounds.

Recent studies have reported specific allelopathic mechanisms of action (Prince et al. 2008). However, so far researchers have investigated the effects of allelopathic compounds on only a few cellular processes, focusing on those areas that are easy to test or have previously been shown to be a target of allelopathy. While this is an obvious place to start, researchers have no way of knowing whether a process identified in this way is the only target of allelopathic compounds or may be part of a more complex physiological outcome. In addition, it can be difficult to distinguish between a direct target of allelopathy versus a symptom of general cell stress. For example, the photosynthetic efficiency of competitors often decreases in response to allelopathic compounds (e.g., Pawlik et al. 2007; Raniello et al. 2007; Prince et al. 2008) but photosynthetic efficiency also decreases in response to nutrient stress (Parkhill et al. 2001), temperature stress (Lesser & Gorbunov 2001), and metal toxicity (Miller-Morey & Van Dolah 2004).

In order to better understand how allelopathy works in the context of organismal complexity, researchers could use "system"-based approaches that utilize whole genome, proteome, or metabolome profiling. One approach for determining how gene expression changes in response to allelopathic compounds could be to use expressed sequence tags (ESTs) from a cDNA library of a target organism susceptible to allelopathy (Dupont et al. 2007). The specific ESTs that are differentially expressed for organisms exposed to allelopathic compounds versus controls may lead to identification of multiple sub-cellular targets, but to date no researchers have used this approach for allelopathic interactions. Similar studies have been used to determine the changes in gene expression of organisms exposed to other kinds of stresses. For example, Kore-eda et al. (2004) found that specific stress genes and a number of genes of unknown function were up-regulated in the ice plant *Mesembryanthemum crystallinum* in response to changes in salinity. Gene expression changes in response to allelopathy could also be determined by transcriptional changes of mRNA using microarrays (Dupont et al. 2007).

Proteomics, the study of protein expression, could be a complementary tool for understanding modes of action of allelopathic compounds. Using 1D or 2D gel electrophoresis coupled with mass spectrometry, the differential expression of proteins in response to a stressor can be determined (Lopez 2007). Although this technique has not been employed for understanding protein responses to allelopathic compounds, differential protein expression has been observed in response to a variety of environmental conditions (e.g., Olsson et al. 2004; Barneah et al. 2006). However, one serious disadvantage of genomic and proteomic approaches described here is their reliance on well-annotated genomic and proteomic databases. For experiments using non-

model organisms, researchers frequently discover differentially expressed genes or proteins with unknown sequences (e.g., Kore-eda et al. 2004; Olsson et al. 2004)), making the results of experiments difficult to interpret. While the genomes of some marine organisms have been sequenced, for example the diatom *Thalassiosira pseudonana* and the sea urchin *Strongylocentrolus purpuratus*, the current lack of genomic data for most marine organisms that are likely targets of allelopathy is a serious limitation (Johnson & Browman 2007). However, as genomes and cDNA libraries of additional organisms are sequenced and annotated, genomic and proteomic techniques will increasingly be appropriate for addressing the cellular processes affected by allelopathic compounds.

The most recently developed "omics" technique, metabolomics, has been applied to only a small number of studies in marine ecology (e.g., Rosenblum et al. 2006; Viant et al. 2006). By using this technique that simultaneously measures hundreds of metabolites, researchers can assess rapid changes in the physiological state of organisms. One major drawback of metabolomic approaches is the difficulty of distinguishing treatment effects from normal background metabolic "noise", thus requiring intensive replication of treatments and controls (Viant 2007). Despite current limitations, the future application of genomic, proteomic, and metabolomic techniques will provide researchers with an unbiased look at the physiological changes caused by allelopathic compounds, allowing deeper insights into the physiological processes that are targeted by allelopathic compounds.

Determining Ecological and Evolutionary Implications of Allelopathic Interactions

The value of understanding mechanisms of allelopathic interactions is limited without an understanding of how allelopathy can influence community structure and how past evolutionary pressure and constraints have shaped the interactions that we see today. Research into allelopathy should incorporate more than pair-wise interactions. Instead, experiments should focus on how community composition changes the production and effects of allelopathic compounds and how allelopathic interactions change the composition of communities. Additionally, the evolutionary forces that shape allelopathic interactions should be considered and tested where possible – for example, regarding the issue of whether the production of allelopathic interaction is an evolutionary stable strategy, whether allelopathy can maintain biodiversity, and whether co-evolution shaped allelopathic interactions.

The effects of allelopathic interactions on community structure

Allelopathic interactions have the potential to both shape and be shaped by community composition. First, the production and effect of allelopathic compounds is not constant but likely changes in response to the presence of specific competitors and microbes. The microbial community can potentially produce or degrade allelopathic compounds (Hulot & Huisman 2004; Piel 2004) and play indirect roles in allelopathic interactions (Smith et al. 2006; Uronen et al. 2007). Second, studies of allelopathic interactions often consider only pair-wise interactions when, in fact, allelopathy has the potential to affect a wide array of competitors, especially in cases when compounds are

exuded into the water column. Changes in the composition of competitor species present in the community by allelopathy can influence the composition of higher trophic levels that prey on these species. Alternatively, higher trophic levels may influence the composition of competitors in a community, minimizing or enhancing allelopathic interactions. In order to get a more accurate picture of the complex web of implications of allelopathy, experiments should seek to understand the full extent of the interactions between production of allelopathic compounds and community structure.

Effects of allelopathy on multiple competitors

Despite the difficulty of working with complex ecological systems, several recent studies have provided insight into the effects of allelopathic compounds on natural plankton assemblages. For example, Fistarol et al. (2003; 2004b) found that while cell-free filtrates of the cultured bloom-forming haptophyte *Prymnesium parvum* and dinoflagellates *Alexandrium* spp. inhibited natural phytoplankton assemblages, not all community members were equally affected. Thus, allelopathy may also change the species composition in a bloom community. In contrast, while some species of Baltic Sea cyanobacteria were found to inhibit the growth of a cryptophyte and a diatom in laboratory experiments (Suikkanen et al. 2004), compounds exuded by the cyanobacterium did not decrease the total density of photosynthetic organisms in a natural plankton community. In fact, filtrates actually stimulated many species of cyanobacteria, as well as some eukaryotic phytoplankton (Suikkanen et al. 2005). Similar experiments have been performed in benthic systems. For example, by measuring coral spat recruitment, Maida et al. (2001) found that the diversity and composition of coral

recruits was significantly different around two potentially allelopathic soft corals and a non-allelopathic control. These examples demonstrate that the knowledge gained from working with natural community assemblages can provide more insight into how allelopathic compounds affect communities than experiments that focus simply on pair-wise species interactions.

Studies that consider the effects of allelopathic compounds on natural assemblages of competitors still face the limitation that small collections or clonal cultures of allelopathic organisms rarely represent the full range of genetic diversity of natural populations. In fact, when assessing the allelopathic effects of phytoplankton, researchers often use a single, genetically identical strain in experiments, despite the fact that a number of recent studies have indicated a surprisingly high degree of genetic diversity present during phytoplankton blooms (e.g., Evans et al. 2004; Gribble & Anderson 2007). One way to overcome this problem is to use field collections as sources of allelopathic substances, which is possible for species that form nearly monospecific blooms, e.g., the dinoflagellate *Karenia brevis* (Steidinger & Haddad 1981). Prince et al. (2008) found that extracellular compounds collected from exudates of a natural *K. brevis* bloom strongly suppressed the growth of three of five competitor species tested in pair-wise lab experiments. However, the allelopathic effects of *K. brevis* blooms varied dramatically over temporal and spatial scales, and bloom intensity was not a good predictor of allelopathic potency (Prince et al. submitted). Researchers assessing allelopathic effects of benthic organisms usually collect multiple organisms for experiments (e.g., de Nys et al. 1991; Thacker et al. 1998), although the genetic relatedness of these individuals is unknown. Secondary metabolite profiles are known to

vary at the individual and population levels for benthic invertebrates (Pettit 1991) and macroalgae (Kubanek et al. 2006; Lane et al. 2007), suggesting that the production of allelopathic compounds could also vary across and within populations. However, population-level variability has not been directly assessed in any studies of benthic allelopathy. While some researchers have collected allelopathic organisms from different populations (e.g., Kubanek et al. 2002), only the average allelopathic effect was reported. Future studies should attempt to understand the role genetic diversity plays in the production of allelopathic compounds, which could also have implication for evolution of allelopathy.

Species composition within marine communities may also influence allelopathic outcomes, for example by altering the waterborne concentration of compounds produced by some phytoplankton. Two studies have demonstrated increased production of anatoxins and microcystins by freshwater cyanobacteria in response to the presence of dinoflagellate or green algal competitors (Kearns & Hunter 2000; Vardi et al. 2002). In contrast, Myers et al. (submitted) found that the presence of phytoplankton competitors led to decreased concentrations of brevetoxins exuded from the dinoflagellate *Karenia brevis*. However, neither anatoxins nor microcystins have been shown to have allelopathic roles, and brevetoxins were rarely responsible for the allelopathic effects of *K. brevis*. In a recent study, Prince et al. (submitted) found that blooms of *K. brevis* were less allelopathic during a year in which the diatom *Skeletonema costatum* was part of the bloom community compared with a year when *S. costatum* was absent. Lab experiments revealed that adding live *S. costatum* cells to live bloom samples significantly reduced the

allelopathic effects of blooms, suggesting that *S. costatum* is able to undermine *K. brevis* allelopathy.

Few studies have investigated how allelopathic interactions change with the presence of specific competitors in benthic systems, although Thacker et al. (1998) determined that the presence of a competitor sponge *Cacospongia* sp. did not induce production of the allelopathic compound 7-deacetoxyolepupuane in the sponge *Dysidea*. Curiously, Thacker et al. (1998) found that sections of live *Cacospongia* sp. exposed to 7-deacetoxyolepupuane contained higher concentrations of organic compounds, although the identity of these compounds and their ecological roles were not determined. The variable nature of allelopathic interactions may be better understood as studies shift away from consideration of competitors as passive targets of allelopathic compounds and toward the view that allelopathy is a dynamic and interactive process.

Influence of bacterial community on allelopathic interactions

The role that bacteria play in allelopathic interactions remains unclear. Although evidence is accumulating that microbial symbionts are responsible for production of many natural products isolated from eukaryotic organisms (Piel 2004), no studies have conclusively demonstrated the role of associated microbes in the production of allelopathic compounds. For example, sponges are believed to use allelopathic compounds to compete for space with corals (Porter & Targett 1988), as well as to defend against overgrowth from (Engel & Pawlik 2000) and to overgrow (Thacker et al. 1998) other sponges. However, nearly 40% of sponge biomass may be made up of associated microbes (Taylor et al. 2007), which have been demonstrated as the source of some

sponge metabolites (Bewley & Faulkner 1998; Piel 2004), making symbiotic bacteria a possible source for sponge allelopathic compounds.

As with benthic macroorganisms, allelopathic phytoplankton are also known to associate with bacteria which have been implicated in the production of natural products (Doucette 1995). Most tests of allelopathy among planktonic organisms have involved use of non-axenic (i.e., bacteria-containing) phytoplankton strains, making it difficult to determine whether observed allelopathic effects were caused by phytoplankton or associated bacteria (e.g., Tillmann & John 2002; Kubanek et al. 2005; Uronen et al. 2007). However, other studies have shown filtrates of axenic phytoplankton to be allelopathic (e.g., Yamasaki et al. 2007a), indicating that at least in some cases eukaryotic plankton are responsible for the production of allelopathic compounds. The potential for a symbiotic microbe to act as producer of allelopathic compounds benefiting its eukaryotic host raises the important question of whether the production of natural products can drive the evolution of symbiosis. Thus, future studies should attempt to determine whether allelopathic compounds are produced by associated bacteria.

In addition to being possible sources of allelopathic compounds, bacteria may play indirect roles in allelopathic interactions. First, heterotrophic bacteria have been hypothesized to degrade allelopathic compounds exuded into the water column, which models predict should limit the effects of allelopathy (Hulot & Huisman 2004). Second, pathogenic effects of some bacteria may lead to a mistaken interpretation of allelopathy being important. For example, Smith et al. (2006) found that macroalgae stimulated the growth of bacteria, which resulted in coral mortality. Had researchers not directly tested whether the proximity of algae to corals stimulated bacterial growth, the results could

have been misinterpreted as an allelopathic interaction between macroalgae and corals. Finally, the microbial community can also benefit directly from allelopathy. The haptophyte *Prymnesium parvum* was shown to cause cell lysis of the competing phytoplankter *Rhodomonas salina*, resulting in an increase of dissolved nutrients, which was shown to boost bacterial biomass (Uronen et al. 2007). Thus, allelopathy may influence marine communities by providing food for mixotrophic and heterotrophic plankton or by increasing the concentration of pathogenic bacteria. However, Uronen et al. (2007) used microbes associated with *P. parvum* and *R. salina* cultures rather than the bacteria that naturally occur during *P. parvum* blooms, making community consequences unclear. Additionally, Uronen et al. (2007) did not consider how bacterial species composition associated with phytoplankton changed due to compounds released from lysed cells. Clearly microbial communities may play complex roles in allelopathic interactions: bacteria have the potential to produce or degrade allelopathic compounds, mediate apparent allelopathic effects, and may be stimulated by allelopathic interactions. The complexity of these interactions begs further investigation into the role of specific microbes involved and the mechanisms mediating interactions using ecologically relevant species and experiments.

Influence of allelopathic interactions on multiple trophic levels

By definition, allelopathic interactions occur between competitors, but allelopathy may indirectly affect higher trophic levels. Because, as primary producers, phytoplankton and some benthic algae form the base of the marine food chain in photic zones, changes in their species composition or abundance caused by allelopathy are likely to impact

consumers. For example, the dinoflagellate *Karenia brevis* and macroalga *Caulerpa racemosa* may use allelopathy to exclude many competitors (Steidinger & Haddad 1981; Piazzi et al. 2001; Kubanek et al. 2005; Raniello et al. 2007), which is likely to impact grazers of benthic and pelagic communities. *K. brevis* has been shown to be nutritionally inadequate to some copepods (Prince et al. 2006) and to cause physiological distress in others (Sykes & Huntley 1987). The effects of *Caulerpa racemosa* on grazers is less clear, but if *C. racemosa* dominates the community, even a small change in grazing patterns could have a dramatic effect on consumers. Although several studies have established that allelopathic compounds released by phytoplankton change the relative abundance of other phytoplankton taxa (Fistarol et al. 2003; Fistarol et al. 2004b; Suikkanen et al. 2004), the effect the altered phytoplankton assemblage on zooplankton grazers is yet unknown. If toxic or unpalatable phytoplankters are less susceptible to allelopathy than palatable species, then consumers could decrease in abundance, which has the potential to affect higher trophic levels and could eventually damage commercially important fisheries. However, no study has tested this hypothesis. Simple experiments that assess the fitness of grazer species on a control phytoplankton assemblage and a phytoplankton assemblage which has been exposed to allelopathic compounds could begin to resolve the indirect effects of allelopathy on consumers.

In some cases, clear distinctions between competition and predation cannot be made. Because mixotrophic algae are able to phytosynthesize as well as use organic carbon sources, they may simultaneously compete with and prey upon other microalgal species. Therefore, allelopathic compounds may enable mixotrophs both to kill competitors that limit their access to resources as well as to ingest prey. For example, the

mixotrophic dinoflagellate *Karlodinium micrum* has been found to produce allelopathic compounds of yet-unidentified molecular structure called karlotoxins, which inhibit the growth of the cryptophyte competitor *Storeatula major*. However, the presence of karlotoxins also increased ingestion of *S. major* by *K. micrum*, indicating that karlotoxins play more than one role (Adolf et al. 2006). Similarly, the mixotrophic haptophyte *Prymnesium parvum* inhibits the growth of competing phytoplankton by the exudation of allelopathic compounds, which may also help *P. parvum* ingest algal prey under nutrient limited conditions (Graneli & Johansson 2003; Graneli 2006). The multiple strategies of mixotrophic algae, which change with different resource availability and community composition, underscore the complexity of allelopathic interactions.

Understanding the evolutionary context of allelopathy

Once allelopathic interactions are placed in the context of ecological communities, the evolutionary pressures that select for or constrain the production of allelopathic compounds can be better understood. To establish why allelopathic interactions between organisms persist over time, it is important to consider the fitness benefit to producing allelopathic compounds. However, because the producer of allelopathic compounds is expected to bear costs, implications of the cost-to-benefit ratio must be explored, focusing on the strategies used by allelopathic organisms to minimize this ratio. Scientists should also consider how co-evolution between competitor species may influence allelopathy and its outcomes, in addition to the patterns in the production and effects of allelopathic compounds we predict as a result of possible co-evolutionary processes.

Fitness benefits of allelopathy

In order for allelopathy to be an evolutionarily stable strategy, the production of allelopathic compounds must provide a fitness benefit to allelopathic organisms. While the benefits of killing competitors may seem obvious in some systems, the effects of allelopathy on the fitness of producers have not been tested. For example, if space is limited in benthic systems, the ability of a sponge to overgrow a competitor (Thacker et al. 1998) or keep from being overgrown (Engel & Pawlik 2000; Kubanek et al. 2002) may allow the allelopathic sponge to survive and reproduce better than those that are overgrown or have no space to settle. However, no studies have clearly linked the production of an allelopathic compound to increased survival, growth, or reproduction. In contrast, the production of compounds that defend against predation have been directly linked to fitness benefits to the producing organism (e.g., Baldwin 1998), and future studies of the benefits of allelopathy should attempt to establish a similar pattern.

In cases where allelopathic compounds are waterborne, the potential benefit to the producer is less obvious than for organisms using surface-mediated allelopathy. In fact, (Lewis 1986) proposed a major theoretical challenge to allelopathy, which he refers to as "the problem of distributed benefits." He argued that because the planktonic environment is not spatially segregated, organisms producing an allelopathic compound should incur the entire cost of this production, while the benefit of allelopathic compounds could be shared by all organisms resistant to allelopathy. If an organism "cheats" by benefiting from allelopathy without paying the cost of production, it will presumably be more fit and eventually outcompete organisms that do produce

allelopathic compounds. What Lewis failed to note, however, is the scale of allelopathic interactions in the plankton. As we mentioned previously, because an individual phytoplankton cell is smaller than the scale of turbulent mixing in the ocean (Fischer et al. 1979), compounds exuded into the water will move away from producers primarily by the relatively slow process of diffusion (Purcell 1977). Thus, it is likely that that in the complex allelopathic landscape of the plankton, benefits are not distributed, but rather remain mostly associated with producers rather than cheaters. The role of spatial segregation in allelopathic interactions among bacteria has been explored in detail, both through models (e.g., Durrett 1997) and experiments (e.g., Kerr et al. 2002). Researchers have found that spatial segregation promotes the persistence of the allelopathic phenotype (Kerr et al. 2002). Similar outcomes have been predicted by models of allelopathic interactions among phytoplankton; lower mixing intensity should also favor plankton that produce allelopathic compounds (Hulot & Huisman 2004). Although we predict that the theoretical challenges to allelopathy in the phytoplankton can be addressed by considering the scale at which interactions occur, the actual benefit to the producing organism has not been conclusively demonstrated.

Role of allelopathy in maintaining biodiversity

Allelopathic interactions may play a role in maintaining biodiversity. Czaran et al. (2002) modeled three phenotypes: one allelopathic, one susceptible to allelopathy but not allelopathic, and one resistant to allelopathy but not allelopathic. In this scenario, susceptible organisms were superior exploitation competitors because they incurred neither the costs of producing nor resisting allelopathic compounds. Resistant organisms

were intermediate exploitation competitors because they incurred the cost of resistance but not production. Allelopathic organisms were the worst exploitation competitors because they carried the cost of production and resistance to allelopathic compounds. In the proposed model, all three phenotypes can persist because although an obvious winner would emerge in any pair-wise interaction, when all three phenotypes are present a rock-paper-scissors situation emerges where no phenotype can out-compete the other two. Such a system has been demonstrated experimentally using three strains of *Escherichia coli*. However, biodiversity was only maintained in a spatially segregated system, because, in well-mixed systems, the susceptible strain was killed by the allelopathic strain, which was then out-competed by the resistant strain (Kerr et al. 2002). While three is the minimum number of phenotypes necessary for biodiversity to be maintained by such a non-hierarchical system, Czaran et al. (2002) predicted through modeling that a stable equilibrium of hundreds of phenotypes with varying degrees of allelopathy and resistance is possible. Because the different phenotypes could be different species or different strains of the same species, Czaran et al. (2002) proposes this situation as one factor that may explain why so many species can exist using the same resources – a dilemma originally termed the "paradox of the plankton" (Hutchinson 1961).

The costs of allelopathy

Attempts to understand the costs of producing allelopathic compounds may benefit from the extensive theoretical consideration of this subject from the chemical defense literature (e.g., Feeny 1976; Rhodes 1979; Coley 1985; Herms & Mattson 1992). Chemical defense theories attempt to explain under which circumstances the benefits of

producing defensive compounds outweigh the costs and how defenses should be allocated in time and space. Much of the discussion is also relevant to the production of allelopathic compounds. Natural selection should favor production or release of allelopathic compounds only when benefits outweigh the costs, and organisms may develop strategies to minimize these costs. The costs of defense can be divided into several categories, including allocation costs (i.e., costs incurred from allocating resources to production of compounds), opportunity costs (i.e., costs associated with loss of competitive ability due to allocating resources to defense rather than growth) and ecological costs (i.e., costs incurred when compounds disadvantage the producer in some other ecological interaction, such as making the producer more vulnerable to pathogens) (Koricheva 2002). While costs of defense against herbivores and predators have been demonstrated (e.g., Baldwin 1998; Mutikainen et al. 2002; Puustinen et al. 2004), few studies have shown that the production of allelopathic compounds are similarly costly. However, in one of very few examples of the ecological costs of allelopathy, the mixotrophic dinoflagellate *Karlodinium micrum* was found to inhibit the growth of competitors (Adolf et al. 2006), but more highly allelopathic strains of *K. micrum* were also more susceptible to infection by the parasitic dinoflagellate *Amoebophrya* sp. (Bai et al. 2007).

When multiple competitors, predators, and pathogens are present, it may benefit an allelopathic organism to use a single compound against several competitors or as a way to inhibit both competitors and to serve additional ecological functions (e.g., as a defense against predation). Multiple functions of allelopathic compounds may minimize the allocation costs, but may also constrain potential co-evolution between an allelopathic

organism and a competitor (cf., Schmitt et al. 1995). Clear examples of compounds that are used for both defense against predation and for allelopathy exist for a handful of marine sponges (Thacker et al. 1998; Kubanek et al. 2002). However, because allelopathic compounds produced by phytoplankton have rarely been identified, it is difficult to determine whether similar situations occur in pelagic systems. Exudates from cultures and blooms of phytoplankton have been shown to suppress laboratory monocultures of multiple competitors (e.g., Kubanek et al. 2005; Tillmann et al. 2007; Prince et al. 2008) as well as multiple species within natural phytoplankton assemblages (e.g., Fistarol et al. 2003; Fistarol et al. 2004b). If multiple species are inhibited by a single allelopathic compound, the allocation cost allelopathy would be reduced, but co-evolution between a single competitor species and the allelopathic species would be constrained. Alternatively, multiple allelopathic compounds with species-specific effects may be produced by organisms. Although maintaining production of multiple compounds is likely to be more energetically costly than producing a single compound for multiple uses, it is possible that the benefits from the resulting evolutionary flexibility outweigh the costs. Questions about the cost-to-benefit ratio of compound production will be difficult to answer without identification of more allelopathic compounds.

Producing or releasing allelopathic compounds only when necessary, such as in the presence of a susceptible competitor or when resources are especially limiting, might minimize both allocation and ecological costs of allelopathy. Studies have documented variability in the allelopathic potency of filtrates or extracellular extracts (e.g., Prince et al. submitted), which may result from such minimization of costs. However, these authors did not find that the presence of susceptible competitors consistently induced production

of allelopathic compounds by *K. brevis*. In contrast, Uronen et al. (2005) found that filtrates of the haptophyte *Prymnesium parvum* are more allelopathic under nitrate and phosphate limited conditions, suggesting that allelopathic compounds may be produced when the benefits likely to be gained from them is greatest (i.e., when nutrients are limited). More research is needed before the costs of allelopathy are understood. If the structures of allelopathic compounds were known, the energy (i.e., ATP used to assemble compounds) and other resources (e.g., nitrogen incorporated into compounds) required to produce allelopathic compounds could be assessed, facilitating efforts to understand allocation costs.

Conclusions

Recent studies have provided several strong examples of pair-wise competitive outcomes mediated by allelopathy within marine systems. Future studies should focus on overcoming the limitations of current methodology as well as incorporating broader and contextual questions of how allelopathic interactions change community structure, and how allelopathic interactions are shaped by evolution. Great strides could be made in understanding allelopathic interactions if methodological concerns were to be addressed. For example, the isolation and structure elucidation of compounds would enable researchers to more easily ask questions about the biosynthesis of allelopathic compounds, the species-specificity of allelopathy, the allocation costs of producing allelopathic compounds, and the rates and concentrations at which these compounds travel through the environment. Understanding the transport of allelopathic compounds through the environment will require a focus on issues of scale and flow but will provide

a more nuanced view of the variability of allelopathy and the conditions that favor it. Finally, genomic, proteomic, and metabolomic techniques should enable researchers to take an unbiased look at the physiological effects of allelopathic compounds on target competitors, providing a more accurate and complete picture of their modes of action.

Future studies should focus on how allelopathic interactions change community structure and are shaped by evolutionary forces in order to make the questions relevant to marine ecology. Recent studies in the plankton have provided evidence that allelopathic compounds can affect whole communities by reducing total phytoplankton biomass, changing relative species abundance, and increasing microbial biomass (Fistarol et al. 2003; Fistarol et al. 2004b; Suikkanen et al. 2005; Uronen et al. 2007). However, questions remain about the roles of associated microbes in the production of allelopathic compounds and the effect of changing phytoplankton assemblages on higher trophic levels. Studies of allelopathy among benthic organisms can provide insights into community structure when the influence of allelopathic interactions on whole communities, rather than pair-wise interactions, are considered. Finally, the facts that allelopathic compounds vary in their production (e.g., Uronen et al. 2005; Prince et al. submitted), can be used for more than one function (e.g., Kubanek et al. 2002; Adolf et al. 2006), and have species-specific effects (e.g., Fistarol et al. 2003; Kubanek et al. 2005), have implications for the evolutionary forces and constraints of allelopathic interactions that should be investigated.

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