SUBLETHAL INTERACTIONS BETWEEN THE HARMFUL ALGA KARENIA BREVIS AND ITS COMPETITORS

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SUBLETHAL INTERACTIONS BETWEEN THE HARMFUL ALGA KARENIA BREVIS AND ITS COMPETITORS

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To my family.

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SUMMARY

Plankton engage in a host of ecological interactions mediated through chemistry, such as detecting and deterring grazers, locating food and prey, and signaling with conspecifics to coordinate behaviors. Many phytoplankton produce and release compounds that inhibit the growth of competitor species, a process called allelopathy. These interactions are being increasingly recognized as critical in structuring planktonic communities, as bottom-up or top down explanations may only partially explain the levels of diversity observed in the plankton. Allelopathy, in particular, may be an important mechanism for species that maintain mono-specific blooms in coastal environments.

In order to understand the role of allelopathy in phytoplankton communities, I investigated how competitor species respond to chemical cues released from the red tide dinoflagellate *Karenia brevis*. *K. brevis* produces a mix of unstable, relatively polar, allelopathic organic molecules that are produced and released at low concentrations (see Chapter 3). The majority of these compounds caused sublethal reductions in competitor growth. In laboratory experiments, these compounds inhibited the growth of competitors *Asterionellopsis glacialis, Skeletonema grethae, Prorocentrum minimum,* and *Akashiwo sanguinea,* although each species was susceptible to a different sub-set of *K. brevis* compounds. At least one competitor was also found to be relatively resistant to *K. brevis* allelopathy. The production of multiple compounds likely benefits *K. brevis* by providing suite of compounds to use against a variety of competitors.

The production of allelopathic compounds, however, varies within and among *K. brevis* strains as described in Chapter 3 of this thesis. In lab-based studies, *K. brevis*

exhibited inter-strain variability in allelopathic potency towards the model competitor *A. glacialis*, although all strains investigated were found to be capable of at least some degree of allelopathy. This suggests that that allelopathy is a genetically fixed trait in *K. brevis* populations. However, there can also be considerable differences among batch cultures of the same *K. brevis* strain, demonstrating a previously underappreciated level of complexity in allelopathy involving *K. brevis*. The basis for this *intra*-strain variability in allelopathy is unknown. Since *K. brevis* blooms are more genetically varied than previously thought, the genotypic make up of *K. brevis* cells in each bloom likely influences the bloom's allelopathic potency and may explain previous observations of variable bloom allelopathy across multiple temporal and spatial scales.

Phytoplankton that are sensitive to *K. brevis* allelopathy are impacted in multiple ways. In the very sensitive competitor, *Thalassiosira pseudonana*, allelopathic compounds ultimately caused a reshuffling of cellular nitrogen pools, altered carbon storage and impaired osmotic regulation as determined using a nuclear magnetic resonance (NMR) based metabolomics approach (see Chapter 2). By characterizing the pool of primary metabolites present in the cell after exposure to *K. brevis* cues, we inferred which metabolic pathways may be affected by allelopathy. For instance, concentrations of betaine and the aromatic metabolite homarine were suppressed, indicating that *K. brevis* allelopathy may disrupt this competitor's ability to osmoregulate. Exposure to *K. brevis* cues enhanced the concentrations of glutamate and the fatty acid caprylate/caprate in *T. pseudonana*, suggesting that protein degradation was enhanced and that energy metabolism was altered. This contrasts with the response to *K. brevis* allelopathy of the diatom *Asterionellopsis glacialis*, which was much more

resistant to chemical cues produced by *K. brevis*, likely through as yet unidentified detoxification pathways.

Although we have gained much insight into interactions between *K. brevis* and other phytoplankton through pair-wise studies, I also demonstrate that in more complex settings, *K. brevis* allelopathy is attenuated (Chapters 3-4). When natural, mixed plankton assemblages were exposed to *K. brevis* compounds, some taxa were not susceptible to allelopathy and in fact the growth of other phytoplankton groups (i.e., diatoms) was stimulated by exposure to *K. brevis* chemical cues. In lab-based studies, cell physiological state was important in dictating the susceptibility of competitors to allelopathy: phytoplankton were most susceptible to *K. brevis* allelopathy when in earlier growth stages (i.e., lag stage) rather than later growth stages. Additionally, the allelopathic potency of *K. brevis* is also influenced by competitor population density: competitors with lower cell concentrations were more susceptible to allelopathy; however cell physiological state may be more important in determining a particular species' sensitivity to *K. brevis* compounds.

Blooms of *K. brevis* initiate offshore on the West Florida Shelf yet little is known about how *K. brevis* allelopathy affects phytoplankton found offshore. In Chapter 4, I seek to determine if habitat is a determinant of phytoplankton sensitivity to *K. brevis* cues. I investigated the impacts of chemical cues from *K. brevis* on competitor species from inshore *vs.* offshore locations with both lab cultures and natural plankton assemblages. Specifically, I exposed two different phytoplankton assemblages from offshore communities in the Gulf of Mexico to chemical cues exuded by *K. brevis*. The offshore communities responded to allelopathy similarly: dominant diatom taxa were

stimulated by exposure to *K. brevis* chemical cues suggesting that allelopathy is not targeted towards members of offshore communities. In order to test whether the stimulatory effects of allelopathy on offshore field assemblages indicated a difference in the sensitivity of these competitors to *K. brevis* allelopathy compared to inshore competitors, we tested the impacts of *K. brevis* chemical cues on four inshore diatoms and four offshore diatoms in lab-based pair-wise experiments. Here, these experiments indicated that chemical cues from *K. brevis* had either neutral or weakly negative effects on offshore diatoms, whereas inshore diatoms were somewhat resistant, but never stimulated, by *K. brevis* cues. This suggests that *K. brevis* allelopathy may be targeted mostly towards both inshore and offshore competitors; a likely scenario since both communities experience highly concentrated *K. brevis* blooms where allelopathic compounds could build up to concentrations capable of inhibiting competitor growth or metabolism.

Overall, my dissertation research provides insight into how species-specific, antagonistic interactions among phytoplankton competitors can affect community structure through direct or indirect mechanisms, highlights the potential role of allelopathy in the maintenance of *K. brevis* blooms, and uses a novel tool set (i.e., metabolomics) to determine the molecular targets of *K. brevis* allelopathy. It further demonstrates that planktonic communities are complex and dynamic ecological systems and that interspecific interactions between phytoplankton can have unexpected, cascading impacts in marine systems.

CHAPTER 1

CHEMICAL ECOLOGY OF THE MARINE PLANKTON*

Introduction

This report summarizes recent research advancements in the chemical ecology of pelagic (open water) marine ecosystems, with an emphasis on allelopathic interactions. With the goal of providing a comprehensive overview of new knowledge in this field, we reviewed articles published in 2006-2008 (inclusive) organized by type of ecological interaction, starting with studies on chemically-mediated intra-species communication, followed by inter-species interactions, and leading up to ecosystem-level effects of plankton secondary metabolites. Major recent research foci of pelagic marine chemical ecology have been on allelopathic effects in competition and the role of algal toxins in predator-prey interactions. There have also been new insights into host-parasite interactions, advances in chemically-mediated mate identification and tracking, and intraspecific signaling, particularly among diatoms using polyunsaturated aldehydes (PUAs).

Before delving into the primary literature, it is worthwhile to point out some relevant recent review articles. For a review on advances in chemical ecology of the marine benthos (bottom-dwelling organisms), see Paul and Ritson-Williams (2008). Cell-

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* Poulson, K.L., Sieg, R.D., & Kubanek, J. (2009). *Natural Products Reports*, 26, 729-745. AND SELECTED PORTIONS OF: Sieg, R.D., Poulson-Ellestad, K.L., & Kubanek, J. (2011). *Natural Product Reports*, 28, 388-399. Reproduced by permission of the Royal Society of Chemistry.

cell communication, allelopathic interactions, and phytoplankton-zooplankton interactions, as well as new advances in the chemical ecology of the benthos were reviewed by Ianora *et al.* (2006). The function of chemical signals in both marine and freshwater pelagic systems, as well as their ability to structure interspecific associations, was well covered in a review by Pohnert *et al.* (2007). A comprehensive, taxonomically-organized review of Antarctic marine chemical ecology, including molecular structures and known ecological functions, was recently published by Avila *et al.* (2008). A review focusing on the ecological roles of volatile organic compounds in freshwater and marine systems was published by Fink (2007). A book chapter on allelopathic interactions in pelagic and benthic communities was written by Granéli and Pavia (2006). A general review of phytoplankton allelopathy, particularly on abiotic and biotic factors that can alter allelopathic interactions with an emphasis on the effects of eutrophication was provided by Graneli *et al.* (2008). In a separate review, Graneli (2006) discussed how allelopathy is used by the toxic haptophyte *Prymnesium parvum.* The effects of Baltic Sea cyanobacterial toxins on multiple ecological scales were reviewed by Karjalainen *et al.* (2007).A general overview of grazing pressures faced by *Phaeocystis* spp., including chemically-mediated predator-prey interactions, has been written by Nejstgaard *et al.* (2007). Although it does not focus specifically on pelagic chemical ecology, a review on dynamic scaling by Zimmer and Zimmer (2008) discussed the proper means to assess the ecological relevance of chemical cues in ecology studies.

Intraspecific signaling

Polyunsaturated aldehydes (PUAs) such as (2*E*,4*E*)-decadienal (**1**)**,** (2*E*,4*E*)-octadienal (**2**)**,** and (2*E*,4*E*)-heptadienal (**3**) are produced by a variety of diatoms (Vardi *et al.* 2006) and other phytoplankton taxa (Hansen & Eilertsen 2007). PUAs have been implicated in a range of functions, including intraspecific signaling and programmed cell death, anti-grazing defenses (Miralto *et al.* 1999), allelopathy (Hansen & Eilertsen 2007), and bacteria-phytoplankton interactions (Ribalet *et al.* 2008). In diatoms, PUAs are produced by the breakdown of unsaturated fatty acids in response to mechanical stress (Pohnert *et al.* 2002).

(2*E*,4*E*/*Z*)-Decadienal (**1**) affects neighboring conspecificis when released by wounded diatoms *Thalassiosira weissflogii* and *Phaeodactylum tricornutum* (Vardi *et al.* 2006). Perception of **1** at the cell surface of diatoms starts a signaling cascade, building up intracellular calcium and nitric oxide production via nitric oxide synthase-like activity, which can lead to cell death. The production of nitric oxide was found to be rapid (occurring within five minutes) and dependent on the concentration of **1** in aqueous medium (Vardi *et al.* 2006). Cell death rates were also high: *P. tricornutum* exposed to 66 μM **1** suffered 90% mortality within four hours. Treatment of cells with a nitric oxide donor (sodium nitroprusside or diethylamine nitric oxide) increased cell death proportionally with nitric oxide accumulation, whereas a nitric oxide synthase inhibitor (*NG*-monomethyl-L-arginine) depressed PUA-dependent cell death (Vardi *et al.* 2006). Pre-conditioning *P. tricornutum* cells with low concentrations of **1** (0.66 μM) increased recovery potential as well as nitric oxide production relative to cells that were not pre-

treated, when the same cells were later exposed to 13 μM **1**. Pre-treated cultures also underwent a six-fold increase in cell density compared to non-pre-treated cells after transfer into media lacking **1**. This suggests that nitric oxide build-up is harmful but diatoms can acclimate to exposure. Interspecific variation in production or susceptibility to PUAs may be involved in bloom succession, or may act as cues for environmental stress (Vardi *et al.* 2006). Vardi *et al.* (2008) characterized a protein belonging to the conserved YqeH subfamily involved in nitric oxide production. Over-expression of the YqeH synthesis gene (PtNOA) in *P. tricornutum* led to increased nitric oxide production and decreased growth, as well as lowered photosynthetic efficiency compared to wild-type controls (Vardi *et al.* 2008). Concentrations of **1** that were sublethal to wild-type cells caused depressed growth in cells that over-expressed PtNOA largely through decreased photosystem II efficiency, suggesting that these mutants were hypersensitive to **1** exposure (Vardi *et al.* 2008). Mutants over-expressing PtNOA increased expression of metacaspases but reduced expression of superoxide dismutase (MnSOD) which, coupled with the involvement of nitric oxide, suggests that PtNOA expression is related to programmed cell death (Vardi *et al.* 2008).

PUAs may be released by non-wounded diatom cells to communicate with conspecifics (Vidoudez & Pohnert 2008). When monitored throughout their growth cycle, cultures of the diatom *Skeletonema marinoi* only released PUAs during late stationary phase but well before cell lysis was prominent. When PUAs were added to cultures during late stationary phase at ecologically-relevant (nanomolar) concentrations, *S. marinoi* cells experienced a temporary increase in growth, followed by a dramatic decline in cell densities (Vidoudez & Pohnert 2008). PUAs added at nanomolar

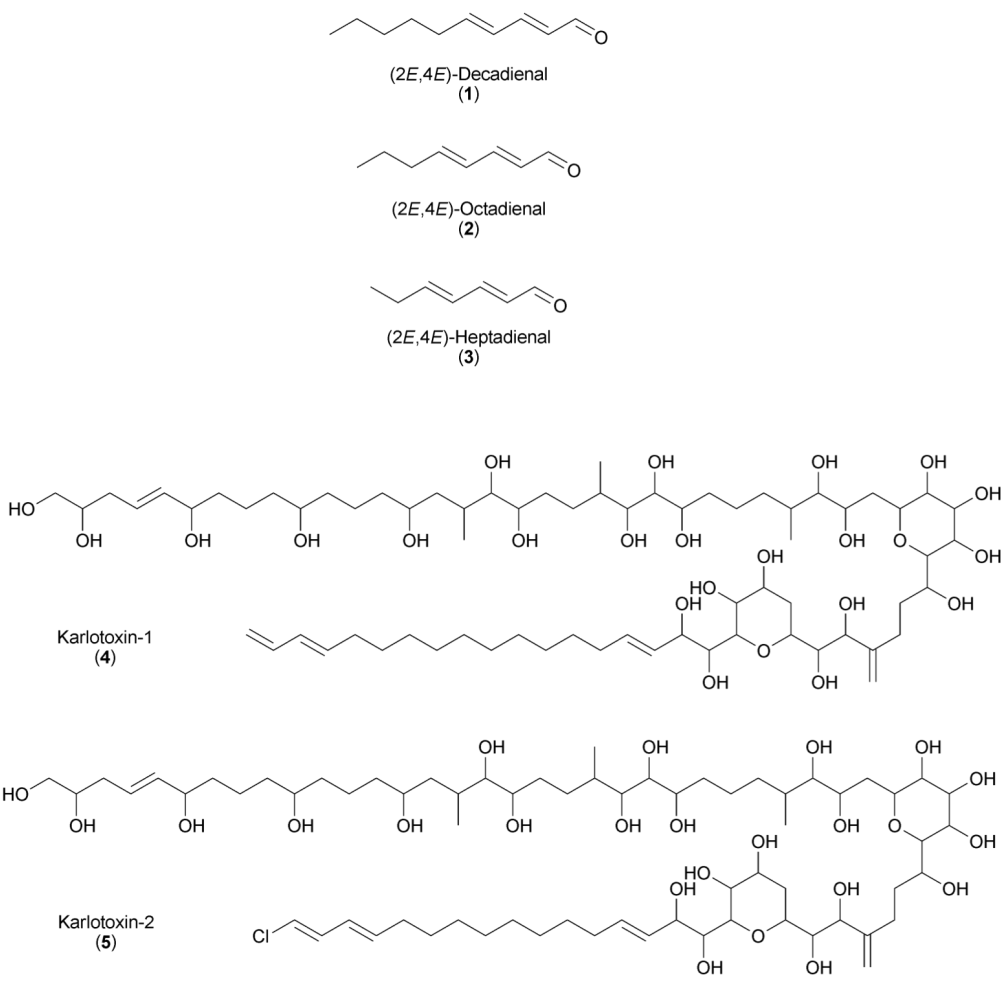

Figure 1.1. Polyunsaturated aldehydes (2*E*, 4*E*)-Decadienal (**1**), (2*E,* 4*E*)-Octadienal (**2**), and (2E, 4E)-Heptadienal (**3**), isolated from diatoms. Chemical structures of Karlotoxin-1 (**4**) and Karlotoxin-2 (**5**) isolated from the dinoflagellate *Karlodinium veneficum*.

concentrations to exponential or early stationary growth phases did not affect *S. marinoi* growth, although micromolar concentrations caused a significant decrease in growth during these two phases (Vidoudez & Pohnert 2008). *S. marinoi* cells that were previously exposed to low PUA concentrations did not respond to later PUA exposure. PUAs could act as sublethal signals because they are released into the environment, their presence in the environment is ephemeral, and they only affect diatoms during specific growth periods (Vidoudez & Pohnert 2008). Although it is not yet entirely clear what message(s) are being relayed by these compounds, it has been proposed that as diatom cells sense deteriorating environmental conditions, PUAs are released to signal for organized bloom termination (Casotti *et al.* 2005), and similar signaling processes have been hypothesized for other phytoplankton groups (Vardi *et al.* 2007). However, the evolution of this strategy is counter-intuitive, requiring group selection arguments that are typically rejected unless cooperating organisms have a high degree of genetic relatedness. Genetic studies of bloom population structure should help shed light on this matter.

The effects of filtrates from the haptophyte *Prymnesium parvum* can also lead to self-imposed cell death. Olli and Trunov (2007) found that *P. parvum* filtrates are toxic to less dense cultures of the same species. However, cells were able to acclimate to filtrates when exposed at low concentrations, which implies that as blooms form, cells associated with the bloom become resistant to the toxins they are emitting (Olli & Trunov 2007). Autotoxicity, therefore, may play a role in algal bloom dynamics. The autotoxic compounds were not identified.

Chemically-mediated switches from asexual to sexual modes of reproduction (a process called mixis) have recently been examined in rotifer populations (Stelzer & Snell 2006). Although populations of rotifers belonging to the class *Monogononta* are primarily composed of asexually-reproducing females, under stressful conditions such as crowding or food depletion a proportion of females within the population undergo mixis, resulting in the production of sexually-reproducing males and females that produce hardy resting eggs (Gilbert 1963). Mixis is induced when rotifer populations reach a threshold, analogous to quorum sensing in cooperative bacteria. Within the *Brachionus manjavacas* (ex *B. plicatilis*) species complex, the mixis induction signal is relatively conserved: mixis was similarly induced by conditioned media from multiple *B. plicatilis* strains, suggesting little divergence in genes encoding the signal over the past 10 million years (Stelzer & Snell 2006). Snell *et al.* (2006) examined the identity of the responsible signal using rotifer-conditioned media coupled with mixis induction assays. They proposed that the signal molecule binds to a receptor in the mother, which triggers her oocytes to become mictic. The incidence of mixis was reduced by the addition of a protease and protected and by protease inhibitors, indicating that the signaling molecule is protein based. Active HPLC fractions that promoted mixis contained a 39 kDa molecule, the N-terminus of which was 100% similar to a steroidogenesis-inducing protein from human ovarian follicular fluid, indicative of the genetic conservation of reproductive hormones. A protein can act as an effective mixis-inducing signal because it allows for high target specificity, low detection limits, and quick signal breakdown (Snell *et al.* 2006).

Mate selection by male *Brachionus manjavacas* rotifers is also chemically-mediated, and appears linked to female age (Snell *et al.* 2007). Males were previously

shown to select mates based upon contact with a glycoprotein on females' corona (Snell *et al.* 1995). In a no-choice assay, male rotifers copulated with young (3 hour old) females significantly more often than with very recently hatched (0.2-1 hour old) or older (6-72 hour old) females (Snell *et al.* 2007). Males couldn't discern virgins from non-virgins, nor could they distinguish between amictic and mictic females. Since younger females have a higher probability of being virgins, selectively mating with younger females whose eggs have not already been fertilized may maximize male reproductive success (Snell *et al.* 2007).

Although the identities of most copepod pheromones remain a mystery, the effects of diffusible female copepod pheromones on male mate-tracking behavior continue to be a focus of signaling studies. Male copepods have been proposed to use both mechanical flow patterns and chemical stimuli such as pheromones to track, capture and identify females. In Y-maze studies, males of the parasitic sea louse *Caligus rogercresseyi* tracked to maze legs containing either juvenile or adult females of the same species over legs that only contained seawater (Pino-Marambio *et al.* 2007). The species-specificity of copepod pheromones has also been addressed using 3D video recordings of copepod behavior (Goetze 2008). Males of three copepod species (*Centrophagous typicus, Centrophagous hamatus,* and *Temora longicornis)* were exposed to females and their tracking behavior was analyzed in a series of no-choice experiments (Goetze 2008). Males displayed non-specific capture behavior, pursuing and capturing heterospecifics and conspecifics at comparable rates. However, post-capture, males became more selective, and released the majority of heterospecific females prior to mating. It appears that dissolved, pre-contact pheromones lack information regarding species identity of the

target female. Contact cues such as surface glycoproteins, or mechanical cues such as genital fitting, may act as more reliable, species-specific signals for copepods (Goetze 2008). As heterospecific encounter rates can be as high as 2,000 encounters m^{-3} d^{-1}, mating attempts with heterospecifics are likely a common and energetically-costly aspect of copepod reproductive behavior (Goetze 2008).

An unusual tracking behavior has been documented in the estuarine copepod, *Oithona davisae* (Kiorboe 2007). Whereas mates of many copepod species track rapidly up a pheromone trail reaching a target female in a matter of seconds, *O. davisae* males spiral around the trail, taking over 30 seconds to capture a female. Male spiraling behavior may be a response to the erratic feeding behavior of conspecific females, characterized by passively sinking, then jumping upwards up to 1 mm every 2 to 5 seconds (Kiorboe 2007). This jumping behavior may create gaps in the pheromone trail if the cue cannot diffuse quickly enough to fill in the gaps between jumps, in response to which males compensate by spiraling around the general area of the pheromone. This tracking strategy seems fitting for oceanic dwellers where chances of mate encounters are low, but since *O. davisae* is often found at high densities in semi-enclosed estuaries and inlets, this costly behavior may be a remnant of an oceanic ancestor. *O. davisae* tracking behavior also makes males conspicuous to predators, potentially leading to increased male predation and creating female-biased sex ratios (Kiorboe 2007).

Host-parasite interactions

Toxin-producing dinoflagellates may incur costs due to their susceptibility to infection by parasites (Bai *et al.* 2007). Blooms of the dinoflagellate *Karlodinium*

veneficum (ex *K. micrum*) around Chesapeake Bay, USA can be ichthyotoxic and are also hosts to the parasitic dinoflagellate *Amoebophrya* sp. In co-culturing experiments using multiple *K. veneficum* host strains, there was a significant positive correlation between host karlotoxin concentration and susceptibility to *Amoebophrya* sp. infection (Bai *et al.* 2007). Even though infection by *Amoebophrya* sp. led to decreased intracellular and extracellular toxin concentrations compared to uninfected controls, it is unlikely that the parasite catabolizes *K. veneficum* toxins. It is more likely that infection led to host lysis and subsequent bacterial degradation of toxins or that infection by *Amoebophrya* sp. inhibited toxin production (Bai *et al.* 2007). Heightened susceptibility to infection could be due to these strains having larger cell sizes or higher cell densities, which would create increased surface area for parasitic attack (Bai *et al.* 2007). *Amoebophrya* sp. may also successfully parasitize *K. veneficum* by having a cell membrane sterol composition similar to its host, which lowers the susceptibility of *Amoebophrya* sp. to the lytic effects of karlotoxins (Place *et al.* 2006) (for a more detailed look at karlotoxin-sterol interactions, see section on constitutive defenses under Predator-prey interactions). Upon host death, toxin release from cells was rapid, implying that the use of parasitic dinoflagellates to mitigate bloom toxicity would not be an effective control strategy (Bai *et al.* 2007). Recently, the molecular structures of karlotoxin-1 (**4**) and karlotoxin-2 (**5**) have been determined, although their absolute and relative stereochemistries remain unassigned (Van Wagoner *et al.* 2008).

Compared to freshwater systems, there has been a relative dearth of studies that examine chemically-mediated tracking towards potential hosts by fish parasites. A few marine studies have examined host-tracking mechanisms in copepod sea lice (Bailey *et*

al. 2006; Pino-Marambio *et al.* 2007). The sea louse *Caligus rogercresseyi*, a known parasite of salmonids, tracked to water conditioned with Atlantic salmon (*Salmo salar*) exudates over either seawater controls or exudates of the copepod predator non-host fish, *Hypsoblennius sordidus,* in a Y-maze study (Pino-Marambio *et al.* 2007). Sea lice also tracked to exudates of rainbow trout, but not to coho salmon exudates, even though both of these species are known hosts for *C. rogercresseyi* (Pino-Marambio *et al.* 2007). Unfortunately, no analysis of specific compounds was conducted.

In a more chemically-focused study, Bailey *et al.* (2006) assessed chemical cues that the sea louse *Lepeophtheirus salmonis* uses to track towards its host, Atlantic salmon (*S. salar*). Using Y-maze behavioral studies, *L. salmonis* larvae tracked towards salmon-conditioned water, lipophilic extracts of salmon-conditioned water, and two purified compounds identified from salmon solid-phase extraction eluates (6-methyl-5-hepten-2-one (**6**) and isophorone (**7**)). Responses to **7** were dose-dependent with maximal responses between 0.01-0.1 mg ml^{-1} (Bailey *et al.* 2006). When added to salmon-conditioned water, 2-aminoacetophenone (**8**) and 4-methylquinazoline (**9**), which were identified in non-host (*Scophthalmus maximus*) conditioned seawater extracts, prevented positive tracking to salmon-conditioned water by juvenile copepods**.** In a related study, *L. salmonis* responded to water conditioned with cubed pieces of *S. salar* flesh with stimulation of antennule neurons followed by movement in the legs and antennules (Fields *et al.* 2007). Neurons were most consistently stimulated by fractions of salmon-conditioned water containing water-soluble compounds of 1-10kDa in size (Fields *et al.* 2007). It appears that parasitic copepods can accurately recognize appropriate salmonid

hosts using specific reliable chemical cues, and that these kairomones vary substantially in molecular structure.

Allelopathy

Most recent studies on the use of inhibitory compounds in competition, referred to as allelopathy, have focused on interactions among phytoplankton (Tillmann *et al.* 2007; Prince *et al.* 2008a). Many allelopathic microalgae are also known to produce potent toxins which can have detrimental effects on vertebrates but are rarely responsible for competitive outcomes between phytoplankton. A common theme is that yet-unidentified, non-neurotoxic metabolites account for the allelopathic effects observed within phytoplankton communities. In many cases, allelopathic compounds have been neither isolated in pure form nor have their structures been elucidated. However, their presence is indicated by the growth-inhibitory nature of phytoplankton filtrates or extracellular extracts. Despite the difficulties in isolating and identifying allelopathic compounds from planktonic organisms, several recent studies have successfully described characteristics of putative allelopathic compounds, and there appears to be a high diversity of molecular structures involved in plankton allelopathy.

Tameishi *et al.* (2009) used filtrates and co-culturing approaches to determine the allelopathic effects of the dinoflagellate *Prorocentrum minimum* on the diatom *Skeletonema costatum*. In three of the four different co-culture treatments, growth of *S. costatum* was inhibited by *P. minimum* cells. Filtrates from late stage *P. minimum* cultures also inhibited the growth of *S. costatum*, and filtrates from early stages of *P. minimum* stimulated *S. costatum* growth. The authors speculate that the cell concentration

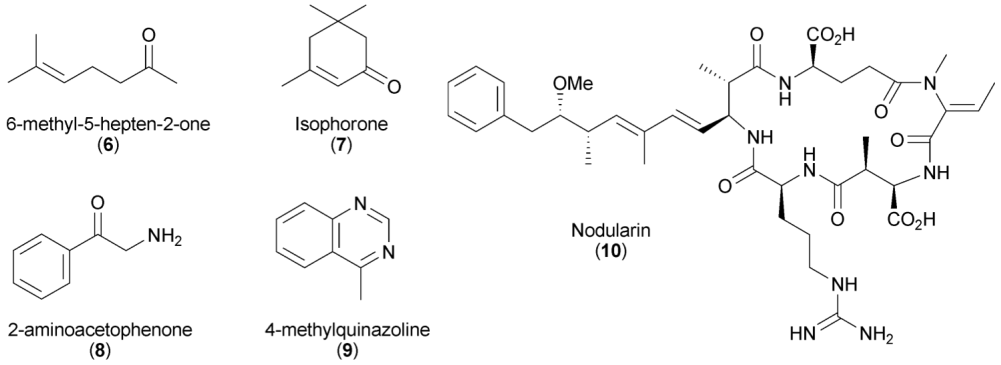

Figure 1.2. Chemical cues from marine fish: 6-methyl-5-hepten-2-one (**6**), isophoronone (**7**), 2-aminoacetophenone (**8**), and 4-methyl-quinazioline (**9**). Nodularin (**10**), a toxin produced by the cyanobacterium *Nodularia spumigena*.

of *P. minimum* was an important factor in determining the allelopathic potency of this species, but it is also possible that physiological differences between cells at different growth stages affected allelopathic compound production and/or release. An >3000 Da allelopathic fraction generated from *P. minimum* filtrate contained little protein and was heat-stable. The allelopathic fraction also contained high quantities of sugars as compared to the non-allelopathic fraction, leading the authors to speculate that *P. minimum* produces large polysaccharides that suppress growth of competitors (Tameishi *et al.* 2009).

The raphidophyte *Heterosigma akashiwo* also releases high molecular weight allelopathic compounds. Yamasaki *et al.* (2010) reported that the allelopathic effects of *H. akashiwo* on diatoms *Thalassiosira rotula* and *Skeletonema costatum* were due to allelopathic polysaccharide protein complexes > 3500 Da based on periodic acid-Schiff staining of SDS-PAGE gels of allelopathic fractions. These compounds were present in *H. akashiwo* bloom waters at concentrations that negatively impacted the growth of *S. costatum* in lab bioassays. Purified polysaccharide-protein complexes adsorbed to the cell surface of susceptible competitor cells, including *H. akashiwo*, *T. rotula* and *S. costatum* cells in the lab, but not to *P.minimum* cells, which appear to be resistant to *H. akashiwo* allelopathy. This suggests that a signaling pathway is initiated by the binding of allelelopathic compounds to specific target receptors on the surface of sensitive cells (Yamasaki *et al.* 2010).

Alexandrium dinoflagellates appear to be broadly allelopathic (see below); however, specific allelopathic compounds have yet to be identified from any

Alexandrium species. Ma *et al.* (2009) partially characterized a suite of high molecular weight compounds (ranging from 5000-500,000 Da) that are likely to be responsible for observed allelopathic effects of *Alexandrium tamarense* on *Rhodomonas salina*. There are two groups of lytic compounds; lipophilic compound(s) were responsible for approximately 98% of the observed allelopathic effects on *R. salina*, whereas more hydrophilic compounds were responsible for 2% of effects. The thermal stability of this suite of compounds would suggest that they could accumulate in seawater at high enough concentrations to affect competitors in the field (Ma *et al.* 2009).

Although there have been several recent examples of high molecular weight allelopathic compounds, in other systems low molecular weight compounds are allelopathic. Prince *et al.* (2010) partially characterized a suite of 500-1000 Da compounds exuded by the Gulf of Mexico dinoflagellate *Karenia brevis* that are allelopathic to the diatom *Asterionellopsis glacialis.* These compounds were found to be relatively polar, either neutral or positively charged, and are likely to contain aromatic functional groups. Low yields of partially purified allelopathic compounds, coupled with difficulties in maintaining allelopathic activity during purification suggests that these compounds are produced in low abundance and unstable, making their subsequent identification and structure elucidation difficult. Identification of putative allelopathic compounds was also attempted using a metabolic fingerprinting approach with extracellular extracts, but this was so far unsuccessful (Prince *et al.* 2010). Producing multiple allelopathic compounds could be advantageous, since multiple competitor species may be present at any time, and may also differ in susceptibility based upon their own physiological state.

Tang and Gobler (2010) investigated allelopathic effects of *Cochlodinium polykrikoides* against 10 competitor species in co-culture experiments. All species experienced significant mortality after 24 h exposure to live *C. polykrikoides*, with *Akashiwo sanguinea* dying within minutes. When exposed to cultures of *C. polykrikoides* separated by a 5 μm screen, all three competitors tested experienced significant mortality after 24 h. However, because filtering *C. polykrikoides* through a 5 μm filter resulted in loss of allelopathic potency, the allelopathic compounds are likely extremely short-lived and live *C. polykrikoides* is necessary for continual release of compounds. Cultures that were incubated with reactive oxygen species-scavenging enzymes peroxidase and catalase lost allelopathic activity, suggesting that the compounds responsible are reactive oxygen species (Tang & Gobler 2010). Unlike in many other study systems, the putative allelopathic compounds produced by *C. polykrikoides* appear to be non-species-specific, and may be responsible for observed ichthyotoxic effects as well as for the negative effects seen on shellfish (Tang & Gobler 2009).

In addition to progress in characterizing allelopathic compounds from phytoplankton, there have been several studies focusing on the ecological context of allelopathic interactions. A recent meta-analysis concluded that allelopathy may only be viable when allelopathic species are present at high population density, such as in established blooms that could use these compounds for bloom maintenance (Jonsson *et al.* 2009). Allelopathy is less likely to be involved in the formation of blooms, because population sizes of phytoplankton are too low to release substantial quantities of allelopathic compounds into the surrounding environment (Jonsson *et al.* 2009).

Dinoflagellates belonging to the genus *Alexandrium* continue to be studied extensively, largely because they produce saxitoxin (**11**) and related compounds that cause paralytic shellfish poisoning in humans and occasionally form large-scale harmful algal blooms (Anderson 1997). Although **11** is not allelopathic, *Alexandrium* spp. and their exudates are (Tillmann *et al.* 2007). Planktonic organisms including chlorophytes, cryptophytes, diatoms, dinoflagellates, and ciliates were each exposed to filtrates from three diverse strains of *A. ostenfeldii* originating from New Zealand, Canada, and Denmark (Tillmann *et al.* 2007). Responses to filtrates included cell lysis, cell elongation, cyst formation, reduced motility, and temporary immobilization with effects depending on the strain of *A. ostenfeldii* to which competitors were exposed (Tillmann *et al.* 2007). Although the allelopathic compounds employed by *A. ostenfeldii* remain unidentified, it appears that outer cell membranes of competitor cells are a frequent target (Tillmann *et al.* 2007). The allelopathic effects were inversely correlated with target cell density, which may be due to a saturation effect dependent on the density of absorbing particles (Tillmann *et al.* 2007). These taxonomically-broad allelopathic effects may help *A. ostenfeldii* form small patches within the water column where they are locally abundant (Tillmann *et al.* 2007).

The allelopathic effects of *Alexandrium* spp. are not limited to *A. ostenfeldii,* and do not appear linked to bacteria associated with the dinoflagellate. In a study by Tillmann *et al.* (2008) cultures of six species (*A. tamarense, A. ostenfeldii, A. lustanicum, A. minutum, A. catenella,* and *A. taylori*) treated with broad-spectrum antibiotics to remove associated bacteria caused lysis of several autotrophic and heterotrophic plankton species. Filtrates of *Alexandrium* spp. treated with antibiotics were also lytic towards the

cryptophyte *Rhodomonas salina* (Tillmann *et al.* 2008). The extent of cell lysis was variable depending on the target species and *Alexandrium* species involved. All *Alexandrium* spp. showed no statistical difference in allelopathic potency whether treated with or without broad-spectrum antibiotics, suggesting that extracellular bacteria are unlikely to be involved in production of allelopathic compounds (Tillmann *et al.* 2008). Although antibiotics removed a majority (up to >99%) of associated bacteria, it is possible that intracellular bacteria may be involved in *Alexandrium* spp. allelopathy (Tillmann *et al.* 2008). Nevertheless, it appears that *Alexandrium* spp. allelopathy is common within the genus, and may play a role in bloom maintenance.

Tillmann *et al.* (2009) described the allelopathic potency of 67 different *Alexandrium tamarense* strains isolated from a single site against *Rhodomonas salina* and *Oxyrrhis marina*. Only two clones were not allelopathic even at the highest cell concentration tested, suggesting that this species is generally allelopathic. *R. salina* was consistently more sensitive than *O. marina* to all *A. tamarense* strains tested. There was also high intraspecific and within-population variability in allelopathic potency of *A. tamarense*, but despite this variation, the authors were unable to relate any of the differences in lytic activity to genetic differences among strains (Alpermann *et al.* 2009; Alpermann *et al.* 2010).

In a related study, Tillmann and Hansen (2009) tested the allelopathic effects of two strains of *Alexandrium tamarense* towards 10 competitor species. When exposed to a non-allelopathic strain of *A. tamarense*, the exponential growth rates of all target species were unaffected. However, when co-cultured with an allelopathic strain, competitor growth rates significantly decreased. Competitor mortality occurred when the allelopathic

A. tamarense cells reached a threshold concentration, which appears to be different for each target species, and may be related to competitor cell size or the total number of competitor cells present relative to *A. tamarense* (Tillmann & Hansen 2009).

Adolf *et al.* (2006) investigated how allelopathy may be a useful strategy to mixotrophic dinoflagellates, which can photosynthesize *and* consume other cells. Karlotoxin-1 (**4**) and karlotoxin-2 **(5**) from the mixotroph *Karlodinium veneficum* were isolated (Bachvaroff *et al.* 2008) and their structures recently elucidated (Van Wagoner *et al.* 2008). Partially purified karlotoxins suppressed growth rates of several raphidophytes, dinoflagellates, and the cryptophyte *Storeatula major*, although for some species, the waterborne concentrations of karlotoxins required to suppress growth (>500 ng/ml) would rarely be found around natural blooms (Adolf *et al.* 2006). These compounds may play an additional role in predator-prey interactions, and their mechanism of action may be linked to the sterol composition of competitors and grazers (see section Predator-prey interactions) (Deeds & Place 2006).

The toxic haptophyte *Prymnesium parvum* is a bloom-forming alga that is allelopathic, capable of immobilizing and lysing competitor cells, and can feed on a range of organisms from bacteria to other phytoplankton. Uronen *et al.* (2007) examined the effects of *P. parvum* filtrates on *Rhodomonas salina* and associated bacterial communities. When *R. salina* was exposed to either cultured *P. parvum* or cell-free *P. parvum* filtrates which contained associated bacteria as well as *P. parvum* exudates*, R. salina* cells were rapidly damaged or lysed, resulting in dissolved organic carbon release within 30 minutes of exposure (Uronen *et al.* 2007). Bacterial biomass increased significantly when *R. salina* was exposed to either of the aforementioned treatments,

suggesting that bacteria can take advantage of this new source of carbon (Uronen *et al.* 2007). For the mixotroph *P. parvum*, there are potential positive direct and indirect effects that arise from the use of allelopathic compounds. Directly, competitor species are removed from the water column, and indirectly, the increase in bacterial biomass creates a potential additional food source for *P. parvum* (Uronen *et al.* 2007).

The dinoflagellate *Karenia brevis* blooms frequently in the Gulf of Mexico, producing neurotoxic brevetoxins (**12-16**) that can lead to massive fish kills and sea mammal mortality(Flewelling *et al.* 2005; Fire *et al.* 2008). These compounds do not appear linked to the allelopathic success of *K. brevis*,(Kubanek *et al.* 2005) although the allelopathic mechanisms of *K. brevis* exudates have recently been investigated. Prince *et al.* (2008a) found that extracellular extracts from natural bloom samples of *K. brevis* inhibited the growth of four (*Amphora* sp., *Asterionellopsis glacialis, Prorocentrum minimum,* and *Skeletonema costatum)* out of five competitor species tested. Cell membranes appeared to be a target of *K. brevis* allelopathy: three competitors (*Akashiwo sanguinea, A. glacialis, and P. minimum*) developed cell membrane damage when exposed to extracellular extracts from *K. brevis* cultures (Prince *et al.* 2008a). All five competitors suffered inhibited photosystem II activity, used as a measure of photosynthetic efficiency. Photosystem II was inhibited by 68% in *S. costatum*, but it is unclear whether photosystem II was a target for allelochemicals or whether cellular stress led to decreased efficiency (Prince *et al.* 2008a). Other hypothesized allelopathic mechanisms, namely disruption of competitor esterase activity or production of iron-sequestering siderophores, did not appear to be mechanisms of *K. brevis* allelopathy

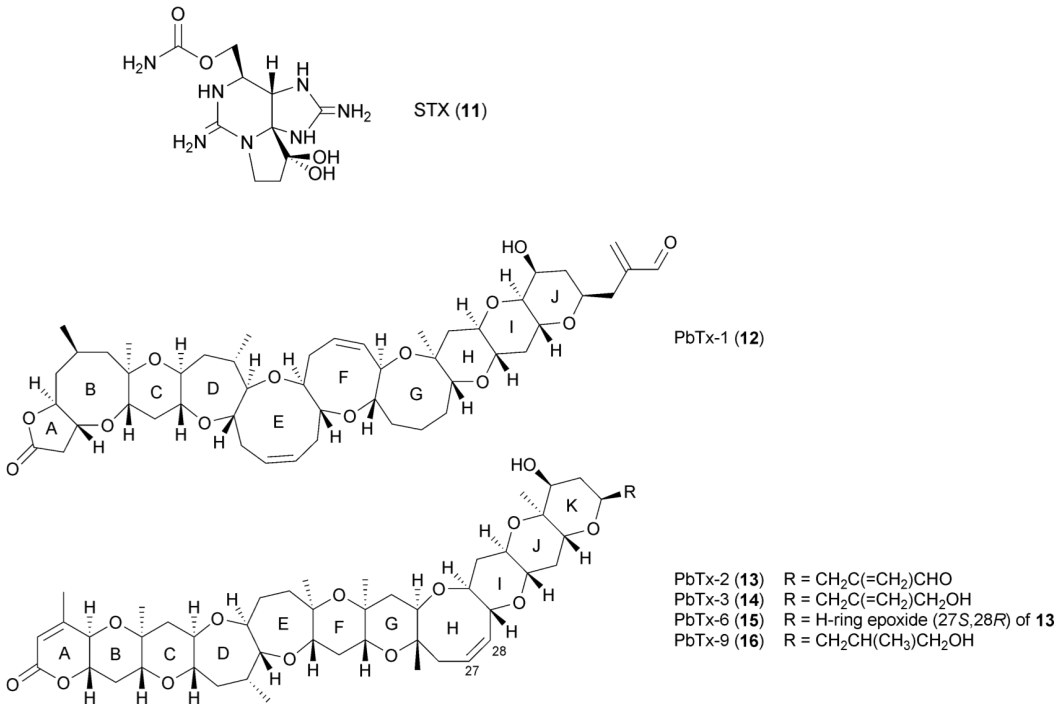

Figure 1.3: Chemical structure the neurotoxin saxitoxin (STX) (**11**), produced by dinoflagellates from the genus *Alexandrium*. Chemical structures of neurotoxic brevetoxins, PbTx-1 (**12**), PbTx-2 (**13**), PbTx-3 (**14**), PbTx-6 (**15**), and PbTx-9 (**16**) produced by the dinoflagellate *Karenia brevis*.

(Prince *et al.* 2008a). Allelopathic potency was variable between culture extracts, possibly due to small differences in growth stage, culture pH, or nutrient limitation.

Some *Karenia brevis* competitors appear to employ strategies to undermine the allelopathic effects of *K. brevis,* which could account for variability from year to year in allelopathic potency of bloom samples (Prince *et al.* 2008b). The diatom *Skeletonema costatum* is one competitor species that may possess such a strategy (Prince *et al.* 2008b). Extracellular extracts of *K. brevis* bloom samples that were co-cultured with *S. costatum* were significantly less allelopathic than extracts from *K. brevis* blooms not exposed to live *S. costatum*. Undermining of allelopathy could be due to *S. costatum* interrupting the biosynthesis or exudation of allelochemicals, metabolizing allelochemicals, or producing compounds that counteract *K. brevis* allelochemicals (Prince *et al.* 2008b). Since it is a superior exploitation competitor, *S. costatum* may also prevent *K. brevis* from acquiring the resources necessary to produce allelochemicals. In laboratory co-culturing experiments, only two of ten phytoplankton species, the diatoms *S. costatum* and *A. glacialis,* reduced *K. brevis* allelopathic potency, suggesting that the ability to undermine *K. brevis* allelopathy is relatively rare within the Gulf of Mexico phytoplankton community (Prince *et al.* 2008b). It is also possible that *S. costatum* produces allelochemicals of its own as a competitive strategy. Yamasaki *et al.* (2007) found that filtrates of bacteria-free cultures of *S. costatum* decreased the growth of its competitors *Heterosigma akashiwo* and *Chaetoceros muelleri.* The resistance of competitors to allelopathic species is likely to be a profitable focus of future research.

Allelopathic potency of *K. brevis* also depends on the identity, cell concentration, and physiological state of competitor species (Poulson *et al.* 2010). Four competitors were differentially sensitive to multiple compounds produced by *K. brevis*, but allelopathic effects were dampened when natural phytoplankton assemblages were exposed to allelopathic *K. brevis* exudates. The presence of *Skeletonema* spp. in the plankton community could explain the lack of allelopathy, as members of this genus have been shown to undermine *K. brevis* allelopathy (Prince *et al.* 2008b). Co-cultures of *S. grethae* and *Asterionellopsis glacialis* responded differently to allelopathic extracts than did monocultures of these species, further indicating that competitors might affect each other's responses to allelopathy from a third party. When *S. grethae* from different growth stages (but at the same cell concentration) was exposed to allelopathic *K. brevis* exudates, *S. grethae* was only significantly inhibited when in lag (early-growth) phase, suggesting that different growth stages of cells in the field are not equally susceptible to *K. brevis* allelopathy. The physiological state of competitor cells therefore plays a large role in determining the potency of *K. brevis* allelopathic compounds (Poulson *et al.* 2010). Although pair-wise studies are informative to characterize plankton allelopathy, it is difficult to extrapolate simple, lab-based studies to what occurs in the field due to the complexity of natural systems.

There have been many studies on the allelopathic effects of freshwater cyanobacteria, but studies involving marine cyanobacteria are somewhat rarer. Nodularin (**10**) is a potent toxin produced by the brackish cyanobacterium *Nodularia spumigena* that promotes liver tumor formation (Ohta *et al.* 1994), and can also bioaccumulate in birds (Sipia *et al.* 2008), zooplankton (Karjalainen *et al.* 2006), and fish (Sipia *et al.* 2006;

Karjalainen *et al.* 2008). The allelopathic effects of toxic *N. spumigena* and non-toxic *Aphanizomenon flos-aquae,* both from the Baltic Sea, were compared using cell-free filtrates of each cyanobacterium (Suikkanen *et al.* 2006). Interestingly, non-toxic *A. flos-aquae* was inhibitory towards the cryptophyte *Rhodomonas* sp., reducing competitor cell numbers by 29% and cellular chlorophyll-a content by 34%. In contrast, toxic *N. spumigena* only reduced competitor cell numbers by 14% and cellular chlorophyll-a content by 12% (Suikkanen *et al.* 2006). Pure **10** added to *Rhodomonas* sp. cultures did not cause a significant change in any *Rhodomonas* growth parameters, suggesting that metabolites other than **10** are responsible for the mild allelopathic effects observed for *N. spumigena* (Suikkanen *et al.* 2006). Alternatively, some of its competitive dominance may be caused by *N. spumigena* having a higher pH tolerance compared to competitor species (Mogelhoj *et al.* 2006). In co-culturing experiments, pH in cultures increased during growth, which could explain eventual *N. spumigena* competitive success (Mogelhoj *et al.* 2006).

Polyunsaturated aldehydes (PUAs) are implicated in allelopathic interactions in Norwegian waters. Two of the dominant phytoplankton in these waters are the haptophyte *Phaeocystis pouchetii* and the diatom *Skeletonema costatum*, both of which can release 2*E*,4*E*-decadienal (**1**) (Hansen & Eilertsen 2007). Three diatom species (*S. costatum, Chaetoceros socialis,* and *Thalassiosira antarctica)* cultured from Austnesfjorden, Norway, were grown with commercially-purchased **1**, and suffered decreased growth in a concentration-dependant manner (Hansen & Eilertsen 2007). However, in field samples, higher *P. pouchetii* densities correlated with higher diatom diversity. Since both *P. pouchetti* and *S. costatum* were frequently the two most common

phytoplankton species in field samples and both can produce **1**, the authors speculated that these species may dominate the community by being somewhat resistant to the effects of **1** at natural bloom concentrations (Hansen & Eilertsen 2007).

In another study, three commercially-purchased PUAs (**1**-**3**) caused a concentration-dependent decrease in the growth rates of six taxonomically diverse phytoplankton species (*Skeletonema marinoi, Dunaliella tertiolecta, Isochrysis galbana, Amphidinium carterae, Tetraselmis suecica* and *Micromonas pusilla*), although the six species did not respond identically to all three compounds (Ribalet *et al.* 2007a). These compounds also disrupted target cell membranes, and degraded cellular chlorophyll. The PUA with the longest alkyl chain (**1**) stunted cellular growth rates more than the other two compounds. Interestingly, one of the target species, the diatom *S. marinoi,* itself produces **2** and **3**, (Wichard *et al.* 2005) and was affected less by these compounds than other target species, suggesting that this diatom is partially resistant to compounds it produces (Ribalet *et al.* 2007a). Often, phytoplankton species that were more susceptible to PUAs were smaller in size and had less-developed cell walls and high lipid content (Ribalet *et al.* 2007a). Diatom cell physiological state can also influence the production of and response to PUAs (Ribalet *et al.* 2007b). Total PUA production was maximized in the diatom *Skeletonema marinoi* when cells were nutrient-limited and in stationary phase. PUA concentrations increased more than three-fold from exponential growth phase to stationary phase if cells were damaged (Ribalet *et al.* 2007b).

Predator-prey interactions

Constitutive defenses

Some secondary metabolites produced by phytoplankton act as constitutive anti-grazer defenses, being produced more or less constantly. While many studies have focused on demonstrating the direct physiological effects of phytoplankton toxicity on consumer species, indirect effects including altering consumer behavior have also been investigated. Behavioral changes such as decreased feeding rates can translate into reduced reproductive success. Exposure to the toxic phytoplankton *Prymnesium parvum* can cause inactivity in the copepods *Eurytemora affinis* and *Acartia bifflosa*, without the copepods actually consuming *P. parvum*, resulting in reduced copepod reproductive success (Sopanen *et al.* 2006). Sopanen *et al.* (2008) found that cell-free filtrates of *P. parvum* also negatively impacted copepod survivability, suggesting that the anti-grazer impacts of *P. parvum* on *E. affinis* are chemically-based. Mixed diets containing *P. parvum* and non-toxic *Rhodomonas salina* reduced copepod feeding activity, but the diets were not as detrimental to copepod health as were *P. parvum* filtrates (Sopanen *et al.* 2008). Although only demonstrated in lab-based studies, impacts of toxic phytoplankton species on copepod grazer behavior may have large implications for copepod population dynamics and reproductive ecology in the field.

Herbivores whose ancestors were exposed to chemically-defended prey may respond differently to chemical defenses than grazers that have a limited shared history with the prey species. Florida estuarine rotifers (*Brachionus ibericus*) were willing to feed on the Florida red tide dinoflagellate *Karenia brevis* in a mixed diet, whereas rotifers

from a Russian inland sea, *B. manjavacas* (ex *B. plicatilus)*, refused *K. brevis* in an identical mixed diet (Kubanek *et al.* 2007). However, Russian *B. manjavacas* fed on mixtures containing *K. brevis* four days into the experiment, indicating an eventual acclimation to the *K. brevis* feeding deterrent. Brevetoxins (PbTx-2 (**13),** -3 (**14**) and -9 (**16**)) were not responsible for the observed effects, but the deterrence has a chemical basis, since lipophilic fractions from *K. brevis* cells were deterrent when coated onto dried yeast particles at natural concentrations. However, the deterrent compound(s) were found to be labile, and thus remain unidentified.

A recent study with the copepod *Acartia tonsa* demonstrated that negative effects of *K. brevis* on copepod egg production and survivability were not due to a chemical deterrent, but were likely caused by the nutritional inadequacy of *K. brevis* as a food source (Prince *et al.* 2006; Speekmann *et al.* 2006). Copepods attempted to compensate for the nutritional inadequacy of *K. brevis*: the highest ingestion rates were observed for copepods fed solely *K. brevis*, but these copepods suffered low survivorship and low egg production rates. Egg production rates were not significantly different between starved copepods and those fed *K. brevis*, indicating that *K. brevis* is not chemically-defended from copepod grazers, but that *K. brevis* is a nutritionally inadequate food source (Prince *et al.* 2006). Speekmann *et al.* (2006) also concluded that *K. brevis* is a low quality food item for *A. tonsa.* When *A. tonsa* was fed sole or mixed diets containing *K. brevis* and the non-toxic dinoflagellate *Peridinium foliaceum*, egg production rates were significantly higher for copepods that were fed *P. foliaceum* versus copepods fed solely *K. brevis* (Speekmann *et al.* 2006). These results, coupled with those of Kubanek *et al.*, (2007)

suggest that *K. brevis* is not chemically-defended against all important grazers, but is still a poor food for zooplankton.

The potential role of domoic acid (**17**) as an anti-grazer defense produced by diatoms of the genus *Pseudo-nitzschia* has recently been examined. Bargu *et al.* (2006) found that krill exposed to abnormally high concentrations of dissolved **17** fed significantly less on a non-toxic food source than krill unexposed to **17**. However, other studies have suggested that *Pseudo-nitzschia* spp. are not chemically-defended against copepods. Olson *et al.* (2006) found that copepod grazing impacts on field populations of *Pseudo-nitzschia* sp. were negligible but this lack of grazing was not attributed to **17**. In a similar field-based study, Olson *et al.* (2008) found no correlation between low grazing rates and particulate or dissolved **17** concentrations in field samples. Due to the lack of recent experimental studies directly testing the impact of **17** on plankton consumers using ecologically-relevant concentrations and exposure methods, little can be concluded about the putative anti-predatory role of this toxin.

Zooplankton grazing on toxic phytoplankton may be affected by prey cell concentrations. Grazing on okadaic acid-producing *Dinophysis* spp. by the copepods *Temora longicornis* and *Centrophages typicus* only occurred when cell densities of *Dinophysis* spp. were low or when other food items were present (Kozlowsky-Suzuki *et al.* 2006). No field-collected copepods positively selected for *Dinophysis* spp. at any cell density, although copepod feeding rates on *Dinophysis* spp. increased when offered a mixture of phytoplankton. Chemical defenses of this genus may prevent copepods from controlling blooms by grazing, except in situations where *Dinophysis* spp. is present at low concentrations. However, in natural systems the spatial distributions of especially

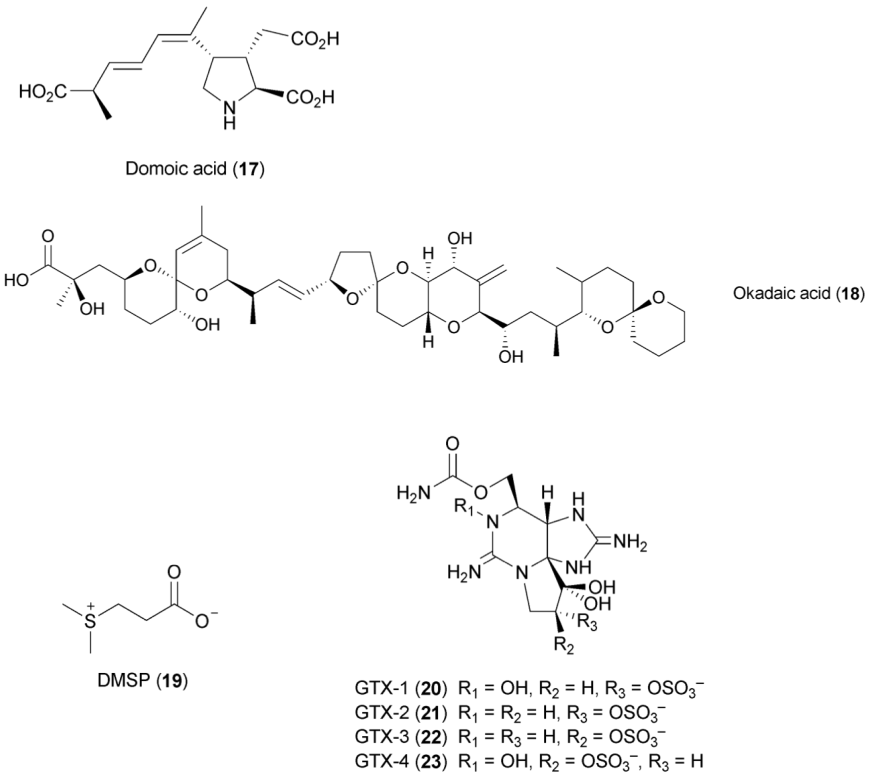

Figure 1.4. Algal metabolites involved in predator-prey interactions. Domoic acid (**17**), produced by the diatoms of the genus *Pseudo-nitzschia,* Okadaic acid (**18**), produced by dinoflagellates of the genus Dinophysis. Dimethylsulfoniopropionate (DMSP) (**19**), which is produced by many phytoplankton species. The gonyautoxins, GTX-1 (**20**), GTX-2 (**21**), GTX-3 (**22**), and GTX-4 (**23**), which are inducible defenses in the dinoflagellate *Alexandrium minutum.*

toxic cells may vary (Lindahl *et al.* 2007) which should be considered when interpreting results from feeding studies. Regardless of its uncertain capacity to serve as an anti-grazer defense, low abundance of okadaic acid (**18**) were also detected in copepods after feeding on *Dinophysis* spp., suggesting that copepods can act as vectors to transfer **18** to higher trophic levels (Kozlowsky-Suzuki *et al.* 2006).

Phytoplankton may also employ chemical defenses for protection against microzooplankton grazers. Lab grazing rates on a non-toxic strain of *Karlodinium veneficum* or the non-toxic cyptophyte *Storeatula major* were double that of grazing on toxic *K. veneficum* strain. The addition of partially purified, waterborne karlotoxins (e.g., **4-5**) from *K. veneficum* reduced grazing pressure by the heterotrophic dinoflagellate *Oxyrrhis marina* on non-toxic *K. veneficum* and *S. major* (Adolf *et al.* 2007). However, since 90% of karlotoxins are cell-bound, the effects of waterborne karlotoxins may not adequately simulate the route of exposure of the toxin to grazers. *O. marina* consumed less non-toxic *K. veneficum* cells when part of a mixed diet containing toxic *K. veneficum* than when offered non-toxic monocultures, suggesting that *K. veneficum* toxins affect *O. marina* feeding behavior. However, this does not exclude the possibility that other, uncharacterized compounds are involved in deterrence. Toxins produced by *K. veneficum* also appear to be allelopathic (see section 4) and may aid *K. veneficum* in prey capture (see section 5.3)(Adolf *et al.* 2006). In a related study, feeding by the copepod *Acartia tonsa* was inversely related to the proportion of toxic *K. veneficum* cells in mixed diets (Waggett *et al.* 2008). Copepod mortality was not influenced by the consumption of toxic

cells, suggesting that *K. veneficum* defenses may deter *A. tonsa* from feeding without killing the copepod.

Susceptibility of *Karlodinium veneficum* grazers to karlotoxins may be related to the sterol composition of grazer cell membranes, with grazers whose membranes are rich in ergosterol and cholesterol hypothesized to be more susceptible to toxicity (Deeds & Place 2006). Fish erythrocytes incubated with dissolved ergosterol or cholesterol were less susceptible to hemolysis by partially purified karlotoxins compared with erythrocytes incubated with gymnodinosterol, suggesting that cholesterol and ergosterol are target molecules for karlotoxin activity (Deeds & Place 2006). Interestingly, gymnodinosterol was found to be the major steroid component of *K. veneficum* cell membranes, which may account for the resistance of *K. veneficum* to its own toxins. The high cholesterol composition of *Oxyrrhis marina* cell membranes may make this grazer vulnerable to cell lysis when exposed to karlotoxins (Deeds & Place 2006). However, the mechanism by which karlotoxins interact with cholesterol or ergosterol is not known.

Lewitus *et al.* (2006) studied how the toxicity of the mixotrophic dinoflagellate *Pfiesteria piscicida* affects trophic interactions. Toxic, moderately toxic, and non-toxic strains of *P. piscicida* were incubated with *Rhodomonas* sp., to determine the impact of *Pfiesteria* strain toxicity on prey consumption. Toxic *P. piscicida* grazed on *Rhodomonas* sp. significantly less than did either the moderately toxic or non-toxic strains, suggesting that more toxic strains are less mixotrophic (Lewitus *et al.* 2006). Sequestration of prey chloroplasts was observed for the less toxic strains. When the three strains of *P. piscicida* were exposed to the ciliate predators *Euplotes woodruffi* and *E. vannus*, the toxic strains were consumed significantly less than the other two strains, leading to the hypothesis that

toxin production may be act as an anti-grazer defense (Lewitus *et al.* 2006). However, toxins from *Pfiesteria* spp. have still not been fully characterized, despite more than a decade of effort by several groups. Moeller *et al.* (2007) reported multiple metal-containing organic toxins from *P. piscicida*, but their complete molecular structures could not be determined due to instability.

Previous studies have shown that dimethylsulfoniopropionate (DMSP; **19**) is an effective defense against microzooplankton grazers, although it does not appear to function as a toxin but rather as a signal to grazers (Strom *et al.* 2003; Fredrickson & Strom 2009). Adding **19** to natural Gulf of Alaska and Puget Sound protist assemblages decreased feeding rates by 28-75% in lab experiments (Fredrickson & Strom 2009). However, **19** reduced feeding rates in only four of 17 field microcosm experiments. These opposing effects were attributed to community microzooplankton acclimating to **19** in microcosm studies due to the longer duration of these studies compared to lab experiments or to grazing inhibition masked by stimulatory effects of **19** on community members not present in the lab-based study (Fredrickson & Strom 2009). DMSP (**19**) appears to have multiple roles as a signaling molecule that can stimulate and inhibit grazing in a concentration-dependent manner, similar to the response of microzooplankton to amino acids (Strom *et al.* 2007). In a related study, addition of valine, cysteine, proline, or serine to cultures of the tintinnid *Favella* sp. reduced feeding rates by 80% relative to controls. Amino acids may make reliable signaling molecules for several reasons: long term exposure to amino acids had no impact on ciliate growth or mortality; low concentrations of amino acids were needed to induce a response; and the observed effects were reversible once the signal was removed. Amino acids with smaller

side chains were also more inhibitory than those with longer side chains, suggesting some chemical specificity of the behavioral response (Strom *et al.* 2007).

Activated defenses

Diatoms are known to produce a variety of chemical defenses that are activated after cellular damage. Ultimately, multiple pathways are used to create compounds from the oxidation of membrane lipids, some of which appear function as anti-predatory defenses (Fontana *et al.* 2007). These compounds include oxypilins, fatty acid hydroperoxides, and polyunsaturated aldehydes (PUAs), some of which act as teratogens interfering with copepod reproduction and development (Miralto *et al.* 1999). Precursor molecules to these anti-predatory compounds are stored by many diatoms as polyunsaturated fatty acids that are enzymatically converted following cell damage defenses (Fontana *et al.* 2007). Egg production and hatching success of the co-occurring copepods *Acartia clausi*, *Calanus helgolandicus*, and *Temora longicornis* were negatively affected after feeding on field-collected *Cerataulina pelagica*, although PUAs were not detected in any samples (Ianora *et al.* 2008). However, when surface seawater samples were re-analyzed for fatty acid derivatives, hydroxyl and keto derivatives of the PUA precursor molecules eicosapentoic acid and docosahexaenoic acid were detected, providing evidence of oxylipins other than PUAs in diatom-dominated field samples (Ianora *et al.* 2008). Low fecundity was also reported for all three copepod species, although egg viability was high. *In situ* fecal pellet production was low, indicating that copepods ate less during a bloom, which may account for low copepod fecundity (Ianora *et al.* 2008). The compounds present during a *C. pelagica* bloom were only partially

characterized, and so it is possible that there were multiple active anti-grazer compounds present in this study.

PUAs may also undermine the nutritional quality of diatoms as food items for copepods. The enzymes that convert fatty acids into PUAs can reduce the nutritional quality of diatom exudates, which in turn may prevent efficient assimilation of diatom fatty acids into copepod tissue. Wichard *et al.* (2007) linked cellular fatty acid depletion with the formation of PUAs in disrupted diatom cells. Diatom enzymes remained active in the foregut of the copepod *C. helgolandicus* indicating that the nutritional quality of diatoms may continue to decline as copepods consume them. Diatom diets supplemented with fatty acids increased the amount of PUAs produced by creating more substrate for the diatom enzymes to convert to PUAs. Because egg hatching success of the copepod *Temora longicornis* was still high despite the increase in PUAs, these compounds may not be directly toxic to this copepod, but may instead reduce the nutritional quality of diatom prey (Wichard *et al.* 2007).

Despite a number of studies concluding that PUAs are responsible for reduced copepod reproductive success, several other studies have rejected this hypothesis. Poulet *et al.* (2006) observed significant decreases in copepod egg production rates within two to three days after incubating *Calanus helgolandicus* females with mixed, natural assemblages of diatoms. This trend was reversible with a change in diet, and no correlation between the presence of diatoms known to produce PUAs and egg production rates was found (Poulet *et al.* 2006). The negative effects on copepod reproduction were attributed to nutritional deficiencies or other unidentified anti-grazer compounds produced by the diatoms, although PUAs were not directly measured (Poulet *et al.* 2006).

In a companion study, Wichard *et al.* (2008) found no correlation between field measurements of PUAs and copepod reproductive parameters, including egg production rates, hatching success, and abnormal larvae development.

The effects of several diatoms and a chryptophyte on the reproductive success of the copepod *Temora longicornis* has also been investigated (Dutz *et al.* 2008). Every diatom tested negatively affected copepod reproduction. Concentrations of total PUAs and polyunsaturated fatty acids, as well as the concentrations of other PUA precursor molecules and sterols, were determined for each prey species. Often, copepods had high fecundity when feeding on PUA-rich diatoms, whereas the fecundity of copepods feeding on PUA-deficient diatoms was low (Dutz *et al.* 2008). Interestingly, the most fertile copepods that consumed non-PUA producing diatoms also experienced the largest reduction in egg hatching success. Reductions in copepod reproductive success were not attributed to nutritional deficiencies of lipids in diatom food nor to PUAs, although the reductions could have resulted from a lack of vitamins and proteins or presence of other deterrent compounds (Dutz *et al.* 2008). Similarly, PUAs from pelagic diatoms appeared to have limited impacts on the benthic copepod *Tisbe holothuriae*, with no observed effects of PUAs on the reproductive success and larval survival (Taylor *et al.* 2007). The importance of PUAs as anti-grazing compounds and their impacts on copepod reproduction clearly cannot be generalized and remain an active area of investigation. Although several studies suggest that PUAs are not involved in diatom-copepod interactions, several of these do not directly measure PUAs. The physiological state (Ribalet *et al.* 2007b) of the diatom or unknown feeding deterrents that are also derived

from the lipid peroxidation pathway may cause some of the detrimental effects on copepods (Fontana *et al.* 2007; Ianora *et al.* 2008).

Induced defenses

Induction of chemical defenses in the presence of grazers has been observed for a few toxic phytoplankton species. Concentrations of cellular gonyautoxins (GTX 1-4; **20-23**) significantly increased in the dinoflagellate *Alexandrium minutum* when co-cultured with the copepod *Acartia tonsa*, compared to *A. minutum* not exposed to copepods (Selander *et al.* 2006). Concentrations of **20-23** increased more in dinoflagellate cells exposed to higher densities of copepods, and to actively-feeding rather than starved copepods (Selander *et al.* 2006). In choice feeding assays, induced *A. minutum* cells were consumed less than non-induced *A. minutum* cells using non-toxic *Prorocentrum micans* as a control food. Because the alternative prey (*P. micans*) was consumed at equal rates when mixed with either induced or non-induced *A. minutum*, it is likely that the copepods rejected induced *A. minutum* and instead consumed the non-toxic control plankton, as opposed to being incapacitated by toxic cells.

The induction of *A. minutum* chemical defenses appears to vary based upon exposure to species-specific consumer cues (Bergkvist *et al.* 2008). Two strains of *A. minutum* (strains no. 83 and CNR A5) were exposed to waterborne cues from the copepods *Acartia clausi*, *Centropages typicus,* and *Pseudocalanus* sp. When *C. typicus* adults were caged away from phytoplankton cells to expose *A. minutum* to predator cues without the risk of consumption, GTX concentrations increased five-fold in strain no. 83 compared to no-copepod controls, whereas for strain CNR A5 GTX concentrations

increased twenty-fold (Bergkvist *et al.* 2008). When exposed to *A. clausi* exudates, only one *A. minutum* strain displayed increased GTX concentrations, and neither strain responded with increased GTXs when exposed to waterborne cues of *Pseudocalanus* sp. Bergkvist *et al.* (2008) offered several hypotheses for the variable induction of GTX production in *A. minutum* when exposed to different grazers. First, *Pseudocalanus* sp. may pose less of a threat to *A. minutum* than do other copepod species, since it is a filter-feeder that cannot efficiently capture large particles like *A. minutum*; thus, *A. minutum* would not strongly benefit from inducing chemical defenses against *Pseudocalanus* sp. It is likely that the induction of anti-grazer defenses evolved due to grazing pressure from a copepod that was capable of feeding on *A. minutum*. The history of exposure to specific consumers could play a role in the induction of toxins as well. *A. minutum*, *C. typicus*, and *A. tonsa* are adapted to warmer waters, whereas *Pseudocalanus* sp. is more adapted to cold waters and rarely encounters *A. minutum* in the field. Since *Pseudocalanus* sp. fed on *A. minutum* but did not induce toxin production, it is likely that the chemical cues received by *A. minutum* came from copepod grazers, and were not alarm cues that were released from the destruction of *A. minutum* cells (Bergkvist *et al.* 2008).

Selander *et al.* (2008) investigated induced GTX production in the same *A. minutum* strains as Bergkvist *et al.* (2008) under nutrient-limiting conditions. Both predator presence and high nitrate concentrations led to increased cellular GTX content. These results support the Carbon-Nutrient Balance Hypothesis (Bryant *et al.* 1983) because paralytic shellfish toxins including **20-23** are alkaloids with a low C:N ratio of ~1.4, which should favor production of nitrogen-rich metabolites when surplus nitrogen is available. The results also support the Optimal Defense Model (Rhoades 1979), since

defenses increase when needed, i.e., in the presence of grazers rather than being wasted in the absence of danger (Selander *et al.* 2008). The authors cautioned that direct demonstrations of paralytic shellfish toxins functioning as anti-grazer defenses have not been demonstrated to date, and the above studies only provide correlative evidence of their function as anti-grazer defenses.

The effects of other *A. minutum* toxins on copepod feeding behavior and reproductive success have also been investigated by Barreiro *et al.* (2006). When a toxic and a non-toxic strain of *A. minutum* were each fed to the copepod *A. clausi*, feeding rates and total consumption of the toxic strain were significantly lower than for non-toxic *A. minutum* controls. Mortality of copepods fed mixed diets was intermediate compared to those fed either toxic or non-toxic strains. Since there was no significant difference in toxin accumulation in copepods fed toxic versus mixed strain treatments, it is likely that the observed intermediate mortality levels were a result of an amelioration effect of non-toxic food within the mixed diet (Barreiro *et al.* 2006). Copepod egg production was suppressed on the toxic diet, whereas egg production rates were similar between non-toxic and mixed diets. Favorable egg production rates in mixed culture treatments may have also been due to an amelioration effect. Alternatively, the low ingestion rates measured for copepods feeding on a toxic diet may have directly led to lower egg production rates (Barreiro *et al.* 2006). Egg hatching success was negatively affected by the toxic diet, and hatching success of the mixed and toxic treatments was not significantly different. Phytoplankton community composition may also determine consumption of toxic species: ingestion and clearance rates by *A. clausi* depended on the presence of other, non-toxic phytoplankton in the community (Barreiro *et al.* 2007).

Alternatively, Estrada *et al.* (2008) found no evidence suggesting that related toxins produced by *Alexandrium catenella* function as anti-grazing defenses in a microcosm study. The authors suggested several hypotheses to explain why negative effects of *A. catenella* may have been missed: concentrations of *A. catenella* cells were lower compared to other studies; toxin concentrations may also have been too low to affect copepods in the microcosm; and the dominant toxins detected in the microcosm were the least potent of the paralytic shellfish toxin suite (Estrada *et al.* 2008).

Ingestion of *Alexandrium* spp. by copepods may depend on cell concentrations as well as toxin composition and concentration (Teegarden *et al.* 2008). When three different *Alexandrium* strains of varying toxicities (high, intermediate, and non-toxic) were offered in mixed diets to four different copepod species, three copepod species did not appear to differentiate between cells of intermediate and high toxicity, but both cell types were consumed less than the non-toxic strain at low cell densities (Teegarden *et al.* 2008). This suggests that these copepods responded to the presence or absence of a toxin, without differentiating between cellular toxin concentrations when overall phytoplankton cell densities were low (Teegarden *et al.* 2008). Moreover, when the overall concentration of *Alexandrium* cells increased, copepods could no longer select for non-toxic prey and instead decreased their overall phytoplankton consumption. At high cell densities of *Alexandrium*, some copepods are likely to reduce overall consumption of phytoplankton rather than only reducing the consumption of toxic cells; therefore grazer biomass may be more important in *Alexandrium* bloom dynamics than the specific response of grazers to phytoplankton toxicity (Teegarden *et al.* 2008).

The effects of microalgal toxins on grazers may also act as selective agents influencing the population genetics and evolution of grazer species (Bricelj *et al.* 2005). Connell *et al.* (2007) found that multiple mutations arising in soft clam *Mya arenaria* populations can confer resistance to saxitoxin (**11**) produced by *Alexandrium* spp. Four resistant genotypes for the saxitoxin-binding sodium channel were found in a survey of *M. arenaria* populations from areas historically known to experience *Alexandrium* spp. blooms, whereas clams from non-bloom areas were typically sensitive to intoxication. An intermediate level of saxitoxin resistance was measured for certain heterozygous genotypes in *in vitro* nerve trunk assays (Connell *et al.* 2007). Sensitive versus resistant phenotypes displayed differences in burrowing and feeding capabilities, toxin accumulation, and survivability, indicating a fitness advantage to resistant phenotypes when exposed to *Alexandrium* toxins (MacQuarrie & Bricelj 2008). The rate of selective pressure that toxins impose on clam populations, whether this selective pressure is variable within clam populations, and the fitness costs for saxitoxin-resistance remain unknown and topics for future study (Connell *et al.* 2007).

Paralytic shellfish toxins may not account for all observed negative effects on susceptible bivalve populations. Extracts of toxic *Alexandrium tamarense* cultures did not affect the immune responses of the clams *Mya arenaria* and *Ruditapes philippinarum*, whereas non-toxic *A. tamarense* extracts negatively impacted hemocyte activity in these clams, which indicates that bioactive compounds other than paralytic shellfish toxins can cause detrimental effects on exposed bivalve populations (Ford *et al.* 2008). The compound(s) responsible remain to be identified.

Chemical cues from a grazer can also induce morphological defenses in phytoplankton. *Phaeocystis globosa* can change its morphology in response to different grazer cues, switching between colonies and single cells in order to defend itself from grazing (Long *et al.* 2007). To avoid predation pressure from larger copepods, *P. globosa* remains as single cells that are too small to be preferred prey. To avoid smaller grazers such as ciliates, the colony morph is advantageous, because it is too big to be consumed by these grazers. *P. globosa* colony formation was suppressed by 70-75% when exposed to chemical cues from a natural copepod dominated mesozooplankton assemblage or from the copepod *Acartia tonsa* feeding on *P. globosa*, although the average number of cells per colony did not change (Long *et al.* 2007). Conversely, cues from the grazing ciliate *Euplotes* sp. stimulated a 25% increase in colony formation in *P. globosa* compared to unexposed controls (Long *et al.* 2007). *P. globosa* can therefore change its morphology to avoid predation by chemically assessing local predation threats. The Antarctic haptophyte *Phaeocystis antarctica* is also capable of inducing morphological defenses in response to grazer cues from a natural mesozooplankton assemblage (Tang *et al.* 2008). Grazer cues were less than 12 kDa in size, based upon diffusion through dialysis membrane (Tang *et al.* 2008). Although specific waterborne chemical cues from ciliates and copepods appear to be responsible for the observed induced morphological changes, these compounds have not yet been identified (Tang *et al.* 2008).

Chemical cues can induce both morphological and behavioral changes in zooplankton. The shell morphology of planktonic larvae of the intertidal snail *Littorina scutulata* changes in response to chemical cues from consumers (zoea larvae of *Cancer* spp.) and from snail larvae consumed by *Cancer* spp. larvae (Vaughn 2007). Snail larvae

exposed to predator exudates had significantly rounder shells and smaller apertures than those not exposed to predator cues, which coincided with significantly higher survival rates compared to unexposed larvae (Vaughn 2007). This is a rare example of morphological defenses in marine zooplankton, despite vast numbers of studies on this topic in freshwater systems. Chemical cues from caged predators can also induce behavioral changes in marine zooplankton (Metaxas & Burdett-Coutts 2006). Urchin (*Strongylocentrotus droebachiensis*) larvae swam at lower average depths when a caged predator (the ctenophore *Bolinopsis infundibulum*) was introduced to the top of a water column, compared with larvae that were not exposed to a predator, suggesting that swimming depth choice may represent an escape response by urchins (Metaxas & Burdett-Coutts 2006). In contrast, oyster (*Ostrea edulis)* larvae did not significantly change swimming depth compared to controls. Behavioral changes in response to a potential chemical cue may be important in minimizing predation upon the pelagic larvae of marine benthic invertebrates (Metaxas & Burdett-Coutts 2006).

Prey Tracking and Recognition

Protozoans can detect and track towards bacterial prey using chemical cues, including cell surface carbohydrates and amino acids. Mohapatra and Fukami (2007) investigated heterotrophic nanoflagellate migration into capillary tubes containing three different marine bacterial species or cellular surface extracts containing bacterial surface compounds and compared both treatments to aged seawater and 0.5 M sodium chloride controls. The highest positive chemotactic response to both surface chemistry extracts and whole cells was measured for the bacterium *Pseudomonas* sp (Mohapatra & Fukami

2007). Clearance rates by heterotrophic nanoflagellates were also measured using these bacteria as prey items, and *Pseudomonas* sp. was ingested at the highest rates (Mohapatra & Fukami 2007). Prey selection by nanoflagellates is not based solely on geometry and size, but also on the surface biochemistry of prey.

Cell surface receptors of marine planktonic protozoa, specifically lectins that allow discrimination among multiple prey types according to prey surface carbohydrates, have recently been investigated. Wootton *et al.* (2007) found a calcium-dependent, mannose-binding lectin from surface protein preparations of the dinoflagellate *Oxyrrhis marina.* Mannose was detected on the surface of prey (*Isochrysis galbana*) cells, indicating that *O. marina* could use mannose-binding lectin to identify *I. galbana* as a prey item (Wootton *et al.* 2007). After mannose-binding lectin functioning was blocked in live *O. marina*, feeding on prey cells was inhibited by 60% and the predator no longer discriminated between mannose-coated beads versus control beads (Wootton *et al.* 2007). Thus, chemoreception at cell surfaces can be used by protozoa to distinguish between different prey cells.

Zooplankton can use a suite of cues found in exudates of phytoplankton to locate prey patches. The response of the predatory dinoflagellate *Oxyrrhis marina* to planktonic thin layers has been observed using lab-generated thin layers containing either live prey (*Isochrysis galbana)* or filtrates from *I. galbana* (Menden-Deuer & Grunbaum 2006). After the introduction of prey to thin layers, more *O. marina* individuals tracked to the thin layer. Swimming speeds and turning rates of *O. marina* also increased, although these effects were less consistent when prey filtrates were added to thin layers than in the presence of live prey (Menden-Deuer & Grunbaum 2006). Since both prey filtrates and

live prey caused *O. marina* to aggregate in thin layers, chemical cues appear to be used in *O. marina* prey tracking, although these kairomones remain unidentified.

Copepods can differentially respond to a variety of physical and chemical cues to gather information about their environment (Woodson *et al.* 2007). The copepods *Temora longicornis* and *Acartia tonsa* responded to velocity gradients and phytoplankton exudates contained within thin layers by increasing swimming speed, turning rates, and residence times in these layers (Woodson *et al.* 2007). These behavioral responses may allow the copepod to effectively search for and locate food items by quickly scanning the area (Woodson *et al.* 2007). Thin layers may help the copepod maintain a desirable position in the water column, and help transport them to new locations based on velocity gradient responses. The interplay between multiple stimulatory cues is important to determine the behavior of zooplankton (Woodson *et al.* 2007).

Prey Capture and Consumption

Karlodinium veneficum can utilize karlotoxins (e.g., **4-5**) to immobilize potential prey. Since karlotoxins are at least 90% cell-associated (Adolf *et al.* 2007), the authors speculated that cell-cell contact is necessary to expose prey to karlotoxins and to immobilize prey cells, which could then be more easily captured and ingested by *K.* veneficum (Adolf *et al.* 2006). When prey cells (*Storeatula major*) were treated with 25 ng/ml mixed karlotoxins and exposed to two different *K. veneficum* strains, prey ingestion rates were significantly higher than when prey cells were not pre-treated with karlotoxins, suggesting that these compounds make prey capture easier for *K. veneficum* (Aldof *et al.* 2006).

Jellyfish are important members of the marine plankton, and recent work has investigated the toxicity of jellyfish venoms used for prey capture (Helmholz *et al.* 2008). Recently, a 27.5 kDa glycoprotein (ClGp1) that is toxic to human HepG2 cells was isolated from the oral arms (mesenteric tentacles) of the blue jellyfish *Cyanea lamarckii*(Helmholz *et al.* 2008). Up to 26.8% of this glycoprotein is composed of carbohydrate portions, and it likely includes mannose and *N*-acetylglucosamine or sialic acid side chains. It is probable that this protein represents one of many glycoproteins present in jellyfish venom (Helmholz *et al.* 2008). Toxins can also be differentially distributed between tentacle types, based upon the ecological function of the tentacle. *C. lamarkii* mesenteric tentacle extracts were seven times more hemolytic and significantly more toxic to human HepG2 cells than fishing tentacle extracts (Helmholz *et al.* 2007). A similar pattern was observed in extracts of mesenteric and fishing tentacles of the lion's mane jellyfish (*Cyanea capillata*). Higher levels of toxicity and hemolytic activity in the mesenteric tentacles indicates that oral arms contribute more to the digestion of prey items than fishing tentacles (Helmholz *et al.* 2007).

Conclusions

The ecological roles of natural products from pelagic organisms are becoming increasingly appreciated. Specifically, allelopathic interactions and predator-prey dynamics have been strong foci of marine plankton chemical ecology research in recent years. Exciting examples of host-parasite interactions among marine planktonic organisms have been documented in the past three years, which complement the larger pool of these types of studies in freshwater plankton systems. In contrast, the importance

of natural products in mutualistic interactions as well as intraspecific communication represents a relatively unexplored avenue for future research. The influence of bacteria on phytoplankton natural product biosynthesis, induction, release, metabolism, and degradation is also under-represented in the literature.

A continued lack of fully-characterized molecular structures, particularly in allelopathy and pheromone studies, remains a hindrance to appreciating the importance of natural products in pelagic communities. Without having specific compounds identified and available in pure form for manipulative experiments and for use as analytical standards, it is difficult to study patterns of production and distribution, mechanisms of action, and the costs and benefits associated with secondary metabolism. However, due to their low natural concentrations, typically high water-solubility, dispersal in large volumes of seawater, and the small size of most planktonic organisms, it is not surprising that these chemical cues are not nearly as tractable as those of benthic marine or terrestrial macroorganisms.

Recent advances in genetics and metabolomics as well as improvements in the sensitivity of analytical instrumentation will aid the discovery of natural products from marine planktonic organisms. Future discoveries of novel natural products will allow researchers to directly test hypotheses about the ecological functions of these compounds in rigorously-designed, ecologically-relevant experiments. Planktonic secondary metabolites can influence the ecology and evolution of organisms at multiple trophic levels within the marine plankton, and their effects can also trickle into other systems. Although chemical ecology involving terrestrial and benthic marine habitats are better-developed fields of study, natural products are clearly crucial in pelagic systems on

multiple ecological scales, and therefore chemical ecology of the marine plankton is an increasingly fruitful area for research.

CHAPTER 2:

METABOLOMICS REVEAL SUBLETHAL EFFECTS OF ALLELOPATHY IN MARINE PLANKTON

Abstract

Competition is a major force structuring marine planktonic communities. The release of compounds that inhibit competitors, a process known as allelopathy, may play a role in the maintenance of large blooms of the red tide phytoplankton *Karenia brevis*, which produces potent neurotoxins that negatively impact coastal marine ecosystems. We employed NMR spectroscopy and mass spectrometry metabolomics to investigate the role of sublethal chemically mediated ecological interactions between the red tide dinoflagellate *Karenia brevis* and two diatom competitors, *Asterionellopsis glacialis* and *Thalassiosira pseudonana*. *K. brevis* is differentially allelopathic to multiple competitors, and caused significant reductions in growth of both diatoms. The impact of *K. brevis* allelopathy on competitor physiology was reflected in the metabolomes of both diatoms. For the more sensitive diatom, *T. pseudonana*, allelopathic compounds enhanced cellular nitrogen cycling, while suppressing central energy metabolism and osmotic regulation, as indicated by the annotation of 15 metabolites whose concentrations were altered by exposure to *K. brevis* allelopathy. *A. glacialis* displayed a more robust metabolism in response to *K. brevis* allelopathy, which may be a result of its frequent exposure to *K. brevis* in the Gulf of Mexico. These chemical cues in the plankton can alter large-scale

ecosystem processes, including primary production, nutrient cycling, and plankton bloom dynamics.

Introduction

Marine phytoplankton are responsible for approximately 50% of global net primary production (Field *et al.* 1998), which ultimately drives the global carbon cycle (Falkowski *et al.* 1998). This primary production provides carbon for higher trophic levels with interactions among species playing critical roles in controlling the flux of biomass and nutrients in the water column (Strom 2008). Chemical cues and signals mediate many interactions among planktonic organisms, including competition (Kubanek *et al.* 2005; Tillmann *et al.* 2007), defense against grazers (Tillmann & John 2002; Teegarden *et al.* 2008), predator detection (Selander *et al.* 2006), prey capture (Sheng *et al.* 2010), and signaling between neighbor cells during bloom events (Vardi *et al.* 2008). Therefore, chemical cues that affect ecological interactions between microalgae are hypothesized to be critical in altering these large scale ecosystem processes.

Allelopathy, the production and release of chemical compounds to inhibit or kill competitor species, is a form of interference competition that alters community composition in terrestrial (Inderjit *et al.* 2011) and benthic aquatic communities (Thacker *et al.* 1998; Rasher & Hay 2010). Allelopathy is also a common structuring force in the plankton, altering species succession (Keating 1977; Vardi *et al.* 2002) and community composition (Fistarol *et al.* 2003; Uronen *et al.* 2007). Additionally, allelopathy may be a successful competitive strategy employed by bloom forming organisms that are potentially weak exploitation competitors (Smayda 1997).

In planktonic systems, allelopathy causes a variety of species-specific physiological effects on target organisms. Some allelopathic interactions result in massive mortality of competitors, either through cell lysis (Ma *et al.* 2009) or initiation of programmed cell death pathways (Vardi *et al.* 2002). However, allelopathy need not be lethal to be effective. Exposure to allelopathic compounds can cause sublethal physiological responses in competitors, such as cyst formation (Fistarol *et al.* 2004) and altered cell swimming behavior (Tillmann & John 2002; Tillmann *et al.* 2007). Often, allelopathic compounds reduce the growth of competitors without inducing mortality (e.g., Suikkanen *et al.* 2006; Ribalet *et al.* 2007).

The red tide forming dinoflagellate *Karenia brevis* is known to be allelopathic towards several competing phytoplankton species (Kubanek *et al.* 2005; Prince *et al.* 2008a). *K. brevis* produces a suite of potent neurotoxins, brevetoxins, responsible for neurotoxic shellfish poisoning in humans as well as fish and marine mammal mortalities during bloom events in the Gulf of Mexico (Landsberg *et al.* 2009), although brevetoxins are not particularly allelopathic (Poulson *et al.* 2010). *K. brevis* produces an additional suite of unstable, polar allelopathic compounds whose molecular structures have not been fully elucidated, although they have been partially characterized (Prince *et al.* 2010). As a relatively weak exploitation competitor (Brand *et al.* 2012), *K. brevis* may use allelopathy to maintain nearly monospecific blooms in near-shore waters (Kubanek *et al.* 2005). Additionally, not all competitors are equally sensitive to the allelopathic compounds produced by *K. brevis* (Kubanek *et al.* 2005). Competitor susceptibility to *K. brevis* allelopathy is at least partly mediated by ecological context. The presence of particular competitor species modulates allelopathic potency (Prince *et al.* 2008b), and competitors

in earlier growth stages are more susceptible to *K. brevis* allelopathy than those in later growth stages (Poulson *et al.* 2010). Allelopathic compounds produced by *K. brevis* cause sublethal reductions in growth and photosynthetic efficiency, and increased cell membrane permeability (Prince *et al.* 2008a), although the exact cellular targets of *K. brevis* allelopathy are unknown.

Metabolomics, the study of all metabolites present in a cell or organism, seeks to understand the functioning of a cell at a snapshot in time (Viant *et al.* 2003; Sardans *et al.* 2011). It can be used to connect genotype to observed phenotypic plasticity and provides an unbiased method for developing *a posteriori* hypotheses about the impact of stressors on cell physiology (Bundy *et al.* 2009). However, no singular method can measure all 200,000 estimated metabolites likely present in a single cell type. Various analytical tools are successful in quantifying different classes of metabolites, and so combining multiple analytical approaches enhances the number and types of metabolites that are successfully profiled. For example, gas chromatography - mass spectrometry (GC-MS) methods are often used to identify and profile low molecular weight primary metabolites, while liquid chromatography - mass spectrometry (LC-MS) methods provide a more flexible platform for characterizing both primary and secondary metabolites of a wide range of polarities and molecular weight ranges (Moco *et al.* 2007). Nuclear magnetic resonance (NMR) spectroscopy, although less sensitive than MS, provides unparalleled structural information without requiring efficient ionization which limits MS methods. Therefore, multiple analytical platforms run in parallel provide complementary information on cell metabolic state (Moco *et al.* 2007).

Metabolomic approaches have been successfully used to investigate sublethal impacts of pollutants on non-model organisms, including salmon (Viant 2007), barnacles (Viant *et al.* 2003), and earthworms (Guo *et al.* 2009). Metabolomics have also been used to investigate impacts of other environmental stressors including nutrient limitation, temperature, and osmotic stress on microbial metabolism (Bolling & Fiehn 2005; Boroujerdi *et al.* 2009; Behrends *et al.* 2010). The effects of ecological interactions on organism metabolism have been less studied using these techniques (see reviews by (Bundy *et al.* 2009; Sardans *et al.* 2011). Most interspecific interactions investigated with metabolomic techniques have been plant-herbivore or pathogen-host relationships (Sardans *et al.* 2011), with very few studying the effects of competition on organism metabolism (but see Scherling *et al.* 2010).

In order to identify potential cellular targets of *K. brevis* allelopathy and better understand the effects of sublethal chemical cues in marine ecosystems, we employed a metabolomics approach, including both NMR spectroscopy and ultra performance liquid chromatography - mass spectrometry (UPLC-MS). With these approaches, we expected to distinguish among the metabolic responses of different competitors to *K. brevis* allelopathy, with the ultimate goal of understanding how *K. brevis* blooms influence community structure and, by extension, ecosystem processes in the Gulf of Mexico.

Materials and Methods

Phytoplankton and general culturing methods

Cultures of the diatoms *Asterionellopsis glacialis* strain CCMP 137 and *Thalassiosira pseudonana* strain CCMP 1335 were grown in artificial seawater (Instant

Ocean, 35 ppt) amended with L1 + Si media (Guillard & Hargraves 1993). Cultures were haphazardly arranged in a Percival incubator set to a 12:12 light:dark cycle with irradiance of 100-145 μmol m^{-2} s^{-1} (Biospherical Instrument QSL2100), at 21°C. Cultures of *Karenia brevis* strain CCMP 2228 were grown in L1 media amended artificial seawater in conditions mentioned above. Growth of cultures was assessed using *in vivo* chlorophyll a fluorescence, with measurements taken at 15:00 h each day, 7 h into the light cycle (Turner Biosystems Trilogy Fluorometer fitted with *in vivo* chlorophyll a module). To directly quantify diatom cell concentrations, samples preserved with Lugol's solution were settled in a Palmer-Maloney chamber for 2-3 minutes, then visually assessed for cell size, and cells were counted using an Olympus IX-50 inverted microscope. Preserved (Lugol's solution) *K. brevis* samples were diluted 1:4 with artificial seawater and cell concentrations measured using a FlowCAM autoimager (Fluid imaging Inc.; 100 μm flow cell, 0.4 mL min^{-1}, autoimage rate of 16 fps). Images of particulate matter were removed prior to determining final cell concentrations. Exponentially growing cultures were used in the following experiments. To calculate the growth of phytoplankton, the following equation was used to convert *in vivo* fluorescence to % growth:

$$\%\ Growth = \frac{Final\ Fluorescence - Initial\ Fluorescence}{Initial\ Fluorescence}\ X\ 100$$

To calculate the specific growth rate, μ, of competitors over the course of the experiment the following equation was used (Wood *et al.* 2005).

$$Specific\ growth\ rate, \mu = \frac{\ln(final\ \ fluorescence - initial\ \ fluorescence)}{Time_{final} - Time_{initial}}$$

Experimental Design

A co-culture experimental design was employed to investigate antagonistic interactions between *K. brevis* and each of two competitor species. The diatoms *A. glacialis* and *T. pseudonana* were each co-cultured with the dinoflagellate *K. brevis* with the latter placed in a permeable dialysis membrane (e.g., Paul *et al.* 2009). This design allowed for ongoing exudation of allelopathic compounds from *K. brevis* through the dialysis tubing over the course of the experiment, but prevented cell contact between species. For controls, competitors were exposed to dialysis tubes filled with L1 media diluted to 65 % of full L1 nitrate, vitamins, and trace metal concentrations, and 90 % of full L1 phosphate concentrations, in an effort to mimic the nutritional environment of exponentially growing *K. brevis* cultures (preliminary experiments indicated that *K. brevis* consumed media constituents leaving the proportions described above once late exponential growth phase was reached; data not shown). After a period of co-culture with live *K. brevis* (treatments) or dilute media (controls), competitor cells were harvested, extracted, and their metabolic profiles compared using ^{1}H NMR spectroscopy and mass spectrometry (see below).

Co-culture preparation

Dialysis tubes (SpectraPor 7; molecular weight cutoff 50 kDa) were prepared for co-culturing by soaking overnight in deionized water followed by rinsing five to eight times. Tubing was cut to approximately 35 cm and briefly autoclaved. Dialysis tubes were knotted at one end and filled with 30-35 mL of either *K. brevis* cultures or dilute

media. To quantify initial *K. brevis* cell concentrations, 1.5 mL of culture from each tube was removed and preserved in Lugol's preservative prior to sealing each dialysis tube with a clip. Each replicate flask containing 1.5 L media received four dialysis tubes containing either *K. brevis* culture (n = 16) or dilute media control (n = 16). *K. brevis* cell concentrations ranged from 8.8-16.3 x 10^3 cell mL^{-1} within dialysis tubes throughout all experiments. After acclimating overnight, 300 mL of exponentially growing competitor culture, either *T. pseudonana* or *A. glacialis*, were inoculated into each flask (1.8 L final volume; n = 15 controls, n = 15 treatments). Additional "blank" control flasks did not receive competitor culture but contained either media or *K. brevis* filled dialysis tubes (n = 1 each). Experimental co-cultures were haphazardly arranged in a walk-in environmental chamber (22 °C) fitted with Philips Universal/Hi-Vision fluorescent bulbs set to a 12:12 light:dark cycle producing irradiance of 75-120 μmol m^{-2} s^{-1}. Flasks were re-arranged daily and swirled three times per day to maximize exposure of competitors to *K. brevis* allelopathic compounds. In order to monitor competitor growth, 3 mL of diatom culture was removed daily, (after 7 h in light period), from each flask for *in vivo* chlorophyll a measurements and preserved in Lugol's preservative to measure cell concentration (see above).

Health of the *K. brevis* cells inside the dialysis tubing was assessed visually each day. *K. brevis* was deemed healthy if phototaxis behavior was observed, culture color remained relatively consistent, and no aggregates formed at the bottom of the tubes. Dialysis tubes with unhealthy *K. brevis* cultures were aseptically removed from the flask, rinsed with sterile deionized water inside and out, refilled with fresh *K. brevis* culture, and returned to the experimental flask. At the end of the experiment 1.5 mL of *K. brevis*

culture from inside each dialysis tube was collected and preserved to measure cell concentration (see above). As expected, no diatom cells were observed inside the dialysis tubing.

Nutrient analyses

Macronutrient concentrations were assessed at the start of the experiment, midway (after six days for *A. glacialis* and after four days for *T. pseudonana*), and on the day of harvest by collection of 15 mL sample from a subset (n = 3 treatment and n = 3 controls) of experimental flasks. Samples were syringe filtered through a 0.2 μm nylon filter and frozen at -20 °C until analysis. Thawed samples were diluted 100 fold with deionized water for colorimetric analysis for nitrates (NO_2^- + NO_3^-), inorganic phosphate, and ammonium concentrations using a Lachat Quikchem 8000 FIA instrument.

Statistical analyses for phytoplankton growth

Unpaired t-tests, or Mann Whitney U tests for non-normally distributed data, were used to determine statistical differences between competitors exposed to *K. brevis* or dilute media controls regarding nutrient concentrations, % growth, and growth rate (GraphPad Prism v.4). To determine statistical differences in the PCA scores of treatment and controls extracts, t-tests were performed in MATLAB v.7.12.0.

Harvest, extraction, and sample processing

Once competitor cultures had reached exponential growth (after 6 days for *T. pseudonana*, after 8 days for *A. glacialis*), *K. brevis* dialysis tubes were removed

aseptically, diatoms from each experimental flask were filtered onto GF/C filters (Whatman #1822-110, muffled for 3 hrs at 450 ℃) (Vidoudez & Pohnert 2012) and immediately dipped into liquid nitrogen to quench cellular metabolism. All competitor cells from a single experiment (n = 15 treatments, n = 15 controls, n = 2 blanks) were harvested within a two to three hour window (within 6-9 hours of light period), to ensure minimal effects of circadian rhythm on metabolism. Treatment and controls cultures were interspersed during the harvesting process. Frozen cells with filters were stored in muffled aluminum foil at -80℃ for a period of days until extraction of metabolites. Cells were extracted using 30 mL of a mixture of ice-cold 3:1:2 methanol/acetone/acetonitrile optimized for extraction of metabolites (data not shown) and ground with a liquid nitrogen cooled mortar and pestle. Filter particulates were removed by centrifugation (5 min at 0 ℃, 1460 *g*) and the supernatant (extract) was transferred to a glass vial while the resulting pellets were further rinsed twice with 10 mL fresh solvent mixture and a final rinse of 3 mL, combined with initial supernatant and dried *in vacuo* using a Thermosavant speedvac vacuum concentrator.

Dried extracts were dissolved with a biphasic mixture of 9:10:15 water/methanol/chloroform to fractionate polar and nonpolar intracellular metabolites (Lin *et al.* 2007; Wu *et al.* 2008). After removal of the aqueous (polar) fraction, the chloroform-soluble fraction was sequentially washed twice with 9:10 water/methanol. All aqueous fractions were pooled and solvents were removed *in vacuo*. Excess inorganic salts were removed by filtration from the aqueous extract by twice triturating with ice-cold methanol, followed by solvent removal *in vacuo.* The chloroform-soluble extracts were stored for future analysis.

In order to compare metabolomes among replicates without the confounding effect of different cell concentrations, for each replicate an amount of polar extract equivalent to 3.98 x 10^8 *T. pseudonana* cells was used for ^{1}H NMR spectroscopy. For LC-MS metabolomics, an extract amount derived from 5.31 x 10^7 cells was used. Because exposure to *K. brevis* caused substantial reductions in *T. pseudonana* growth, in order to achieve adequate extract concentrations for ^{1}H NMR spectroscopy, aliquots of some treatment extracts were pooled resulting in n = 9 treatment spectra from 15 treatment extracts. Extracts from two control cultures were also pooled in order to achieve the minimum extract concentration described above (resulting in n = 14 control spectra). Extracts were pooled based on similar final fluorescence measurements, based on the prediction that cultures that reached similar population densities were in comparable metabolic states.

For the experiment with *A. glacialis*, an aliquot of polar extract derived from 7.96 x 10^7 and 2.65 x 10^7 cells was used for ^{1}H NMR spectroscopy and LC-MS, respectively. Three treatment replicates that were contaminated with greater than 100 cell mL^{-1} *K. brevis* outside of the dialysis tubing were removed from metabolomic analysis. A few samples were not included in the analysis due to poor water suppression (see NMR spectral data acquisition) resulting from excess inorganic salts (final replicate numbers: n = 9 treatments, n = 11 controls).

NMR spectral data acquisition

^{1}H NMR spectra were acquired for all diatom polar extracts that fulfilled the criteria discussed above. Each polar extract was dissolved in 700 µL of 0.2 mM sodium

phosphate buffer (pH = 7.1) in 90% H_2O and 10% D_2O (99.9 % atom D_2O, Sigma Aldrich) containing 0.5 mM or 0.25 mM of 3-(trimethylsilyl)propionic-2,2,3,3-d_4 acid (TMSP) as an internal standard. Samples were gently centrifuged (~5 min) to pellet salt precipitate and filtered (Fisherbrand, 0.2 μm Teflon) into 5 mm NMR tubes. Spectra were collected on a Bruker Avance 500 MHz DRX NMR spectrometer equipped with a 5 mm broadband direct probe, with an excitation-sculpting gradient pulse program for water suppression as follows: 11 μs (90°) pulse, 22 μs (180°) pulse, and 2 ms (108°) shaped pulse (Hwang & Shaka 1995). The spectral width was 5.5 kHz, with a relaxation delay of 20 μs. For each sample, 256 scans were compiled to gather adequate signal for analysis.

NMR spectral processing

Spectra were imported into MATLAB Version 7.12.0 and preprocessing was performed in NMRLab (Gunther *et al.* 2000) and PROMETAB 3.3 (Viant 2003). Spectra were aligned to the chemical shift of the internal standard (TMSP) at 0.00 ppm, manually phased, and baseline corrected. Spectral regions around TMSP (-0.5-0.5 ppm), water (4.6-5.0 ppm for *A. glacialis*, 4.0-4.9 ppm for *T. pseudonana*) and residual methanol (3.3-3.5 ppm) were removed before noise filtering. For the *A. glacialis* experiment only in which a small number of *K. brevis* cells escaped from the dialysis tubing, we removed signals caused by *K. brevis* cell contamination (identified by their presence *K. brevis* blank culture extracts), in order to not confound intracellular *K. brevis* and *T. pseudonana* metabolites. These regions included: 1.41-1.42 ppm, 2.65-2.67 ppm, 2.93-2.95 ppm, 2.98-3.02 ppm, 4.40-4.15 ppm. Spectra were then binned (0.005 ppm), partial quotient normalized to account for slight differential dilution among samples, generalized log

(glog) transformed to account for variance among signals within each sample reducing bias towards highly concentrated metabolites, and mean centered. The lambda values for glog optimization were obtained using a set of five quality control extracts generated with the above methods with a single large batch culture of each diatom species (Parsons *et al.* 2007). Lambda for *T. pseudonana* was 9.8068×10^{-8} whereas a lambda value of 5.7289×10^{-9} was used for *A. glacialis* samples.

Mass spectrometry data acquisition

For metabolomic analysis of polar fractions, we utilized UPLC-MS approach. Separation solvents were H_2O + 0.1% acetic acid and acetonitrile. A Waters Xevo G2 quadrupole time-of-flight mass spectrometer was equipped with an Acquity UPLC BEH C_{18} silica column (2.1 x 50 mm, 1.7 μm) with ionization in positive and negative modes. For positive ion mode, the capillary voltage was 2.3 kV with sampling cone and extraction cone voltages at 45 V and 4 V, respectively. For negative mode, the sampling cone voltage was -45 V and the extraction cone voltage was -4V. Source and desolvation temperatures were 120°C and 350 °C, respectively, with the desolvation gas flow set to 650 L hr^{-1}.

Statistical analysis and metabolite annotation

Principal component analysis (PCA) was used to examine differences in diatom metabolomes between treatment and controls of the same species (MATLAB with PLS Toolbox v.7.03). After PCA, the loadings plots were used to ascertain which metabolites within extracts were responsible for distinguishing between *K. brevis* exposed and media

exposed cultures. Database (Human Metabolome Database (Wishart *et al.* 2007), Chenomx Profiler) and literature searches were used to annotate spectra and tentatively identify metabolites. Representative extracts from each experiment were used to collect 2D NMR spectral data (standard HSQC and TOCSY experiments, 500 MHz) to aid in annotation. The Kyoto Encyclopedia of Genes and Genomes (KEGG), was also used to aid in identification of particular pathways involving critical metabolites.

Results

Karenia brevis shows sublethal allelopathy towards both diatom competitors

After six days of co-culture physically separated by dialysis membrane, we observed a strong negative effect of *K. brevis* allelopathy on the growth of *T. pseudonana* (Figure 2.1). *Thalassiosira pseudonana* growth was reduced by 85 % overall, (Figure 2.1B, n = 15, $p < 0.0001$), with growth suppression and subsequent population decline observed after four days of co-culture. Allelopathy led to a negative specific growth rate of *T. pseudonana* during the last 3 days of the co-culture (unpaired t-test with Welch's correction, $p < 0.0001$), indicating cell death (Figure 2.1A). These allelopathic effects took at least three days to manifest, as *K. brevis* had no effect on the specific growth rate of *T. pseudonana* during the first three days of the co-culture (unpaired t-test, $p = 0.23$).

Karenia brevis was also allelopathic towards *A. glacialis* over the course of eight days of co-culture (Figure 2.2), as reflected in a significant reduction in the specific growth rate for exposed *A. glacialis* populations (unpaired t-test, n = 15, p = 0.029). The overall growth of *A. glacialis* was reduced by 35 % when exposed to *K. brevis*

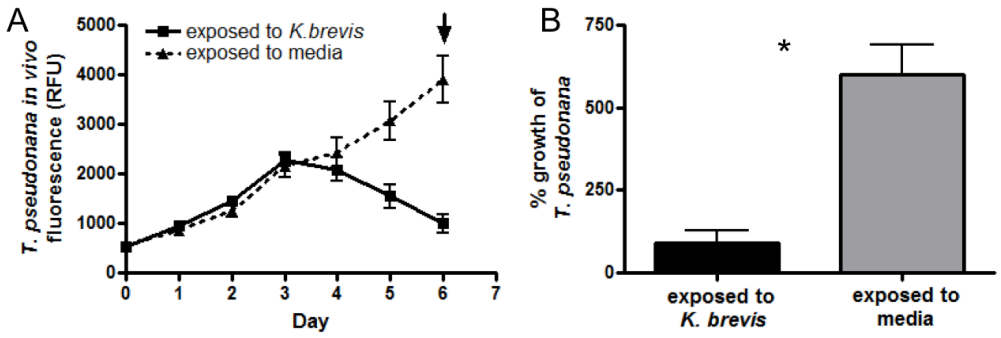

Figure 2.1. Effects of exposure to live *Karenia brevis* on the growth of *Thalassiosira pseudonana.* A) *T. pseudonana in vivo* fluorescence (arrow indicates day of harvest for metabolomics) and B) % growth after 6 days. The growth of *T. pseudonana* was significantly reduced by exposure to *K. brevis*, n = 15, $p < 0.0001$, unpaired t-test with Welch's correction, indicated by asterisk (*). Error bars represent ±1 S.E.M.

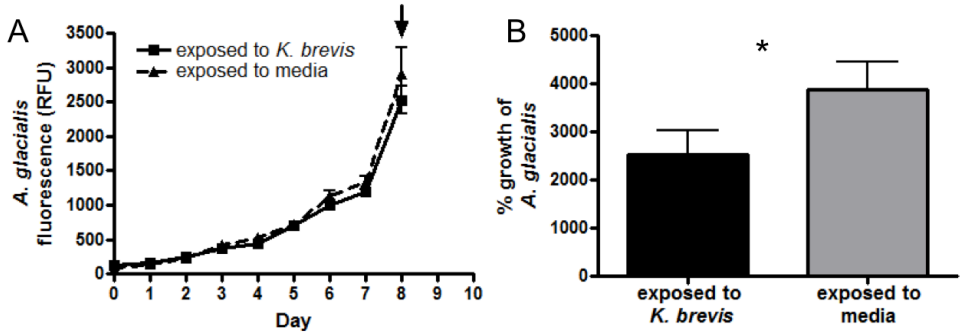

Figure 2.2. Effects of exposure to live *Karenia brevis* on the growth of *Asterionellopsis glacialis.* A) *A. glacialis in vivo* fluorescence (arrow indicates day of harvest for metabolomics) and B) % growth after 8 days. The growth of *A. glacialis* was significantly reduced by exposure to *K. brevis*, n = 15, $p = 0.011$, Mann Whitney U test, indicated by asterisk (*). Error bars represent ±1 S.E.M.

allelopathy (Figure 2.2B, n = 15, $p = 0.011$). However, negative growth was not observed, indicating a weak allelopathic effect of *K. brevis* on *A. glacialis*.

Final macronutrient concentrations did not differ between treatment and control *A. glacialis* cultures, suggesting that the negative effects of *K. brevis* on *A. glacialis* growth were not a result of exploitation competition (unpaired t test, n = 3, $p = 0.13$ for nitrates and $p = 0.81$ for phosphate). For phosphate, treatment cultures averaged 18.5 ± 1.4 μM (± 1 S.D.) whereas mean phosphate concentrations for control cultures was 18.1 ± 2.7 μM. For nitrate, mean concentrations were 938 ± 57 μM and 792 ± 120 μM for treatments and controls, respectively. At the time of harvest in the *T. pseudonana* experiment, nitrate concentrations were not different between treatment and control cultures (896 ± 36 μM in treatments, 815 ± 110 μM in controls, n = 3, $p = 0.29$), however phosphate concentrations were 28 % lower in co-cultures (15.1 ± 2.7 μM) compared to controls (21.1 ± 0.4 μM, n = 3, $p = 0.021$). Phosphate was not limiting in these cultures since concentrations were greater than 15 μM after 6 days of co-culture, indicating that the negative effects of *K. brevis* on competitor growth were most likely mediated by allelopathy rather than exploitation competition.

Thalassiosira pseudonana **metabolism is substantially altered by allelopathy**

Effects of *K. brevis* exposure on *T. pseudonana* metabolism were clearly discernable by ^{1}H NMR spectroscopic profiles analyzed by principal component analysis, which successfully separated the metabolomes of *T. pseudonana* exposed to allelopathy from that of *T. pseudonana* grown alone (PC1, 31.7% of variance, n = 9-14, $p < 0.0001$)

(Figure 2.3A). Concentrations of several metabolites within *T. pseudonana* cells, including dihydrouracil, glycerate, glucose, myo-inositol, betaine, and the amino acid alanine were suppressed by *K. brevis* allelopathy, indicating impacts on multiple cellular pathways (Figure 2.4). Specifically, these metabolites participate in a wide variety of metabolic pathways, including pyrimidine catabolism (Voet *et al.* 2004), photorespiration (Kroth *et al.* 2008), carbon and central energy metabolism (Kroth *et al.* 2008) and osmotic regulation (Keller *et al.* 1999) (Table 2.1). Notably, exposure to *K. brevis* reduced concentrations of the aromatic osmolyte homarine by 80% in *T. pseudoanana* cells (unpaired t-test, n = 9-14, $p < 0.0001$). Concentrations of a lesser number of metabolites were enhanced by *K. brevis* allelopathy, including acetate, the fatty acid caprate/caprylate, dimethylamine and/or sarcosine, as well as amino acids glutamate and proline, indicating an additionally effect of *K. brevis* allelopathy on fatty acid biosynthesis/metabolism (KEGG) and nitrogen metabolism (Hockin *et al.* 2012) (Table 2.1, Figure 2.4). Abundances of several unidentified metabolites with ^{1}H NMR chemical shifts at 2.0-3.0 ppm and 1.60 ppm (with a TOCSY correlation at 2.25 ppm) were also enhanced by exposure to *K. brevis*.

Asterionellopsis glacialis metabolome is subtly impacted by *Karenia brevis* allelopathy

K. brevis allelopathy produced a more subtle effect in the metabolome of *A. glacialis*, as profiles of the polar metabolites were significantly separated by the 5th principal component (PC) of a five PC model (Figure 2.3B, n = 9-11, $p = 0.033$). Loadings on PC5 suggest that the abundances of few aliphatic metabolites were enhanced

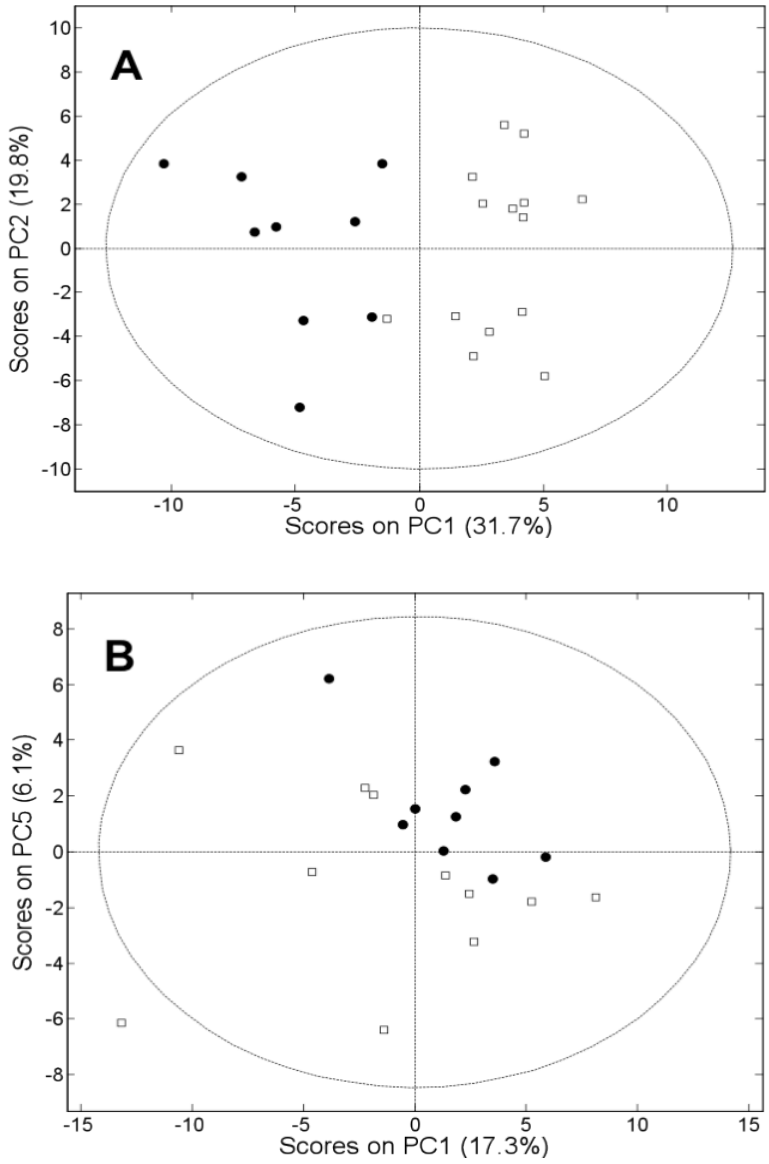

Figure 2.3. Effects of *Karenia brevis* allelopathy demonstrated by changes in the metabolomes of competitor diatoms. A) PCA scores plot of ^{1}H NMR spectral data for *Thalassiosira pseudonana* exposed to *K. brevis* (filled circles) or dilute media control (empty squares), showing significant separation along the 1st principal component (unpaired t-test, n = 9-14, $p < 0.0001$). B) PCA scores plot of ^{1}H NMR spectral data of *Asterionellopsis glacialis* exposed to live *K. brevis* (filled circles) or dilute media control (empty squares). There was a significant separation of PCA scores along the 5th principal component (unpaired t-test, n = 9-11, $p = 0.033$).

by exposure to allelopathy, specifically glycerophosphocholine and lactose (or other polysaccharides, with metabolite NMR signals at 3.00-4.44 ppm, and a doublet at 4.45 ppm attached to a carbon with ^{13}C NMR signal at 105.9 ppm). *K. brevis* allelopathy, however, did not significantly reduce the concentrations of lactose in *A. glacialis* cells (unpaired t-test, n = 9-11, $p = 0.71$), indicating that this metabolite is not a biomarker for allelopathy on its own. We tentatively identified several other metabolites from a variety of pathways, including acetate, glutamate, dimethylglycine/sarcosine, dehydroascorbic acid, and betaine; however they do not appear to be impacted by *K. brevis* allelopathy as their signals did not appear in the loadings on PC5. This suggests that most metabolic pathways in *A. glacialis* maintain robust in response to *K. brevis*.

Discussion

Metabolomics is a powerful tool for understanding sublethal ecological interactions

The diatom *A. glacialis* appears to maintain a relatively robust metabolism in response to *K. brevis,* despite mild allelopathic effects on growth (Figures 2.2-2.3). The metabolism of *T. pseudonana*, however, is greatly altered by *K. brevis* allelopathy, which causes a lethal effect after four days of co-culture (Figures 2.1, 2.3). In response to *K. brevis* allelopathy, *A. glacialis* cells experienced increased polysccharide and glycerophosphocholine concentrations indicative of enhancement in carbon metabolism and increased storage products for use in central energy metabolism (KEGG). This is in contrast to *T. pseudonana* where carbon metabolism was severely disrupted by *K. brevis* allelopathy. Since *A. glacialis* growth was reduced with subtle metabolic responses to

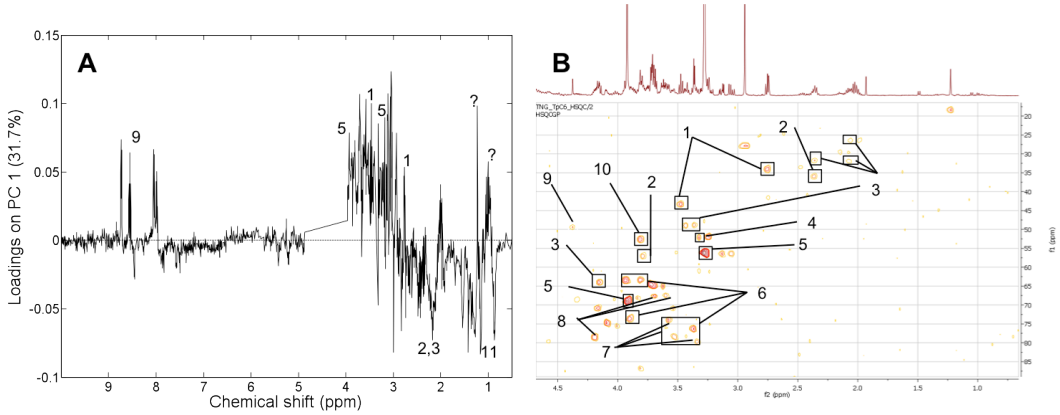

Figure 2.4 Biomarkers for *Karenia brevis* allelopathic effects on *Thalassiosira pseudonana*. A) PCA loadings plot for principal component 1. ^{1}H NMR signals with positive loads represent chemical shifts of metabolites with decreased concentrations after exposure to *K. brevis*, while negative loadings show metabolites with enhanced concentrations due to *K. brevis* allelopathy. B) Representative 2D HSQC NMR spectrum of *T. pseudonana* used to aid in identification of metabolites. Potential annotated metabolites include: dihydrouracil (1); glutatmate (2); proline (3); taurine (4); betaine (5); glucose (6); myo-inositol (7); gylcerate (8); homarine (9); alanine (10), caprate/caprylate (11). Spectral information for 6 and 7 overlap in one region of the spectrum.

Table 2.1 Candidate metabolites identified from ^{1}H and HSQC NMR spectra of *Thalassiosira pseudonana* exposed to *Karenia brevis* allelopathy. Chemical shifts in bold indicate observed signals in *T. pseudonana* extracts, while non-bold shifts indicate signals not observed due inadequate sensitivity or overlap in that spectral region. Chemical shift values that were not observed are reported from either Boroujerdi *et al*. 2012 or the human metabolome database (HMDB). Metabolic pathways reported from KEGG.

Metabolite	^{1}H chemical shift (ppm)	^{13}C chemical shift (ppm)	Loading on PC1	Effect of *K. brevis*	Metabolic Pathway/ Function
Acetate	**1.93**	26.2	negative	enhanced	central energy metabolism
Dimethylamine[1]	**2.71**	37.3	negative	enhanced	glycine metabolism
Sarcosine[1]	**2.72**, 3.59	35.6, 53.5	negative	enhanced	glycine metabolism
Caprate/Caprylate	**0.85, 1.27, 1.53, 2.16**	39.5, 27.9, 30.9, 23.9, 33.2, 14.6	negative	enhanced	Fatty acid biosynthesis, biosynthesis of secondary metabolites
Glutamate	**2.07**, 2.14, **2.35**, **3.78**	**31.7**, 29.9, **36.1**, **57.4**	weak negative	enhanced	amino acid metabolism, energy metabolism
Proline	**2.05**, **2.07**, **2.36**, **3.35**, **3.43**, **4.15**	**26.5**, **31. 7**, **31. 6**, **48.8**, **49.1**, **63.9**	weak negative	enhanced	amino acid metabolism, urea cycle, osmoprotectant
Glycolate[2]	**3.92**	**63.1**	---	---	photorespiration
Taurine	**3.29**, 3.43	**51.7**, 38.3	weak positive	suppressed	amino acid metabolism
Alanine	1.45, **3.80**	19.1, **53.6**	positive	suppressed	amino acid metabolism
Betaine	**3.28**, **3.91**	**56.2**, **68.8**	positve	suppressed	glycine metabolism, osmoprotectant
Dihydrouracil	**2.74**, **3.48**	**34.0**, **43.1**	positive	suppressed	pyrimidine catabolism
Glucose[2]	**3.33**, **3.36**, **3.53**, **3.59**, **3.57**, 3.76, **3.79**, **3.87**, **3.92**, 3.93, 4.66, 5.25	**79.6**, **76.3**, **78.3**, **75.9**, **75.0**, 74.0, **63.5**, **74.0**, **63.1**, 73.6, 98.9, 95.1	positive	suppressed	central energy metabolism
Myo-inositol[2]	**3.33**, **3.59**, **3.57**, 3.75, 3.75, **4.08**	**79.6**, **75.9**, **74.0**, 75.7, 73.9, **74.5**	positive	suppressed	central energy metabolism
Glycerate	**3.70, 3.79, 4.17**	**67.7, 67.7, 77.8**	strong positive	suppressed	photorespiration
Homarine	**4.37**, 7, 8.04, **8.55**, **8.73**	**49.3**, 130.2, 129.0, **149.5**, **148.6**	strong positive	suppressed	osmoprotectant

[1] indicates metabolites that share observed chemical shifts
[2] indicates metabolites that share multiple observed chemical shifts

K. brevis allelopathy, it is possible that important biomarkers in *A. glacialis* cells were not detected due to the relative insensitivity of ^{1}H NMR spectroscopy. Alternatively, it is possible that most individual cells in the population of *A. glacialis* in each experimental flask were not impacted by *K. brevis* allelopathy, and only a relatively small number of cells responded to allelopathic compounds. *A. glacialis* may also upregulate stress response or detoxification proteins that would result in a more robust metabolism when exposed to allelopathy (B. Nunn, unpublished), and future proteomics work will investigate whether these types of proteins contribute to the partial resistance of *A. glacialis* to *K. brevis* allelopathy.

The sensitivity of *Thalassiosira pseudonana*to allelopathy may be due to its lack of historical exposure to *K. brevis* blooms. This diatom strain (CCMP 1335) was isolated from the North Atlantic Ocean, whereas *A. glacialis* (CCMP strain 137) was originally isolated from the Gulf of Mexico where *K. brevis* has bloomed during most years of the last half-century (Walsh *et al.* 2006). *A. glacialis* may have evolved partial resistance to *K. brevis* allelopathy due to its frequent exposure, and its robust metabolome may be a result of adaptations in the cellular target(s) of allelopathy that mitigate its impacts, allowing *A. glacialis* cells to function relatively well despite modest growth inhibition. For instance, resistance in some populations of the soft clam *Mya arenia* to paralytic shellfish toxins of the dinoflagellate *Alexandrium* spp. is conferred by a single amino acid mutation in the clam sodium channel, minimizing binding of these toxins to sodium channels (Bricelj *et al.* 2005). What is unknown is whether these small, but ecologically important genotypic differences among populations provide relatively normal metabolic

functioning in response to stress, such as toxin exposure. Nevertheless it is clear that the species-specific effects of *K. brevis* allelopathy are demonstrated in the metabolic responses of competitors.

The impacts of *K. brevis* allelopathy on naïve competitor assemblages may be greater than the impacts observed on assemblages from the Gulf of Mexico. Rare expatriate blooms of *K. brevis* have been observed in the Atlantic Ocean (Steidinger 2009), thus exposing a number of naïve competitors and grazers to *K. brevis* allelopathic and antifeedant compounds (Turner & Tester 1989). Exposure history also affects feeding preferences of mesozooplankton grazers, including rotifers and copepods, which are more likely to consume and tolerate chemically defended algae if they have had previous exposure (Turner & Tester 1989, Kubanek *et al.* 2007). Copepod populations co-occurring with *Alexandrium fundyense* have also evolved resistance to this toxic alga compared to populations that do not regularly encounter *A. fundyense* (Colin & Dam 2002; Colin & Dam 2007), demonstrating that exposure history is important when investigating ecological interactions among planktonic organisms. Additionally, the potential for climate change to alter relative abundance (Hinder *et al.* 2012) and/or the geographic ranges of plankton populations (Thomas *et al.* 2012) may increase the likelihood of exposing *K. brevis* to naïve competitors, potentially enhancing the ability of *K. brevis* to benefit from allelopathy. In terrestrial systems, history of exposure to allelopathy can alter competitive outcomes in terrestrial systems (Callaway & Ridenour 2004), such that plant populations previously exposed to allelopathic competitors have co-evolved resistance, unlike populations with limited exposure (Jensen & Ehlers 2010; Lankau 2012).

Allelopathy rearranges cellular metabolism in sensitive competitors

Analysis of the *T. pseudonana* metabolome (Table 2.1) reveals that *K. brevis* allelopathy impacts multiple cellular targets, with the possibility that metabolic pathways are reshuffled in order to compensate for negative effects of allelopathy. With only metabolomic data, it is difficult to determine causal relationships within metabolic networks (Wienkoop *et al.* 2008). For instance, metabolites may increase in concentration when rates of production are increased or when catabolic processes are suppressed, although this uncertainty should be alleviated by combining metabolomic data with other "omics" technologies (e.g., proteomics), resulting in a more complete representation of cellular processes (Fiehn & Weckwerth 2003; May *et al.* 2008; Fernie *et al.* 2012). Regardless, it is clear that sensitive competitors exposed to allelopathy operate in a functionally compromised metabolic state.

Allelopathy altered carbon and energy metabolism in *T. pseudonana*, such that the breakdown of starches was impeded while internal stores of fatty acids were not metabolized, as evidenced by a decrease in glucose and an increase in fatty acid concentrations in response to allelopathy (Table 2.1). Alternatively, reduced concentrations of glucose and other sugars in *T. pseudonana* (Table 2.1, C. Jones, *unpublished*) may have resulted from enhanced glycolysis. Preliminary proteomics work indicated that abundances of acetyl CoA dehydrogenase responsible to the first step in fatty acid metabolism, as well as at least one glucosamylase enzyme which catalyzes starch breakdown, were reduced (B. Nunn, *unpublished data*), supporting the hypothesis that *K. brevis* allelopathy prevents *T. pseudonana* cells from utilizing internal carbon stores. Allelopathy also reduced the abundance of RuBisCO, the enzyme responsible to

carbon fixation (B. Nunn, *unpublished*), which may indicate that cells experiencing allelopathy become carbon limited.

In *T. pseudonana*, there was a substantial reshuffling of internal nitrogen stores in response to allelopathy, as evidenced by enhanced glutamate concentrations in *T. pseudonana* exposed to *K. brevis*. Glutamate can be produced from pyrimidine catabolism (KEGG) such that increased concentrations of this metabolite could indicate nitrogen scavenging via nucleotide catabolism in *T. pseudonana,* as observed in nitrogen limited *Arabidopsis thaliana* (Zrenner *et al.* 2009). When nitrogen starved, *T. pseudonana* upregulates protein catabolism enzymes (Hockin *et al.* 2012) and cellular levels of ubiquitin were also more abundant in *T. pseudonana* when exposed to *K. brevis* allelopathy (B. Nunn *unpublished*). Therefore, it is likely that protein and pyrimidine catabolism are enhanced by *K. brevis* allelopathy, leading to decreased dihydrouracil concentrations and a larger store of free glutamate inside the cell. Although nitrate concentrations were not different between control and treatment cultures and were replete throughout the experiment (suggesting that *T. pseudonana* was not nitrogen limited in this experiment), it is possible the *K. brevis* allelopathy interferes with the ability of *T. pseudonana* to utilize nitrate from the media.

Competitors exposed to *K. brevis* allelopathy may experience oxidative stress. Glycerate concentrations were reduced in *T. pseudonana* exposed to *K. brevis* indicating that photorespiration was disrupted, which would likely increase oxidative stress (Allen *et al.* 2008; Kroth *et al.* 2008). These cells may have responded to increased oxidative stress by reducing the abundance of photosynthetic machinery, according to preliminary proteomics analysis (B. Nunn *unpublished*). Reduced myo-inositol concentrations (Table

2.1) in response to *K. brevis* allelopathy may indicate increased use of this metabolite for other functions (including signaling, stress response, and phosphorus storage) or utilization as a respiratory substrate to alleviate the cell of oxidative stress (Allen *et al.* 2008).

Osmotic regulation is impacted by *K. brevis* allelopathy, as evidenced by decreased concentrations of homarine and betaine, known organic osmolytes in marine planktonic organisms (Dickson & Kirst 1986; Keller *et al.* 1999). *T. pseudonana* may respond to *K. brevis* allelopathy by compensating for impacted osmotic regulation by enhancing or maintaining concentrations of both proline and dimethylamine (Table 2.1) in an attempt to replace homarine and betaine as osmolytes. Glycine metabolism was disrupted, as evidenced by reductions in betaine, homarine concentrations with concomitant increases in sarcosine/dimethylamine concentrations (Table 2.1, KEGG), suggesting that inhibited photorespiration may have downstream effects on osmotic regulation (Keller *et al.* 1999; Boroujerdi *et al.* 2012). Additionally, *K. brevis* exudates have been shown to increase competitor cell membrane permeability through an unknown mechanism (Prince *et al.* 2008a). Presumably, more permeable cells would leak osmolytes and other metabolites, while allowing for diffusion of inorganic ions across plasma membranes, potentially reducing the need for organic osmolytes. Errera and Campbell (2011) postulated that brevetoxins might interact with as yet unidentified sodium channels in *K. brevis* and open these channels to enhance ion transport out of the cell. If waterborne brevetoxins had a similar effect on putative sodium channels in *T. pseudonana* (Taylor *et al.* 2012), this would also enhance the movement of inorganic ions into *T. pseudonana* cells, and reduce need for organic osmolytes. Previous studies have

indicated that brevetoxins are unlikely to be responsible for allelopathic effects of *K. brevis* on most competitors (Kubanek *et al.* 2005; Prince *et al.* 2010) and are unlikely to have caused increased cell permeability of competitors in previous studies (Prince *et al.* 2008a). Nevertheless, it is possible the decreased homarine and betaine concentrations are a response to greater abundances of inorganic ions present in *T. pseudonana*, either from increased cell membrane permeability and/or opened sodium channels.

Karenia brevis produces a suite of small, polar allelopathic compounds (Prince *et al.* 2010) with differential potency against co-occurring competitors (Poulson *et al.* 2010). Additionally, several phenotypic responses to *K. brevis* allelopathy have been previously reported, including cell membrane disruption and reduction in photosynthetic efficiency (Prince *et al.* 2008a). The particular allelopathic compounds responsible for each of these observed effects are unknown, and it is possible that each allelopathic compound has unique cellular targets. The observation that allelopathy impacted multiple, disparate pathways in *T. pseudonana* suggests that multiple cellular pathways must compensate for the impacts of *K. brevis* allelopathic compounds, ultimately which altering cellular nitrogen recycling, osmotic regulation, photorespiration, carbon storage and central energy metabolism.

***Karenia brevis* allelopathy may lead to programmed cell death**

One might predict that that the inability of *T. pseudonana* to resist *K. brevis* allelopathy leads to programmed cell death (PCD). Both oxidative stress and carbon limitation can induce PCD in plankton (Vardi *et al.* 2008), and we observed population decline of *T. pseudonana* after four days of exposure to *K. brevis* indicating that

allelopathy was lethal to some members of the population. Bidle and Bender (2008) observed sub-cellular morphological changes in *T. pseudonana* cells undergoing programmed cell death. Since we did not observe obvious morphological changes in *T. pseudonana* or *A. glacialis* cells exposed to *K. brevis,* PCD induced by allelopathy would likely have been at the very initial stages. Future proteomic work will determine if abundances of enzymes involved in PCD pathways, including caspaces, metacaspaces, and superoxide dismutases, are increased in response to allelopathy (Vardi *et al.* 2006).

A. glacialis may be able to successfully scavenge reactive oxygen species (ROS) after exposure to allelopathy, whereas *T. pseudonana* may not, again highlighting a potential allelopathy resistance mechanism present in *A. glacialis*. Preliminary proteomic analysis indicates that ascrobate peroxidase was downregulated in *T. pseudonana* when exposed to *K. brevis* allelopathy. Decreased abundances were also observed for the protein mucin19 (B. Nunn, *unpublished*). Mucins, glycoproteins involved in signaling that are expressed at cell surfaces, also demonstrate known anti-apoptopic activity (Singh & Hollingsworth 2006; Workman *et al.* 2009). Future proteomic analysis may also reveal whether ROS scavenging enzymes, for instance catalases, ascorbate peroxidases, and mitochondrial alternative oxidase, are upregulated in *A. glacialis,* and suppressed in *T. pseudonana,* when exposed to allelopathy (Mittler 2002; Fernie *et al.* 2012). Ascorbate peroxidase genes have been found to be upregulated in iron limitated *T. pseudonana*, whereas mucin genes were downregulated, demonstrating a balance between ROS reducing mechanisms and the initiation of PCD pathways in stressful situations (Thamatrakoln *et al.* 2012). This balancing act ultimately leads to stress acclimation, which may be disrupted by allelopathy.

Future metabolic profiling using lipid-soluble extracts from these competition experiments may also aid in determining whether *K. brevis* allelopathy initiates PCD in *T. pseudonana*, through identifying PCD biomarkers such as sphingomyelin, diacyglycerol, phosphatadylserine (Bidle & Bender 2008; Thamatrakoln *et al.* 2012). In addition, diatom lipid biochemistry mediates a number of important ecological interactions, including grazing (Miralto *et al.* 1999), susceptibility to viral infection (Vardi *et al.* 2009), and toxin sensitivity (Deeds & Place 2006). Understanding how allelopathy alters this biochemistry will shed light on how competitive interactions may have cascading ecological effects.

Conclusions

Metabolomics is an under-utilized tool for investigating the impacts of sublethal ecological interactions on organism physiology. In the plankton, metabolomic analysis reveals responses of competitors that are sensitive to allelopathy, and can distinguish between physiological responses of different competitors. These physiological responses of phytoplankton to *K. brevis* can provide insight into the biological factors involved in the maintenance of *K. brevis* blooms, as well as the impacts these blooms may on ecosystem processes including primary production and the flux of biomass and nutrients in the water column. This interdisciplinary, systems biology approach provides an unbiased opportunity to establish novel, testable hypotheses towards understanding the mechanisms by which allelopathy alters species composition in plankton communities, and thus the roles of chemical cues in mediating important ecological interactions.

CHAPTER 3:

ALLELOPATHIC COMPOUNDS OF A RED TIDE DINOFLAGELLATE HAVE SPECIES-SPECIFIC AND CONTEXT-DEPENDENT EFFECTS ON PHYTOPLANKTON*

Abstract

The use of chemical compounds to suppress the growth of competitors is a strategy known as allelopathy that can be readily observed with many phytoplankton species in lab studies. However, it is unclear how these allelopathic interactions are altered when the complexity of the system is increased, more closely mimicking natural conditions. In the current study, we conducted lab experiments to decipher how the identity, abundance, and growth stage of competitors affect the outcome of allelopathic interactions with the red tide dinoflagellate *Karenia brevis*. Multiple chemical compounds produced by *K. brevis* were found to inhibit the growth of four phytoplankton competitors, although these competitors were susceptible to different combinations of compounds. We found that physiological state and cell concentration of competitors were important determinants of allelopathy, with early stage (lag phase) cells more vulnerable to allelopathic effects than later growth stages for the diatom *Skeletonema grethae*. Despite being allelopathic to multiple competitors in the lab, in a microcosm experiment

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* Poulson, K.L.[1], Sieg, R.D.[1], Prince, E.K., & Kubanek, J. (2010). *Marine Ecology-Progress Series*, 416, 69-78. AND SELECTED PORTIONS OF: Prince, E.K.[1], Poulson, K.L.[1], Myers, T.L., Sieg, R.D., & Kubanek, J. (2010). Characterization of allelopathic compounds from the red tide dinoflagellate Karenia brevis. *Harmful Algae*, 10, 39-48. [1]Denotes co-first authorship.

using plankton field assemblages extracellular extracts of two strains of *K. brevis* had no effects on some taxa while stimulating growth of some diatoms, suggesting that in a species-rich ecological community under oligotrophic conditions, the relative importance of *K. brevis* allelopathy may not be as high as most lab studies predict.

Introduction

In planktonic systems, the use of chemical compounds to kill or slow the growth of competitors, a process known as allelopathy, may confer a selective advantage to phytoplankton that are weak exploitation competitors (Adolf *et al.* 2006, Tillmann *et al.* 2008, Poulson *et al.* 2009). Many groups of phytoplankton, including dinoflagellates (Kubanek *et al.* 2005, Adolf *et al.* 2006, Tillmann & Hansen 2009), haptophytes (Uronen *et al.* 2007) raphidophytes (Yamasaki *et al.* 2009), diatoms (Hansen & Eilertsen 2007, Ribalet *et al.* 2007), and cyanobacteria (Suikkanen *et al.* 2006) are allelopathic towards co-occurring species. However, the effectiveness of phytoplankton allelopathy is affected by both biotic and abiotic factors, making it difficult to draw conclusions from simplified lab studies. The presence of specific competitor species can induce the production of allelopathic compounds (Vardi *et al.* 2002) or undermine the effectiveness of allelopathy (Prince *et al.* 2008b). Abiotic influences, such as nutrient concentrations, salinity, light intensity, and temperature, can affect allelopathic potency, as found for the haptophyte *Prymnesium parvum* (Graneli & Salomon 2010). Additionally, allelopathic potency can vary among strains, as shown with the dinoflagellates *Alexandrium ostenfeldii* (Tillmann *et al.* 2008), *Karenia brevis* (Kubanek *et al.* 2005) and *Karlodinium veneficum* (Adolf *et*

al. 2006), making it difficult to predict allelopathic outcomes of genetically diverse blooms.

Although allelopathy is becoming increasingly appreciated based upon laboratory results, the relevance of allelopathy in ecological settings has been challenged, particularly in the process of bloom formation (Flynn 2008, Jonsson *et al.* 2009). A few micro- and mesocosm studies have shown that allelopathic effects can be observed within complex plankton communities, but these effects may be dampened depending on environmental conditions (Fistarol *et al.* 2004, Suikkanen *et al.* 2005). When studying allelopathy, pair-wise interactions between competitors are often investigated using extracellular extracts or cell-free filtrates (e.g. Kubanek *et al.* 2005, Prince *et al.* 2008a, Yamasaki *et al.* 2009), but it is unlikely that outcomes in a biodiverse plankton community will be accurately predicted from the sum of these pair-wise interactions. Additionally, most studies have utilized crude extracts or cell-free filtrates, which do not allow researchers to test for the presence of multiple allelopathic compounds of varying potency released by cells. However, in order to efficiently identify allelopathic compounds or the mechanism(s) by which these compounds affect competitors, simpler pair-wise laboratory experiments are invaluable. Overall, more field-based and multi-species studies of allelopathy and its role in community and bloom dynamics are needed in order to complement mechanistic lab-based investigations.

Karenia brevis is an allelopathic red tide dinoflagellate that blooms most years in the Gulf of Mexico (Tester & Steidinger 1997). Although neurotoxic brevetoxins produced by *K. brevis* cause massive fish kills (Landsberg *et al.* 2009), have been shown to accumulate in shellfish (Plakas *et al.* 2002), and trophic transfer of these compounds

can result in marine mammal mortality (Flewelling *et al.* 2005), these toxins do not appear to be responsible for allelopathy towards most phytoplankton competitors (Kubanek *et al.* 2005, Prince *et al.* 2008a). Previous studies showed that extracellular extracts and filtrates from both *K. brevis* cultures and blooms were allelopathic against multiple competitors, although some species were resistant (Kubanek *et al.* 2005, Prince *et al.* 2008a). *K. brevis* produces multiple allelopathic compounds (Prince *et al.* 2010) which may allow *K. brevis* to suppress several competitors simultaneously. To further explore this hypothesis, we investigated the species-specificity of allelopathic compounds against a suite of susceptible phytoplankton. Additionally, we investigated whether allelopathy is a common trait in *K. brevis* by comparing the allelopathic potency among multiple *K. brevis* strains, as well as the variability in allelopathy within strains.

Although multiple studies have demonstrated the allelopathic effects of *Karenia brevis* against individual competitor species, the relative importance of *K. brevis* allelopathy against a backdrop of other competitive interactions is unknown. To investigate this, a microcosm experiment was conducted in which the inhibitory effect of *K. brevis* extracts was tested against a natural, non-bloom Florida plankton assemblage. Additionally, since *K. brevis* allelopathy can be undermined by other competitor species (Prince *et al.* 2008b), we investigated how species composition, growth stage, and cell concentration of competitors influences allelopathic potency.

Materials and Methods

Phytoplankton culturing

Non-axenic clones of the diatoms *Asterionellopsis glacialis* (strain CCMP 137), *Skeletonema grethae* (CCMP 775) and *Amphora* sp. (CCMP 129); the dinoflagellates *Akashiwo sanguinea* (CCMP 1740), *Prorocentrum minimum* (CCMP 695), and *Karenia brevis* (CCMP 2228, 2229 and the axenic 2281 hereafter "2228," "2229," and "2281," respectively) were obtained from the Provasoli-Guillard National Center for Culture of Marine Phytoplankton (CCMP). Additional strains of *K. brevis* (TxB3 and TxB4, hereafter "TxB3" and "TxB4," respectively) were obtained from Texas A&M University. All cultures were maintained at 22 °C with a 12:12 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} (Biospherical Instruments Inc, model QSL2100). All phytoplankton cultures were grown in L1 + silicate media made with filtered natural seawater from Maine, USA (CCMP). Growth curves and cell concentrations were generated using an Olympus IX-50 inverted microscope with a Palmer-Maloney settling chamber on culture samples preserved with acidified Lugol's solution.

Generation of extracellular extracts

To obtain extracellular extracts of *Karenia brevis* cultures used in high-performance liquid chromatography (HPLC) and subsequent species-specificity experiments (see below) a mixture of three adsorbent resins that remove lipophilic

organic molecules from aqueous media were added to *K. brevis* cultures while in exponential growth stage (3.5×10^3 - 1.6×10^4 cells mL^{-1}; *sensu* Prince *et al.* 2006). This method allows for the extraction of lipophilic compounds released by cells into the media without extracting intracellular material. For all other experiments, a modified protocol was used to reduce the potential for false positives. These extracellular extracts were obtained by adding two resins (XAD -7 and HP-20, Supelco) to cultures of *K. brevis* while in mid to late exponential growth stage ($9.8 \times 10^3 – 3.0 \times 10^4$ cells mL^{-1}), and incubated for 12-15 h. In order to clean the resins prior to addition, both resins were rinsed once with HPLC grade acetone, and eight times with HPLC grade methanol (1 h per rinse). The resins were removed with gentle filtration through Nitex nylon mesh and rinsed with sterile seawater to remove any cells potentially remaining on the resins. Compounds were eluted from the resins using three rinses of HPLC grade methanol after a seawater and deionized water rinse. An extract of 0.2 μm filtered, sterile Maine seawater (CCMP) was also generated in this manner for controls. Seawater was stored at 5 °C in the dark until use. A 48 h allelopathy bioassay using *Asterionellopsis glacialis* was performed as described below to determine which *K. brevis* cultures were allelopathic. The six most allelopathic extracts of *K. brevis* 2228 were combined to yield enough extract for use in mesocosm studies. Preliminary tests of these extracts demonstrated that *K. brevis* 2228 was allelopathic against *A. glacialis*, but extracts of neither *K. brevis* TxB3 nor seawater inhibited *A. glacialis* growth (data not shown).

Comparison of allelopathic potency among multiple strains of *Karenia brevis*

To investigate the degree of variability in allelopathic compound production among populations of *Karenia brevis*, we collected extracellular extracts (as described above) of five representative *K. brevis* strains: CCMP 2228, CCMP 2229, CCMP 2281, TxB3, and TxB4. To explore batch-level variation in allelopathic potency within each strain of *K. brevis*, we repeated this process with six individual batch cultures (100 mL each, in L1 media) of each strain. Cultures were allowed to reach exponential phase before extraction, after ten days of growth.

Bioassay and statistical analysis

To determine the potency of each strain, we exposed the competitor diatom *Asterionellopsis glacialis* to extracellular extracts, and assessed growth after 48 h using *in vivo* fluorescence, measured with a Turner Design TD-700 fluorometer (calibrated with chlorophyll *a*) as described in Prince *et al.* 2008a and b. Briefly, culture tubes filled with 11 mL of Li + Si media were inoculated with 1 mL of *A. glacialis* culture and allowed to acclimate overnight. Following acclimation, a 12 mL equivalent of extract dissolved in DMSO (i.e. the amount of extract from 12 mL of *K. brevis* culture) was added to each replicate tube ($n = 7$ per treatment). A 12 mL equivalent of MESW water extract was also added to control *A. glacialis* culture tubes. Tubes were then blocked, such that each block contained one replicate exposed to each strain and control extract. The growth of *A. glacialis* cultures within each tube was assessed using *in vivo* fluorescence, and % growth was calculated using equation 1:

$$\%\ growth\ of\ competitor(48\,h) = \frac{final\ fluorescence - initial\ fluorescence}{initial\ fluorescence} \times 100$$

In order to determine variation of allelopathic potency within each strain, we normalized the growth of *A. glacialis* relative to the control from each block, using equation 2:

$$\%\ growth\ relative\ to\ control = \left(1 - \frac{\%\ growth\ of\ control(48\,h) - \%\ growth\ of\ treatment(48\,h)}{\%\ growth\ of\ control(48\,h)}\right) \times 100$$

A 2-way ANOVA was used to compare allelopathic potency among and within *K. brevis* strains, with the fixed factor of and strain and random factors of culture batch (GraphPad Prism v.4).

Purification of allelopathic compound(s) exuded by *Karenia brevis*

HPLC fractionation of extracellular *Karenia brevis* extracts

To test the species-specificity of *Karenia brevis* allelopathy, a pooled crude extracellular extract of *K. brevis* strain 2228 was tested against five phytoplankton using the assay described below. Allelopathic compounds from *K. brevis* 2228 were purified from extract using high performance liquid chromatography (HPLC). We utilized a C_{18} reversed-phase silica column (Zorbax SB-C18, 4.6 x 250 mm, 5 μm particle size) with a Waters 1525 binary HPLC pump set to a flow rate of 1 mL min^{-1}. To detect UV absorbance of compounds in the extracts, we used a Waters 2487 dual wavelength detector set to 215 and 254 nm, operated by Waters Breeze software. The separation method utilized a mobile phase of methanol/water (1:3) ramped to 100% methanol over 60 min, resulting in nine fractions (A-I) which were each tested for allelopathic potency against the sensitive competitor *Asterionellopsis glacialis* using the assay described

above. Two fractions (G, F) that were allelopathic towards *A. glacialis* were further fractionated by the same HPLC method, resulting in six yet-unidentified compounds (1-6) used to determine the species specificity of *K. brevis* allelopathic compounds in assays against multiple competitors.

Testing species-specificity of allelopathy

Small cultures (3 mL each) of competitors were used in bioassays to determine the species specificity of *K. brevis* allelopathic compounds. Replicate tubes with 2.8 mL of L1 or L1 + Si media were inoculated with 200 µL of phytoplankton culture (*Asterionellopsis glacialis, Amphora* sp., *Skeletonema grethae*, or *Prorocentrum minimum*). With the slower growing *Akashiwo sanguinea*, tubes containing 2.0 mL media were inoculated with 1.0 mL culture. Assays using crude extracts were conducted using natural concentrations of extract, (i.e., the amount of extract derived from 3 mL of *K. brevis* cultures dissolved in 5 µL dimethylsulfoxide (DMSO)) which was added to treatment tubes. Solvent control tubes received DMSO only (n = 7 tubes per treatment). To determine growth after 48 h, *in vivo* fluorescence of competitor cultures in each tube was measured. To test for the species specificity of individual HPLC fractions, and because *K. brevis* allelopathic compounds are known to be labile (Prince *et al.* 2010), each replicate received twice the natural concentrations of *K. brevis* compounds. Three or four individual treatments were tested against each control and cultures were inverted daily. Approximate cell concentration in each experimental tube was assessed after 48 h using *in vivo* fluorescence (after 96 h for *A. sanguinea*). Percent growth of competitors in each tube was calculated using equation 1. In order to compare between treatments from

different blocked groups, normalized growth was calculated to result in growth relative to controls using equation 2.

Statistical analyses

The percent growth of each treatment after 48 h was compared within groups using a 1-way analysis of variance (ANOVA) with a Tukey HSD post-hoc test using Graphpad Prism v.4 ($p \leq 0.05$) (Zar 1999). After running 1-way ANOVA on a single block, equation 2 was used to plot growth data from multiple blocks in a single figure, but this normalized data was not analyzed in the 1-way ANOVA. In figures where significance is denoted by an asterisk, treatment growth is significantly different relative to control growth in their respective blocks.

Plankton community response to *Karenia brevis* allelopathy

Collection of natural non-bloom plankton assemblages

Natural plankton assemblages were collected from a pier on St. Charles Island, Florida (29.6725 °N, 84.8633 °W, salinity 23 ppt, sea surface temperature 16.5 °C) in December 2009, using a conical phytoplankton net (10 μm mesh, 1 m depth vertical tow, n = 6 tows). Mesozooplankton were removed by filtering samples through 150 μm Nitex nylon mesh. *Karenia brevis* was not observed in these samples. After each tow, concentrated plankton samples were brought to twice natural densities using glass fiber filtered Gulf of Mexico seawater.

Microcosm preparation

To determine how allelopathy alters plankton community structure, Florida assemblages were exposed to *Karenia brevis* extracts from either the non-allelopathic TxB3 strain or the allelopathic 2228 strain. Controls were exposed to seawater extracts. The six diluted tow samples were each split into three treatment bottles (400 mL, $n = 6$ replicates per treatment). Each replicate within a treatment came from a separate tow. Bottles were incubated at Georgia Tech (22 °C, 12:12 h light/dark cycle). Extracts of seawater or *K. brevis* equivalent to 400 mL of culture were added to replicate bottles using DMSO as a carrier solvent (667 μL per bottle).

Data collection and analysis

Replicate bottles were sampled prior to addition of extract and after 48 h exposure. To measure changes in community structure, 5 mL aliquots of acid Lugol's preserved samples were settled in Utermöhl settling chambers for 20 minutes, and diatoms, dinoflagellates, and microzooplankton were enumerated. Other plankton groups were not used in analysis due to inadequate abundances. Diatoms were further classified into pennate and chain diatoms. Genera of diatoms from a subset of bottles were identified.

The growth of chain diatoms, pennate diatoms, dinoflagellates, and microzooplankton over 48 h was used to determine the effects of *Karenia brevis* extracts on the assemblage. Prior to analysis, growth of each plankton group was converted using equation 3:

$$\%\, growth\, of\ \ plankton\, group\,(48\,h) = \frac{cell\, concentration\,(48\,h) - cell\, concentration\,(0\,h)}{cell\, concentration\,(0\,h)} \times 100$$

The percent growth of each plankton group after 48 h across treatments was compared using a 1-way ANOVA with a Tukey's HSD post-hoc test ($p \leq 0.05$). Paired *t*-tests were conducted to compare mean cell densities of the different plankton groups in a given treatment between 0 h and 48 h. All statistical analyses were conducted using JMP 8 (SAS).

Testing allelopathic outcomes with phytoplankton co-cultures

To test whether competitors could influence each other's susceptibility to allelopathy, crude extracellular extracts of *Karenia brevis* were tested for allelopathic activity against *Asterionellopsis glacialis* and *Skeletonema grethae* in co-culture, using a 48 h allelopathy assay. Cultures were grown to mid-exponential growth phase (3.0×10^5 cell mL^{-1} and 1.4×10^5 cells mL^{-1} for *S. grethae* and *A. glacialis*, respectively), and exposed to *K. brevis* 2228 or seawater extract at natural concentrations as described for the species-specificity assay. Tubes contained *S. grethae* monocultures, *A. glacialis* monocultures, or a co-culture of *A. glacialis* and *S. grethae* (5 mL culture per tube, $n = 8$ per treatment). To prepare co-culture treatments, 2.5 mL of each competitor was added to each replicate tube resulting in a final total cell concentration similar to that in monocultures. Cultures were incubated for 48 h and measured for changes in fluorescence. After each measurement, an aliquot of culture from each tube was preserved to determine cell concentrations. Percent growth of cultures was calculated using equation 1. Statistical differences in growth were determined using an unpaired t-test.

Effects of competitor cell concentration and growth stage on *Karenia brevis* allelopathic potency

To determine if the susceptibility of *Skeletonema grethae* to *Karenia brevis* allelopathy is contingent on *S. grethae* cell concentration, various cultures of *S. grethae* were exposed to *K. brevis* 2228 extracts. Three cell concentrations of *S. grethae* were used in this experiment: "High" (average concentration $(1.50 \pm 0.26) \times 10^6$ cell mL^{-1}), "Medium" (average concentration $(4.14 \pm 0.22) \times 10^4$ cell mL^{-1}), and "Low" (average concentration $(5.57 \pm 1.23) \times 10^3$ cell mL^{-1}). *K. brevis* and seawater extracts were added at natural concentrations ($n = 10$ for all treatments). Cell concentration was approximated via fluorescence, and percent growth over 48 h was calculated using equation 1. An unpaired t-test was used to compare growth of *S. grethae* at a particular concentration exposed to *K. brevis* extract from cells exposed to seawater extract. A 1-way ANOVA with a Tukey HSD post-hoc test ($p < 0.05$) was used to compare the growth of different densities of *S. grethae* exposed to seawater or *K. brevis* extract.

To test whether cell growth stage is important in determining the susceptibility of *Skeletonema grethae* to *Karenia brevis* 2228, *S. grethae* cultures were grown until reaching either lag, exponential or stationary growth stages and then centrifuged three times (5 min, 4,000 rpm) to pellet cells, rinsing with sterile seawater between spins to ensure the removal of excess nutrients from media. The cells were re-suspended in sterile seawater, aliquots were transferred into tubes, and brought up to a final cell concentration that mimicked that of cultures in lag phase ($(5.41 \pm 3.90) \times 10^5$ cell mL^{-1}, 3 mL final volume), ensuring that tubes contained *S. grethae* cells of differing physiological state at equal cell concentrations. A new batch of *K. brevis* 2228 extract was generated for this

experiment, and either 2228 or seawater extracts were added, growth was measured, and statistical analyses were run as in the cell density experiment above.

Results

Karenia brevis allelopathy is variable within and among strains

At least one batch of every *K. brevis* strain was allelopathic towards *A. glacialis*, but strains varied in allelopathic potency and frequency (Figure 3.1). The effects of both batch and strain were significant (2-way ANOVA, $p < 0.0001$ for both) as well as the interaction of batch and strain (2-way ANOVA, $p < 0.0001$). *K. brevis* strain 2228 was generally allelopathic, with five out of six batch cultures significantly inhibiting *A. glacialis* growth compared to controls (Figure 3.1; $p < 0.001$ for all, 2-way ANOVA with Bonferroni correction). This strain was also the most potent and caused negative growth of *A. glacialis*, indicating *A. glacialis* cell death had occurred. Strain 2229 reduced *A. glacialis* growth by an average of 78% across six batches compared to controls (Figure 3.1). Five of six *K. brevis* 2229 cultures were significantly allelopathic ($p < 0.001$-0.05) suggesting that strain 2229 was as consistently allelopathic as strain 2228, but perhaps not as potent. Half of the batch cultures of strain 2281, our only axenic strain of *K. brevis,* were inhibitory towards *A. glacialis* ($p < 0.01$-0.001) and caused a mean 35% reduction of *A. glacialis* growth across all batches. One batch extract of *K. brevis* 2281 significantly stimulated the growth of *A. glacialis* ($p < 0.05$). Of the two strains originally isolated from the Texas coast, TxB4 was rarely allelopathic, only one of the six cultures significantly inhibited the growth of *A. glacialis* ($p < 0.001$; all others $p > 0.05$), resulting in an average 40% reduction of *A. glacialis* growth. Strain TxB3 was moderately

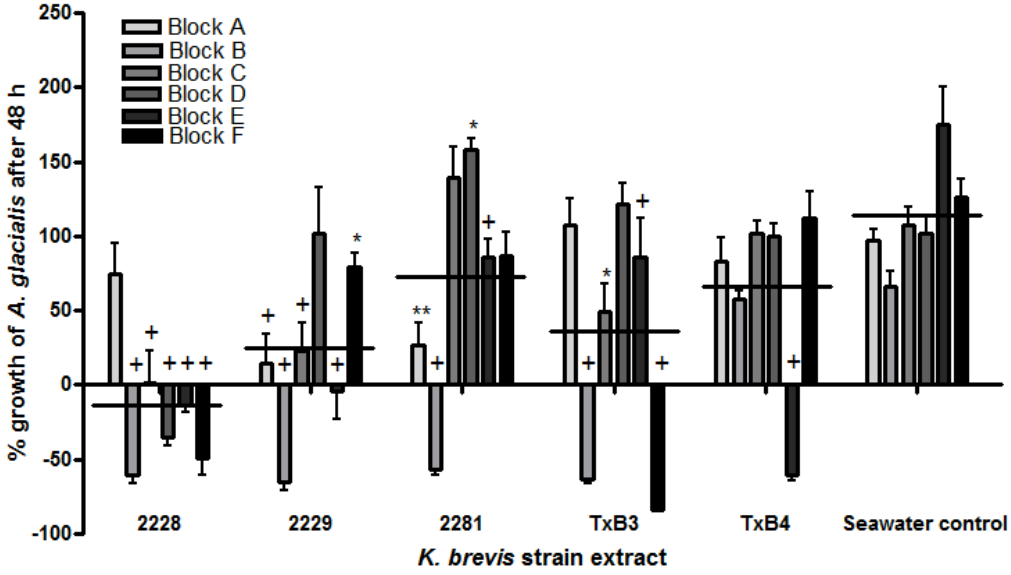

Figure 3.1. Allelopathic effects of extracellular extracts of multiple *K. brevis* strains on the growth *A. glacialis* over 48 h. Solid line indicates average % growth of *A. glacialis* across all batches. Symbols (+, **, *) indicate significant difference in % growth from control within the same block (2-way ANOVA with Bonferroni post test, $n = 7$, "+" = $p < 0.001$, "**" = $p < 0.01$, "*" = $p < 0.05$). Error bars in this and all subsequent figures indicate one standard error.

allelopathic; four of six cultures were inhibitory ($p < 0.001$-0.05), causing a 68% reduction in growth of *A. glacialis* versus controls). The extract of at least one batch of each strain caused cell death of *A. glacialis* (i.e. negative growth values) compared to controls indicating that, while variable, all strains of *K. brevis* examined are capable of producing allelopathic compounds.

Karenia brevis is allelopathic to multiple phytoplankton species

Multiple competitors were inhibited by *Karenia brevis* 2228 extracellular extracts (Figure 3.2). Diatoms *Amphora* sp., *Asterionellopsis glacialis*, and *Skeletonema grethae* all experienced decreased growth relative to controls when exposed to allelopathic *K. brevis* extracts for 48 h, with *A. glacialis* and *S. grethae* suffering negative growth whereas *Amphora* sp. growth was impaired but still positive (all $p < 0.001$ vs. controls; Figure 3.2). Dinoflagellates *Akashiwo sanguinea* and *Prorocentrum minimum* appeared to be the most sensitive of the competitors tested, with mortality occurring over 48-96 h ($p < 0.001$ for both; Figure 2). *K. brevis* was therefore allelopathic towards all five competitor species tested.

Species-specificity of allelopathic compounds

K. brevis exudates clearly contain multiple allelopathic compounds, encompassing a range of polarities and potencies. Of the nine partially purified fractions from the first round of HPLC purification of extracellular *K. brevis* extracts three

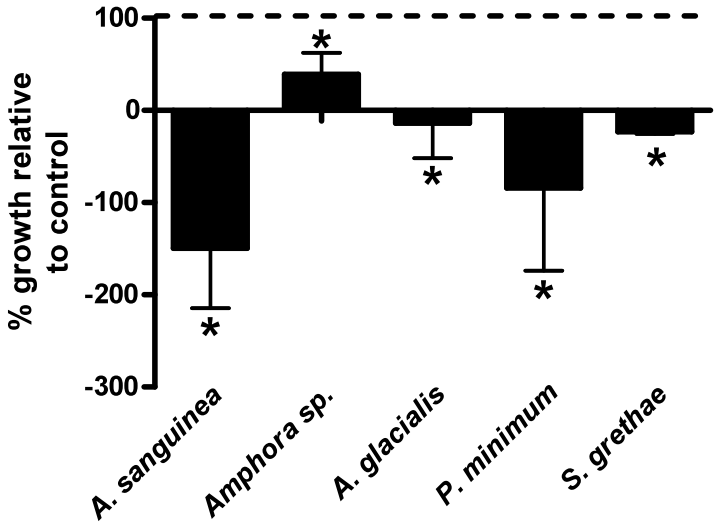

Figure 3.2 Effects of *Karenia brevis* 2228 extracellular extracts on growth of five Gulf of Mexico phytoplankton species over 48 h (except *Akashiwo sanguinea*, studied over 96 h). Dashed line in this and subsequent figures indicates growth equivalent to controls. Asterisks (*) indicate growth significantly different from controls (1-way ANOVA with Tukey post hoc, $p \leq 0.05$, $n = 7$, except $n = 6$ for *A. sanguinea)*.

(fractions A, F, and G) were allelopathic towards *Asterionellopsis glacialis* (Figure 3.3). The reconstituted fraction (ALL) killed *A. glacialis* cell as evidenced by a 156% reduction in growth relative to controls ($p < 0.001$). HPLC fraction A was the most polar and most potent, as it killed *A. glacialis* cells (110% inhibition relative to controls, $p < 0.001$). Fraction A eluted from the reversed-phase HPLC column with 25-48% aqueous methanol. Two allelopathic fractions (F and G) caused sublethal reductions in *Asterionellopsis glacialis* growth, by 25% and 22%, respectively ($p < 0.05$; Figure 3.3B). These fractions eluted from the reversed-phase HPLC column with 85-95% aqueous methanol, indicating they were more lipid-soluble than fraction A. Brevetoxin B (PbTx-2) eluted with HPLC fraction C (concentration 2.7 ng mL^{-1}culture) was not allelopathic ($p > 0.05$, Figure 3.3B). No brevetoxins were detected fractions A, F, and G, confirming that brevetoxins are generally not allelopathic (e.g. Kubanek *et al.* 2005, Prince *et al.* 2008b).

Further HPLC purification of the above allelopathic fractions yielded six compounds (1-6), with variable allelopathic effects against four competitor species (Figure 4). The diatom *Amphora* sp. was not significantly inhibited by either of thes fractions (data not shown). Compound 2 was allelopathic to *Asterionellopsis glacialis, Skeletonema grethae*, *Prorocentrum minimum*, and *Akashiwo sanguinea* (77%, 73%, 54%, and -356% growth relative to controls, respectively). Sensitivity to other *K. brevis* compounds varied among competitor species with, for example, *A. glacialis* suffering negative effects from compounds 1, 2, 4, and 5 whereas *S. grethae* was harmed by compounds 2, 5, and 6 (Figure 3.4).

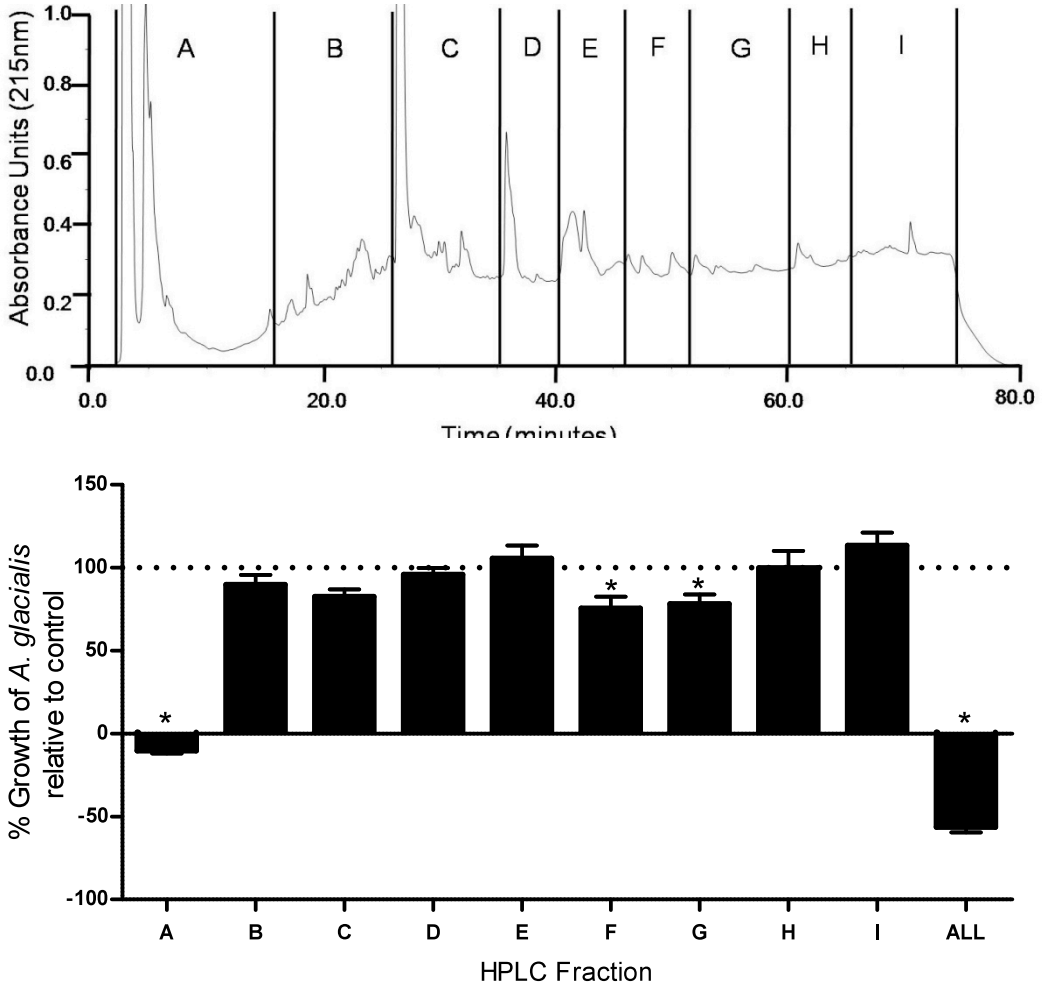

Figure 3.3. HPLC fractionation of allelopathic compounds from *Karenia brevis.* (Top) C_{18} reversed-phase HPLC-UV chromatogram. (Bottom) Suppression of diatom *Asterionellopsis glacialis* exposed to HPLC fractions A-I. Dashed line represents growth equivalent to controls. "ALL" represents a treatment recombining all HPLC fractions at natural concentrations. Asterisks (*) indicate significant inhibition of growth relative to controls (1- way ANOVA with Tukey post-hoc test; $p \leq 0.01$, $n = 7$).

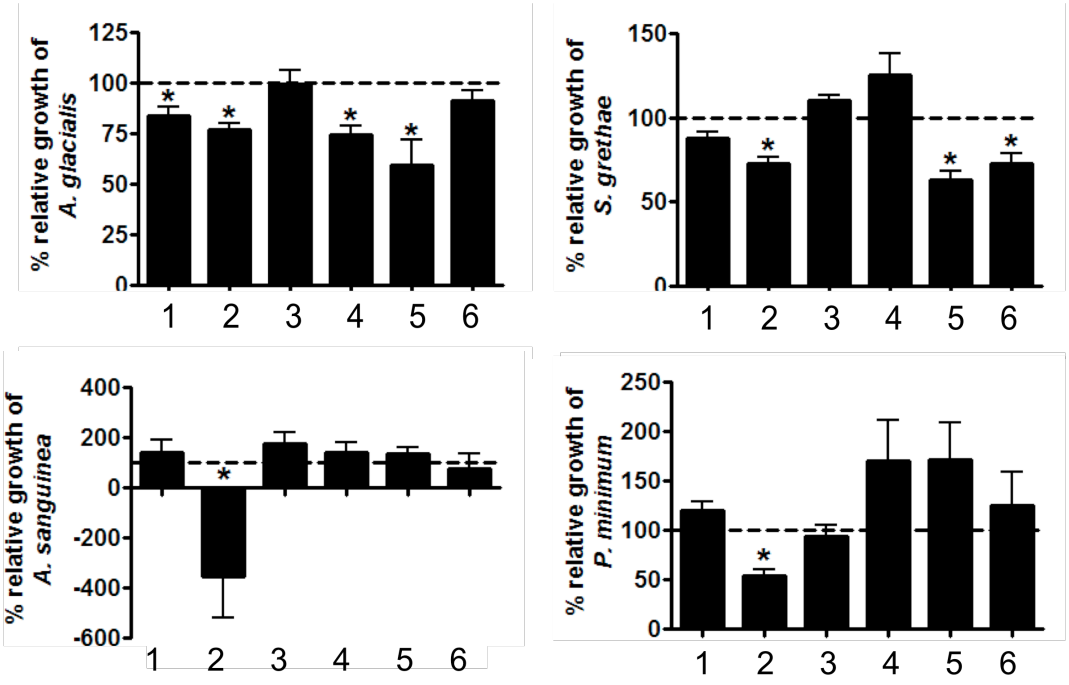

Figure 3.4. Allelopathic effects of lipophilic *Karenia brevis* 2228 compounds 1-6 on (top, left) diatom *Asterionellopsis glacialis,* (top, right) diatom *Skeletonema grethae,* (bottom, left) dinoflagellate *Akashiwo sanguinea,* (bottom, right) dinoflagellate *Prorocentrum minimum.* Asterisks (*) indicate growth significantly different ($p \leq 0.05$) from controls after 48 h for *A. glacialis*, *S. grethae*, and *P. minimum*, and after 96 h for *A. sanguinea* ($n = 7$ for all).

Plankton community responses to *Karenia brevis* allelopathy

In the microcosm study using a natural < 150 μm plankton assemblage from the Gulf of Mexico, *Karenia brevis* allelopathy was not evident. Contrary to lab-based results, allelopathic *K. brevis* 2228 extracts did not significantly kill or suppress growth of any phytoplankton groups analyzed (Figure 3.4). When comparing the effects of *K. brevis* vs. seawater extracts on growth of diatoms over 48 h, *K. brevis* 2228 was neither allelopathic nor stimulatory towards chain or pennate diatoms (Figure 3.5), and *K. brevis* TxB3 was significantly stimulatory to chain diatoms, but did not affect pennate diatoms (Figure 3.5). Pennate diatoms grew substantially over 48 h in all treatments, including those exposed to seawater extracts (Figure 3.5). Chain diatoms grew when exposed to *K. brevis* extracts, but not when exposed to seawater controls (Figure 3.5). Concentrations of dinoflagellates and microzooplankton were not significantly altered regardless of the *K. brevis* extract to which they were exposed, although microzooplankton exposed to only seawater extract decreased in abundance (Figure 3.5).

At the start of the microcosm experiment, chain diatoms of the genus *Skeletonema* were the dominant diatoms observed, accounting for 72% of diatom cells counted. After 48 h, *Skeletonema* spp. were still the dominant diatoms in all treatments, accounting for 53-80% of diatom cells. Other major diatoms belonged to the genera *Pseudo-nitzchia, Chaetoceros, Coscinodiscus,* and *Cylindrotheca*, although none of these groups ever accounted for more than 10% of the total diatom community at either time point (data not shown). Overall, diatoms from the Florida assemblage were not harmed by exposure to *Karenia brevis* extracts (Figure 3.5).

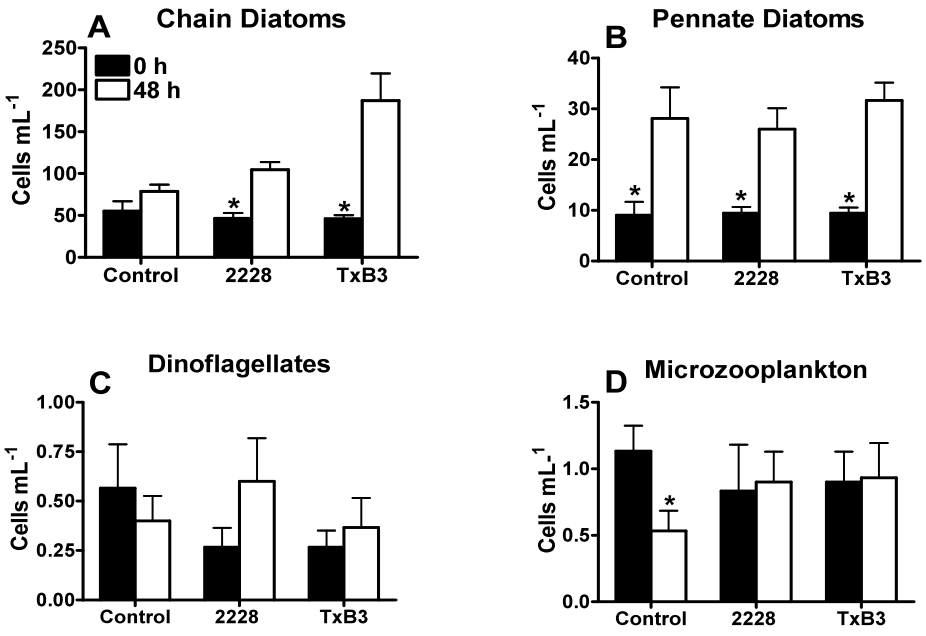

Figure 3.5. Population densities of a natural Florida plankton assemblage of (A) chain diatoms, (B) pennate diatoms, (C) dinoflagellates, and (D) microzooplankton after 48 h exposure to seawater extracts (control), *Karenia brevis* allelopathic extracts (2228), or *K. brevis* non-allelopathic extracts (TxB3). Statistical differences in growth after 48 h determined by t-test ($p < 0.05$, $n = 6$) and are indicated by an asterisk.

Competitor co-cultures respond differently to *Karenia brevis* allelopathy

In an experiment utilizing pooled *Karenia brevis* 2228 extract that was previously shown to be allelopathic to *Asterionellopsis glacialis* (data not shown), growth of the diatom *A. glacialis* in monoculture was inhibited by 76% compared to controls ($p < 0.001$), but *Skeletonema grethae* in monoculture was not affected (Figure 3.6A). When exposed to the same *K. brevis* 2228 extract in co-culture, growth of both *A. glacialis* and *S. grethae* was suppressed ($p < 0.0001$, Figure 3.6A), with proportions of *A. glacialis* and *S. grethae* remaining unchanged in both treatments and controls (Figure 3.6B).

Importance of competitor cell concentration and growth stage in *Karenia brevis* allelopathy

When exposed to *Karenia brevis* 2228 extracts used in the co-culture experiment, growth of *Skeletonema grethae* at low and medium initial cell concentrations was significantly inhibited after 48 h ($p = 0.046$ and 0.0023, respectively, Figure 3.6C). Cells at initial concentrations similar to that used in the monocultures of the co-culture experiment above were not affected by exposure to *K. brevis* 2228 extracts ($p = 0.46$, Figure 3.6C). Significant differences between the growth of *S. grethae* control cultures at all three concentrations were also detected (1-way ANOVA, $p < 0.05$, Figure 3.6C).

Growth of *Skeletonema grethae* at different growth stages was differentially affected by the addition of *Karenia brevis* 2228 extracts, even when initial cell

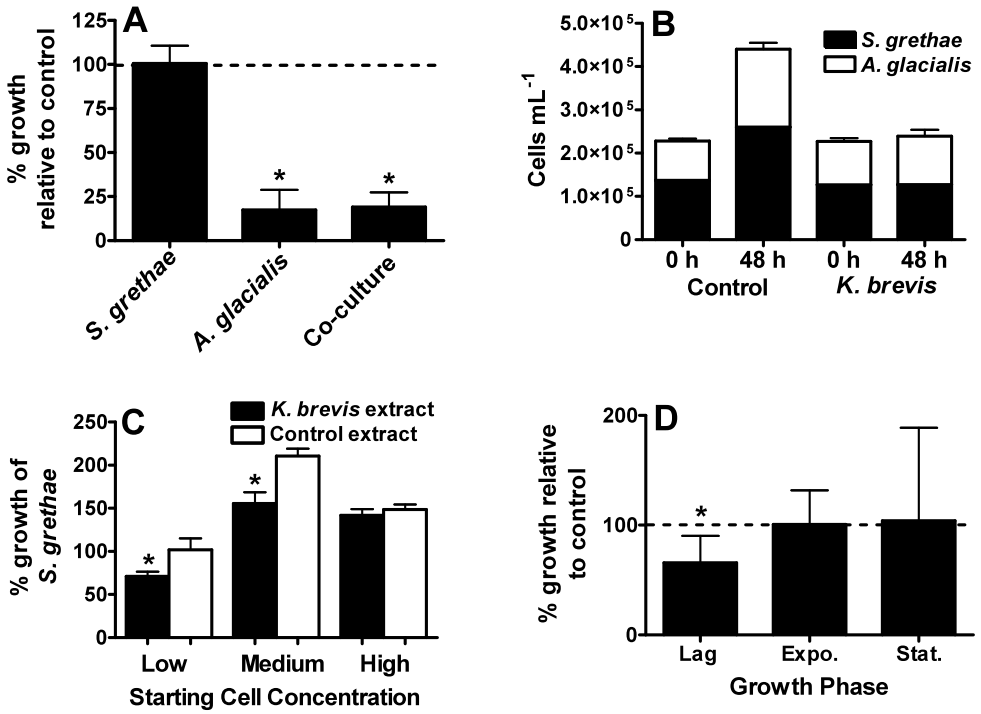

Figure 3.6. Effects of *Karenia brevis* 2228 extracellular extracts after 48 h exposure on A) monocultures and co-cultures and B) co-cultures only of *Asterionellopsis glacialis* and *Skeletonema grethae*. C) Effects of *K. brevis* 2228 extracts after 48 h exposure on *S. grethae* of varying starting cell concentrations. D) Effects of *K. brevis* 2228 extracts on *S. grethae* cells at different growth stages normalized to the same cell concentration. Asterisks (*) represent significant differences between treatments and controls (unpaired t-test, $p < 0.05$).

concentration of *S. grethae* was held constant (Figure 3.6D) . Growth of *S. grethae* in lag phase was significantly inhibited by *K. brevis* 2228 extracts ($p = 0.028$, unpaired t-test), whereas growth of *S. grethae* from both exponential and stationary growth phases were not significantly inhibited, when compared to controls (Figure 3.6D).

Discussion

Multiple *Karenia brevis* strains are allelopathic towards at least one competitor

Each *K. brevis* strain we tested exhibited allelopathy towards *A. glacialis*, but potency and frequency of allelopathic effects varied widely by strain. This variation among strains suggests that all strains have the genes to produce allelopathic compounds. In a study by Tillmann *et al.* (2010), 65 of 67 strains of *Alexandrium tamarense* were allelopathic towards *Rhodomonas salina,* indicating that allelopathy is a common, and perhaps important, trait in *A. tamarense* populations. There was more variation in the potency of these same *A. tamarense* strains against *Oxyrrhis marina*, which was more resistant to allelopathy than *R. salina*, also indicating a species-specific effect of the allelopathic compounds produced by *A. tamarense* (Alpermann *et al.* 2010). Unlike *A. tamarense*, however, *K. brevis* exhibits greater inter-strain allelopathic variability. Of the *K. brevis* strains tested, 2228 and 2229 were the most potent and most commonly allelopathic (Figure 3.1) whereas strain 2281 appeared least potent, although half of the cultures examined were inhibitory towards *A. glacialis.* Strains isolated from Texas waters, TxB3 and TxB4 were also allelopathic against *A. glacialis*.

Inter-strain variability in toxin production appears common among phytoplankton. Studies have demonstrated significant inter-strain (or intraspecific)

differences in phytoplankton toxin production, including with *K. brevis* (Errera et al. 2009, 2011) and *Alexandrium ostenfeldii* (Tillmann *et al.* 2007). In some cases intraspecific differences can be greater than interspecific variation in toxin production (e.g., Thessen *et al.* 2009). These inter-strain differences in *K. brevis* allelopathy potency could be due to variable concentrations of allelopathic compounds between strains, differences in the identity of individual of allelopathic compounds produced by each strain or a combination of both (Alpermann *et al.* 2010). This suggests that genetically diverse blooms of *K. brevis* (Henrichs *et al.* 2013) may vary in allelopathic potency depending upon the dominant genotypes present, and in fact, *K. brevis* blooms have been shown to differ in potency between years (Prince et at 2008b).

Perhaps more striking than the inter-strain variation in allelopathic potency is that allelopathy varies greatly among batch cultures of the same *K. brevis* genotype. Although small differences in pH, salinity, cell concentration, or physiological state of *K. brevis* could have existed between the batches tested here, cultures of all strains were grown in identical nutritional and light regimes. Additionally, the allelopathic potency of each culture did not correlate with final *K. brevis* cell concentration as measured by *in vivo* fluorescence, ($r^2 = 0.029$, $p = 0.37$ for all cultures) indicating that differences in cell concentration between culture flasks were not responsible for the observed variation in potency. Previous studies have found no correlation between *K. brevis* cell concentration and allelopathic potency of blooms or cultures of *K. brevis* (Prince *et al.* 2008b). Similar differences in allelopathic potency between batch cultures of *Alexandrium ostendfeldii* have been reported, although causes for this have not been fully investigated (Tillmann *et al.* 2007).

It is likely that allelopathic compounds are not produced by bacteria in the media but rather by *K. brevis* itself. Three of six culture extracts from the axenic strain 2281 significantly reduced *A. glacialis* growth versus controls, and one culture stimulated *A. glacialis* growth (Figure 3.1). Moreover, the allelopathic potency of the non-axenic strain TxB4 is indistinguishable from that of 2281, and the allelopathic effect of TxB4 was driven by only one allelopathic culture that caused 60% mortality of *A. glacialis* inoculum (Figure 3.1). Therefore, while 2281 was not particularly potent compared to strains 2228 and 2229, strain 2281 was more commonly allelopathic than TxB4 (Figure 3.1).

Bacteria may, however, influence *K. brevis* allelopathy. Although stock cultures of *K. brevis* strain 2281 have been maintained axenically, the cultures used for this experiment were not tested for the presence of bacteria before extraction. It is possible that different bacterial communities associated with each *K. brevis* strain could exist. How they would impact allelopathic potency is unclear but could include bacterial production of compounds, or that bacterial presence could induce the production of compounds in *K. brevis*. Associated bacteria could also supply necessary metabolite precursors for allelopathic compound production in *K. brevis*, or vice versa. For instance, a tight association with bacteria is crucial for high levels of domoic acid production by *Pseudo-nitzschia* spp., (Bates *et al.* 1995, Bates *et al.* 2004). However, it is unlikely that vast differences in bacterial community cultures would occur between batch cultures of the same *K. brevis* strain inoculated at the same time. Thus, it is more likely for differences in bacterial communities between strains to explain inter-strain variability in allelopathic potency. Together, these data suggest that the production of allelopathic

compounds by *K. brevis* is a genetically fixed trait, while expression of the allelopathic phenotype within each strain is likely dictated by unknown epigenetic, ecological (e.g. Prince *et al.* 2008) and/or abiotic factors.

Different *Karenia brevis* compounds are allelopathic to different competitors

Karenia brevis produces a suite of allelopathic compounds, some of which inhibit multiple competitors and others that are allelopathic only towards certain species (Figures 3.3, 3.4). Purification of several compounds from *K. brevis* 2228 extracellular extracts led to one compound (2) that was allelopathic towards four of the five phytoplankton species tested, including two diatoms and two dinoflagellates (Figure 3.4). Additionally, compound 5 inhibited the growth of the diatoms *Skeletonema grethae* and *Asterionellopsis glacialis,* Figure 3.4A, B). *S. grethae* was also inhibited by compound 6, whereas *A. glacialis* was inhibited by compounds 1 and 4. The diatom *Amphora* sp. was not inhibited by any *K. brevis* HPLC fractions collected (data not shown), despite being inhibited by crude extracellular extracts of *K. brevis,* which could suggest synergistic effects of *K. brevis* compounds against certain competitors or decomposition of compounds that are allelopathic towards *Amphora* sp. These results demonstrate that not all diatom species are equally susceptible to the same allelopathic compounds (Figure 3.4A, B). In contrast, the dinoflagellates tested in this study (*Akashiwo sanguinea* and *Prorocentrum minimum*) were only inhibited by a single compound (2, Figure 3.4C, D). To date we have not been successful in identifying the full molecular structures of allelopathic compounds from *K. brevis*, although lipophilic compounds employed in the current study have molecular weights of 500-1000 Da and possess aromatic functional

groups (Prince *et al.* 2010). Since *K. brevi*s is likely to be exposed to multiple competitor species in a natural assemblage, it could be beneficial to produce multiple allelopathic compounds in order to ensure a competitive advantage.

Species-specific allelopathic effects appear to be common in the plankton. For instance, the dinoflagellate *Karlodinium micrum* produces karlotoxins (Bachvaroff *et al.* 2008) that inhibit some competitors, but has not effect on others (Adolf *et al.* 2006). Allelopathic *Alexandrium* spp. had species-specific impacts in both field (Fistarol *et al.* 2004, Hattenrath-Lehman and Gobler 2011), and lab based studies (Tillmann & Hansen 2009). These examples suggest that competitors vary in susceptibility to filtrates or crude extracts containing multiple compounds released by an allelopathic species, but they do not tease apart whether this is due to compounds that target different competitors, or to inherent differences in competitor susceptibility to a single allelopathic compound. The present study demonstrates that different compounds are responsible for the varying susceptibility of different species to *K. brevis* allelopathy.

In a complex ecological community, the allelopathic effects of *Karenia brevis* are dampened

Although experiments with individual competitor species demonstrated that *Karenia brevis* 2228 is allelopathic towards multiple competitors (Figures 3.2, 3.4), these extracts did not kill or significantly inhibit the growth of phytoplankton in a natural community assemblage (Figure 3.5). The non-allelopathic *K. brevis* strain TxB3 significantly stimulated the growth of chain diatoms (Figure 3.5). It is possible that extracts from *K. brevis* 2228 (previously shown to be allelopathic against *Asterionellopsis*

glacialis) had a slight allelopathic effect on the plankton assemblage, but stimulation from trace amounts of organic nitrogen, phosphorous, or vitamins extracted with *K. brevis* exudates may have been sufficient to outweigh inhibitory effects. When exposed to extracts from the non-allelopathic TxB3 strain, such a stimulatory effect would be more obvious as seen in Figure 3.5A, since there would be no counter-acting allelopathic effect. These stimulatory effects would be expected to be more pronounced when studying a resource-limited natural assemblage compared to a lab culture grown under nutrient-replete conditions. Similar results were found in another microcosm study, in which filtrates from three cyanobacterial species were stimulatory towards multiple community members, whereas in lab-based studies, these competitors were inhibited (Suikkanen *et al.* 2005). This highlights the difficulty in separating the effects of exploitation competition and interference competition (i.e., allelopathy) in more complex natural settings, as opposed to highly controlled lab experiments.

The identity and population density of the dominant community member may affect the overall outcome of *Karenia brevis* allelopathy

The ability of some competitors to undermine *Karenia brevis* allelopathy (Prince *et al.* 2008b) could also explain the lack of observed allelopathy for the natural assemblage used in this study. Our Florida phytoplankton plankton assemblage was dominated by members of the genus *Skeletonema,* one of which (*S. grethae*) was previously shown to reduce the allelopathic potency (towards *S. grethae*) of *K. brevis* cultures and field samples by an unknown mechanism (Prince *et al.* 2008b). Among 2005 and 2006 Florida field collections all dominated by *K. brevis*, the presence of

Skeletonema sp. appeared to be associated with less allelopathic samples (Prince *et al.* 2008b). However, whether *Skeletonema* protects other phytoplankton from *K. brevis* allelopathy is unknown. In the current study, the lack of an inhibitory effect of *K. brevis* extracts towards the natural phytoplankton assemblage could have been due to *Skeletonema* spp. undermining *K. brevis* allelopathy towards the phytoplankton assemblage at large. Since our Gulf of Mexico field assemblages were dominated *Skeletonema* spp., we wanted to test how allelopathic outcomes are affected by interactions among competitor species.

To determine how phytoplankton are affected by allelopathy when in the presence of other phytoplankton species, we tested allelopathic effects of *Karenia brevis* on co-cultures of *Skeletonema grethae* and another susceptible competitor which may undermine *K. brevis* allelopathy as well, the diatom *Asterionellopsis glacialis* (Prince *et al.* 2008b). In co-culture, both diatoms were similarly inhibited by *K. brevis* 2228 extracts, although in this experiment *S. grethae* was not inhibited when grown alone (Figure 3.6A-B). Rather than implying a protective effect with allelopathy being undermined by one of these species, the enhanced susceptibility of *S. grethae* in co-culture may be related to the initial population density of this diatom, since initial cell concentrations of *S. grethae* grown alone were twice that of *S. grethae* grown in co-culture with *A. glacialis*.

Competitor population density and physiological state drives *Karenia brevis* allelopathic outcomes

The population density of competitors is known to be important in allelopathic interactions. Allelopathic effects of the dinoflagellate *Alexandrium ostenfeldii* were lower when competitor population density was high, suggesting a possible saturation effect whereby individual cells adsorb or absorb allelopathic compounds, mitigating their damage towards other cells (Tillman *et al.* 2007). Similarly, lytic effects of the mixotrophic haptophyte *Prymnesium parvum* towards the dinoflagellate *Oxyrrhis marina* were decreased in response to increased *O. marina* cell concentrations (Tillmann 2003). In the current study, *Skeletonema grethae* of higher initial concentration was less susceptible to *Karenia brevis* allelopathic extracts than *S. grethae* of low or medium cell concentration (Figure 3.6C), complementing the findings of Tillman *et al.* (2007). However, cell concentrations of *Skeletonema* spp. within our natural plankton assemblage were lower than any of the concentrations used in the experiment in which we varied initial *S. grethae* concentration, yet field-collected chain diatoms (dominated by *Skeletonema* spp.) were resistant to *K. brevis* 2228 allelopathy. Additionally, when exposed to *K. brevis* TxB3 extracts, the growth of chain diatoms in the assemblage was stimulated, perhaps from trace amounts of organic nutrients and vitamins extracted from the culture media. This stimulatory effect of *K. brevis* extracts suggests that diatoms were nutrient-limited in the field, and therefore in a physiological state analogous to stationary growth. If stationary phase phytoplankton are less susceptible to *K. brevis* allelopathy than more physiologically active cells, this could explain the lack of observed allelopathy in the microcosm experiment.

In support of the hypothesis that growth stage is important to sensitivity towards allelopathy, *Skeletonema grethae* was most sensitive to *Karenia brevis* 2228 extracts in

lag phase, and in this experiment not sensitive in exponential growth or stationary phase (even though cultures were normalized to the same starting cell concentrations; Figure 3.6D). This indicates that varying physiological states associated with these different growth stages are important factors in determining *S. grethae* sensitivity to allelopathy. *S. grethae* cells may produce and release stage-specific compounds that defend cells from *K. brevis* allelopathic compounds. *Skeletonema marinoi* produces a wide variety of metabolites at specific growth stages, and exudates of this species have been shown to affect the growth and cellular functions of other phytoplankton species (Barofsky *et al.* 2009, Paul *et al.* 2009). The possibility that *S. grethae* also produces stage-specific compounds that are capable of defending against or undermining *K. brevis* allelopathy warrants further study. Alternatively, cells in lag phase may suffer from a trade-off between certain cellular functions and the ability to defend themselves from allelopathic compounds produced by *K. brevis*. Finally, the difference in cell surface area-to-volume ratio of cells and/or chains of *S. grethae* at these different growth stages may also dictate how susceptible these cells are to allelopathic compounds. Specifically, allelopathic compounds may more rapidly contact potential cellular targets of smaller *S. grethae* cells or chains. It may also benefit *Karenia brevis* to produce multiple compounds if competitor cells from natural assemblages are in different physiological states and these different cells vary in their sensitivity to allelopathy.

Conclusions

The dinoflagellate *Karenia brevis* produces multiple compounds that are allelopathic towards several phytoplankton species, but it is evident that competitors are susceptible to slightly different suites of compounds. Additionally, multiple strains of *K. brevis* are allelopathic to at least one competitor, but allelopathic potency is highly variable within and among *K. brevis* strains. Competitor growth stage and cell concentration appear to play important roles in determining the effectiveness of *K. brevis* allelopathic compounds, with cells in lag phase more susceptible to *K. brevis* allelopathy than cells in later growth stages. The relative resistance of stationary phase cells may explain why phytoplankton in the nutrient-limited microcosm experiment did not suffer allelopathic effects of *K. brevis*. It may be advantageous for *K. brevis* to produce multiple allelopathic compounds if a diverse chemical arsenal provides protection against a variety of phytoplankton competitors under a range of ecological conditions. However, the effects of *K. brevis* allelopathy in the field may be mild relative to the growth inhibition observed in nutrient-replete lab experiments. Although pair-wise lab experiments can be useful in permitting a deeper understanding of mechanistic aspects of competitive interactions, they may not accurately predict ecological outcomes in the field when one considers the complexity of multi-species interactions.

CHAPTER 4:

LOCATION, LOCATION, LOCATION: HOW SENSITIVE ARE OFFSHORE PHYTOPLANKTON TO KARENIA BREVIS ALLELOPATHY?

Abstract

The bloom-forming dinoflagellate *Karenia brevis* produces a suite of allelopathic compounds that inhibit the growth of several phytoplankton competitors in laboratory experiments. However, it is less clear how allelopathy affects competition in the field, including whether allelopathic compounds impact *K. brevis* bloom dynamics. We investigated the extent to which phytoplankton species typically found offshore in the Gulf of Mexico, where *K. brevis* blooms initiate, are sensitive to *K. brevis* allelopathy. Natural assemblages of offshore phytoplankton dominated by diatoms were largely resistant to *K. brevis* allelopathy, even experiencing slight stimulation of growth from exposure to *K. brevis* chemical cues. When tested in pair-wise lab experiments, four diatom species found offshore in the Gulf of Mexico exhibited a similar degree of resistance to *K. brevis*. In addition, four diatom species whose near shore habitats are prone to *K. brevis* blooms were not at all stimulated by *K. brevis* exudates, and exhibited similar resistance trending toward sensitivity to allelopathy relative to offshore species. Overall, Gulf of Mexico phytoplankton that co-occur with *K. brevis* blooms in both near shore and offshore environments responds similarly to *K. brevis* allelopathy. Therefore, *K. brevis* may benefit from allelopathy in either environment when concentrated blooms occur.

Introduction

Karenia brevis is a red tide dinoflagellate that blooms almost annually in the Gulf of Mexico (Tester & Steidinger 1997), producing a suite of neutotoxins, brevetoxins (Baden 1989), responsible for fish and marine mammal mortality events (Flewelling *et al.* 2005). Brevetoxins also cause neurotoxic shellfish poisoning in humans (Landsberg *et al.* 2009). Allelopathy, the production and release of compounds to inhibit competitors, may be one mechanism that *K. brevis* uses to maintain large, nearly monospecific blooms in the Gulf of Mexico (Kubanek *et al.* 2005). Although *K. brevis* is infamous for its toxicity towards vertebrates, its allelopathic effects are attributed to a separate suite of unstable compounds, distinct from brevetoxins, that inhibit the growth of other phytoplankton (Prince *et al.* 2010).

Researchers have questioned whether allelopathy can function as a viable competitive mechanism in the plankton, often citing the rapid dilution of allelopathic compounds as a major drawback to this competitive strategy (Lewis 1986; Flynn 2008). It seems unlikely that allelopathy facilitates bloom formation since in early bloom stages, concentrations of algal cells are too low to release substantial concentrations of allelopathic compounds (Jonsson *et al.* 2009). In contrast, phytoplankton in the vicinity of large, established blooms are more likely to be exposed to high doses of allelopathic compounds. At high cell concentrations associated with bloom maintenance, allelopathic compounds may accumulate in the water column, presenting sensitive competitors with an inhospitable environment for growth.

K. brevis blooms are impacted by physical forces that concentrate blooms from offshore initiation sites, moving *K. brevis* populations near shore via wind driven currents (Walsh *et al.* 2006; Hetland & Campbell 2007). In the eastern Gulf of Mexico, blooms often form 18-74 km offshore on the West Florida Shelf (Steidinger & Haddad 1981), accumulating along shelf fronts and moving inshore as blooms progress (Tester & Steidinger 1997). Near shore, very dense blooms (>1,000,000 cell L^{-1}) can persist for months (Vargo 2009) where the environment is greatly influenced by land runoff that supplies near shore blooms with nutrients (Brand & Compton 2007). Dense near shore blooms of *K. brevis* frequently encounter competing phytoplankton including the diatoms *Asterionellopsis glacialis* and *Skeletonema* spp. (Turner & Hopkins 1974; Badylak *et al.* 2007), both of which are known to be sensitive to *K. brevis* allelopathy (Prince *et al.* 2008; Poulson *et al.* 2010). Previous studies investigating *K. brevis* allelopathy have typically focused on such inshore competitors to *K. brevis* and have not investigated how plankton present in offshore environments respond to *K. brevis*.

Because blooms of *K. brevis* initiate on the West Florida Shelf, our objective was to determine if competitors present offshore are susceptible to *K. brevis* allelopathy, contrasting their responses with those of inshore phytoplankton. Since allelopathy is likely most effective when an allelopathic species congregates at high population density (Jonsson *et al.* 2009), we surmised that *K. brevis* allelopathy could be targeted towards inshore competitors. Alternatively, because plankton community members found offshore rarely encounter dense populations of *K. brevis* compared to inshore community members (Brand and Compton 2007), offshore competitors may be more susceptible to allelopathic compounds given that they are less likely to have evolved resistance.

To test these competing hypotheses, we used lab-based experiments to compare the susceptibility of eight diatom species (four inshore and four offshore) to *K. brevis* allelopathy. We also measured responses of natural phytoplankton assemblages sampled from offshore habitats to *K. brevis* allelopathic compounds previously shown to inhibit growth of inshore diatoms.

Materials and Methods

Organisms

Phytoplankton were obtained from the National Center for Marine Algae (NCMA) and stock cultures were maintained in natural Maine seawater purchased from NCMA (35 ppt) amended with L1 + Si media (Guillard & Hargraves 1993). Diatoms used were *Asterionellopsis glacialis* strain CCMP 137, *Chaetoceros affinis* strain CCMP 159, *Leptocylindrus danicus* strain CCMP 1856, *Odontella aurita* strain CCMP 1796, *Rhizosolenia* cf. *setigera* strain CCMP 1694, *Skeletonema costatum* strain CCMP 775, *Stephanopyxis turris* strain CCMP 815, and *Thalassiosira* sp. strain CCMP 1055 (see Table 1 for geographic origin of strains). The dinoflagellate *Karenia brevis* strain CCMP 2228 and strain TxB3 (obtained from L. Campbell) were grown in the same conditions above, in L1 media without silicates. Strain 2228 was isolated from the Mote Marine Laboratory dock in Sarasota, Florida (NCMA); whereas strain TxB3 was isolated off of S. Padre Island, Texas (Errera *et al.* 2010). Cultures were grown at 21°C in a Percival incubator with irradiance of 100-145 μmol m^{-2} s^{-1} (Biospherical Instrument QSL2100) set to a 12:12 light/dark cycle. For some experiments, cultures were grown in an

environmental chamber at 22 °C outfitted with fitted with Philips Universal/Hi-Vision fluorescent bulbs set to a 12:12 light:dark cycle with an irradiance of 75-120 μmol m^{-2} s^{-1}.

Growth of cultures was monitored using *in vivo* fluorescence with a Turner Trilogy fluorometer equipped with an *in vivo* fluorescence module in relative fluorescence mode. Counts of either live cells or cells preserved with Lugol's solution were performed with an inverted Olympus IX50 inverted microscope using a Palmer-Maloney settling chamber or an Utermöhls settling chamber for less concentrated samples (see field assemblage experiments below). To enumerate *K. brevis* cells, a FlowCAM autoimager was used with diluted samples preserved with Lugol's solution (Fluid imaging Inc.; 100 μm flow cell, 0.4 mL min^{-1}, autoimage rate of 16 fps). Growth and was calculated using equation 1:

$$\%\ growth = \frac{final\ fluorescence - initial\ fluorescence}{initial\ fluorescence}\ X\ 100$$

To normalize the growth of phytoplankton relative to controls in the following experiments, equation 2 was used:

$$relative\ \%\ growth = \left(1 - \frac{\%\ growth\ control - \%\ growth\ treatment}{\%\ growth\ control}\right) X\ 100$$

To calculate the specific growth rate, μ, of phytoplankton during periods of exponential growth the following equation was used:

$$Specific\ growth\ rate, \mu = \frac{\ln(fluorescence_I - fluorescence_F)}{Time_I - Time_F}$$

where $fluorescence_I$ is in vivo fluorescence of phytoplankton at the initiation of exponential growth, $fluorescence_F$ is in vivo fluorescence at the end of exponential

growth phase, $Time_I$ is the day exponential growth begun and $Time_F$ is the day in culture plankton exited exponential growth phase (Wood et al. 2005).

Extraction of allelopathic compounds from *Karenia brevis* cultures

Extracellular extracts of cultures of *K. brevis* 2228 and TxB3 were generated for use in experiments with natural plankton assemblages in 2011 (see below). Cultures were extracted in exponential phase after 7-10 d of growth using a method optimized from Poulson *et al.* (2010). Briefly, organic compounds exuded from *K. brevis* cells (grown as 1.1 L batch cultures) were extracted using a mixture of adsorbent lipophilic resins (Diaion HP20/Amberlite XAD 7 in a 1:1 mixture). Cultures were incubated with resins for 12-15 h before gentle sieving to separate cells from resin beads, allowing removal of extracellular compounds from *K. brevis* cultures without *K. brevis* cell lysis. Compounds were eluted from the resins with HPLC grade methanol after sterile artificial seawater (35 ppt) and deionized water rinses. Simultaneously, sterile seawater and L1 media were also extracted to generate negative control extracts. Elution solvent (methanol) was removed with by rotary evaporation or a Thermosavant SpeedVac concentrator. Dried extracts were stored at -20 °C until use.

To test the allelopathic potency of *K. brevis* extracts described above, we used a growth inhibition assay with the near shore diatom *A. glacialis,* known to be sensitive to *K. brevis* allelopathy (Kubanek *et al.* 2005; Poulson *et al.* 2010). *A. glacialis* was exposed to natural concentrations of *K. brevis* extract, i.e., extract (dissolved in dimethylsulfoxide, DMSO) from 3 mL of *K. brevis* culture was added to 3 mL of *A. glacialis* culture in L1 + Si media, in 6 mL culture tubes. Growth of *A. glacialis* was calculated based on *in vivo*

fluorescence, using equation 1. Extracts were deemed allelopathic if they significantly reduced the growth of *A. glacialis* compared to *A. glacialis* treated with a seawater control extract in a 1-way ANOVA with Tukey post test. *K. brevis* extracts found to be allelopathic towards *A. glacialis* were used in the natural assemblage experiment in 2011 (see below).

Offshore plankton assemblage experiments

Field assemblage experiment with *Karenia brevis* extracellular extracts (2011)

To determine the impact of *K. brevis* allelopathy on offshore competitors, a natural plankton assemblage was exposed to extracellular extracts of *K. brevis* 2228 and TxB3. The experiment was performed in 2011 off the Louisiana coast, while aboard the R/V Endeavor (Cruise EN496; station 017; 28° 51.16' N, 88° 29.55' W; approximately 65 km offshore; surface salinity 22.6 psu, surface temperature 30.3 °C). The plankton community was sampled using a bucket from surface water in early afternoon. The community was gently sieved through a 300 μm mesh to remove mesograzers and large colonies of *Trichodesmium* into 18 polycarbonate bottles. Phytoplankton assemblages (125 mL each) were exposed to natural concentrations of *K. brevis* or L1 media control extracts (i.e., extract generated from 125 mL of culture or media dissolved in 210 μL DMSO carrier solvent) (n = 6 each). Replicate bottles were placed haphazardly on deck top incubators with flow-through water to maintain ambient temperature and light regimes.

At times 0 h and 42 h, 25 mL of each plankton assemblage was sampled and preserved in Lugol's solution for cell counts. For enumerating phytoplankton, 5 mL of

each preserved sample was placed in an Utermöhl settling chamber and settled overnight before light microscopy. For each sample at least 150 cells were enumerated, identified when possible to the genus level (Thomas 1997). Growth was calculated for the most prevalent members of the community using equation 1. To compare the effects of *K. brevis* compounds on competitor growth, a 1-way ANOVA with Tukey post test was used.

Field assemblage experiment with live *Karenia brevis* (2012)

To determine the impact of chemical cues from live *K. brevis* on the plankton community structure in offshore habitats, an additional experiment was performed on the West Florida Shelf in June of 2012. An offshore plankton assemblage was exposed to *K. brevis* cells while physically separated by dialysis membrane, allowing exchange of waterborne chemical cues but without contact between *K. brevis* and members of the plankton assemblage. Unlike in the 2011 experiment, this design also allowed for replenishment of *K. brevis* cues over the course of the experiment, which is important since *K. brevis* allelopathic compounds are labile (Prince *et al.* 2010).

Plankton assemblages were collected while onboard the R/V Endeavor (cruise EN509; station 016; 22° 55.29' N, 87° 01.56' W; approximately 290 km from shore within an eddy of Mississippi plume water; surface salinity 34.6 ppt; surface temperature 27.8 °C). Plankton were collected with a conical phytoplankton net in mid-morning (10 μm mesh, ~1 m depth vertical tows, n = 4 tows). For each replicate, 20 mL of concentrated community mix was added to 200 mL of GF/F filtered surface seawater

(collected from this station) in 250 mL polycarbonate bottles. *Trichodesmium* colonies and copepods were removed by visual picking, although small mesograzers remained.

For treatments, late exponential phase *K. brevis* 2228 cells were concentrated using a 5 μm regenerated cellulose membrane (Millipore) with an Amicon ultrafiltration unit. Cultures were slowly filtered with constant gentle stirring until approximately 10 % of the initial volume remained. This concentrated *K. brevis* culture was then diluted to a cell concentration of ~$1.5x10^3$ cell mL^{-1} (similar to natural bloom concentrations) GF/F filtered seawater to remove excess nutrients. Previously cleaned, sterilized, and cut dialysis tubing (SpectraPor 7, 50 kDa molecular weight cutoff) was knotted at one end and filled with 20 mL of *K. brevis* culture and clipped shut. *K. brevis* filled dialysis tubes were then added to bottles containing plankton assemblages. For controls, 20 mL dilute media (ultra-filtered to 10% volume, then reconstituted to initial volume with filtered seawater) was added to dialysis tubes and placed in bottles containing plankton assemblage (n = 7 for both treatment and controls). Contents of dialysis tubes for both treatments and controls were replaced after 24 h in order to ensure that *K. brevis* cells were healthy throughout the experiment. Bottles were incubated in the same manner as the 2011 experiment and community structure was assessed as above.

Lab experiments testing sensitivity of offshore and inshore phytoplankton to *Karenia brevis* allelopathy

To decouple the responses of offshore diatom species to *K. brevis* chemical cues from competition among species in the mixed communities, we conducted lab experiments in which the sensitivity of diatoms to *K. brevis* was assessed, again with

physical separation of species but continual exchange of chemical cues. We considered the following four species to be representative of "offshore" communities, based on isolate source, personal observations, and personal communication (with C. Heil): *Leptocylindrus danicus*, *Chaetoceros affinis*, *Rhizosolenia setigera*, and *Stephanopyxis turris*. Species representative of inshore communities were: *Asterionellopsis glacialis*, *Odontella aurita*, *Skeletonema grethae*, and *Thalassiosira* sp. To allow exchange of waterborne chemical cues while avoiding direct contact between *K. brevis* and competitor cells, *K. brevis* was cultured inside a 50 mL "cage," with a 5 μm nylon mesh on one end, inserted into a 100 mL pyrex bottle.

To construct each cage, the closed, tapered end of a polystyrene centrifuge tube was cut off and 5 μm nylon mesh was heat-sealed the now open end. A small hole was drilled in the side (at the 30 mL mark) to accommodate a long needle (600 μm inner diameter) in order to sample cultures without disrupting the cage. The cage was then was suspended from the top of a bottle representing one experimental unit. When the media level equilibrated, 3 mL of exponentially growing *K. brevis* (strain 2228) was inoculated inside the cages of treatment replicates. For controls, cages were inoculated with 3 mL of L1 media diluted to 65% full media, to mimic growing *K. brevis* culture. Approximately 20 mL of media was inside the cage at the start of the experiment, with the total volume of 80 mL in each bottle. The biovolume of *K. brevis* and each competitor tested were within one order of magnitude of each other at the beginning of the experiments (Table 4.1). Bottles were placed haphazardly in an incubator.

To monitor the growth of competitors in response to *K. brevis* chemical cues exchanged through the cage, 3 mL of competitor species culture was sampled through a

Table 4.1. Initial and final cell concentrations of phytoplankton used in lab based pair-wise experiments.

Competitor Species	Strain (CCMP)	Isolate Source	Initial cell concentration (cell mL^{-1})		Final cell concentration (cell mL^{-1})	
			K. brevis	Competitor	*K. brevis*	Competitor
Asterionellopsis glacialis	137	Gulf of Mexico	$4.2x10^3$	$1.1x10^4$	$3.0x10^4$	$2.6x10^4$
Chaetoceros affinis[1]	159	Gulf of Mexico	$4.0x10^3$	$1.4x10^4$	$3.7x10^4$	$1.0x10^5$
Leptocylindrus danicus[1]	1856	Gulf of Mexco	$5.1x10^3$	$5.4x10^3$	$1.1x10^4$	$1.1x10^4$
Odontella aurita	1796	Gulf of Mexico	$2.2x10^3$	$3.2x10^4$	$1.6x10^4$	$2.3x10^5$
Rhizosolenia cf. *setigera*[1]	1694	Arabian Sea	$3.3x10^2$	$3.6x10^3$	$4.6x10^3$	$4.5x10^4$
Skeletonema grethae	775	Gulf of Mexico	$2.7x10^2$	$7.0x10^4$	$6.3x10^2$	$1.4x10^6$
Stephanopyxis turris[1]	815	Gulf of Mexico	$4.5x10^2$	$4.2x10^2$	$7.1x10^3$	$3.0x10^3$
Thalassiosira sp.	1055	U.S. Virgin Islands, Caribbean Sea	$3.4x10^2$	$9.7x10^4$	$1.8x10^3$	$1.6x10^6$

[1]Denotes "offshore" species

needle every other day, over a period of 8 to 10 d. Every other day, cages were gently lifted to mix cultures and ensure transfer of waterborne chemical cues through the cage mesh and bottles were rearranged haphazardly in the incubator. *In vivo* fluorescence was measured as a proxy for cell concentration. Since *R. setigera* cells measure greater than >300 μm, this species was sampled by removing the cage and sampling through a sterile pipette in order to avoid breaking cells. On the first and last day of the experiment, 3 mL of *K. brevis* culture was sampled and preserved in Lugol's solution for later enumeration as described earlier.

To compare the growth rates between treatment and controls in each pair-wise interaction, non-linear or linear regression was used to determine effects of *K. brevis*. For most cases, exponential growth curves or linear regression was used to fit the fluorescence data. To statistically compare growth rates between treatments and controls, F tests were performed as in Kubanek *et al.* (2005), which compared the slopes and initial cell concentrations (or Y intercepts) of the growth curves. In order to determine if there were any differences in cell concentration between treatments and controls over the course of the experiments, growth curves generated from fluorescence data in the co-culturing assays were analyzed using 2-way repeated measures ANOVA with Sidak's multiple comparison post test because of the lack of independent replicate measurements at different time points. Time and treatment were considered fixed factors. Total percentage growth was calculated with equation 1 using cell concentrations determined from visual counts of plankton samples taken at initial and final time points (see above); percentage growth normalized to controls was calculated with equation 2. Comparison of

percentage growth and mean normalized growth of inshore and offshore competitors was done using an unpaired t test.

Results

Responses of offshore plankton assemblages to chemical cues from *Karenia brevis*

In two separate years at different offshore locations within the Gulf of Mexico, phytoplankton assemblages dominated by diatoms were largely resistant to allelopathic compounds of *K. brevis* (Figures 4.1-4.2). Extracellular extracts used in the first experiment with offshore assemblages were generated from two *K. brevis* strains originally cultured from Gulf of Mexico blooms, and were confirmed in lab experiments to be allelopathic towards at least one near shore diatom, *A. glacialis* (2228 caused 38 % growth reduction, TxB3 caused 75 % reduced growth of *A. glacialis*). Exposure for 42 h to each of these mixtures of *K. brevis* allelopathic compounds caused slight stimulatory effects on centric diatoms ($p = 0.050$; Figure 4.1A). Among the centric diatoms, growth of the most abundant genus, *Chaetoceros,* was not significantly enhanced by *K. brevis* chemical cues relative to media extract controls whereas growth of the genus *Leptocylindrus* was stimulated relative to controls ($p = 0.57$ for *Chaetoceros*; $p < 0.0001$ for *Leptocylindrus;* Figure 4.1C, D). The enhancement of *Leptocylindrus* spp. growth by extracts of *K. brevis* strain TxB3 was most dramatic: populations increased by 182% relative to media controls ($p = 0.0009$) while exposure to compounds from the other *K. brevis* strain (2228) marginally stimulated the growth of *Leptocylindrus* spp (106 % enhancement of growth versus controls; $p = 0.080$; Figure 4.1D). Additional diatom

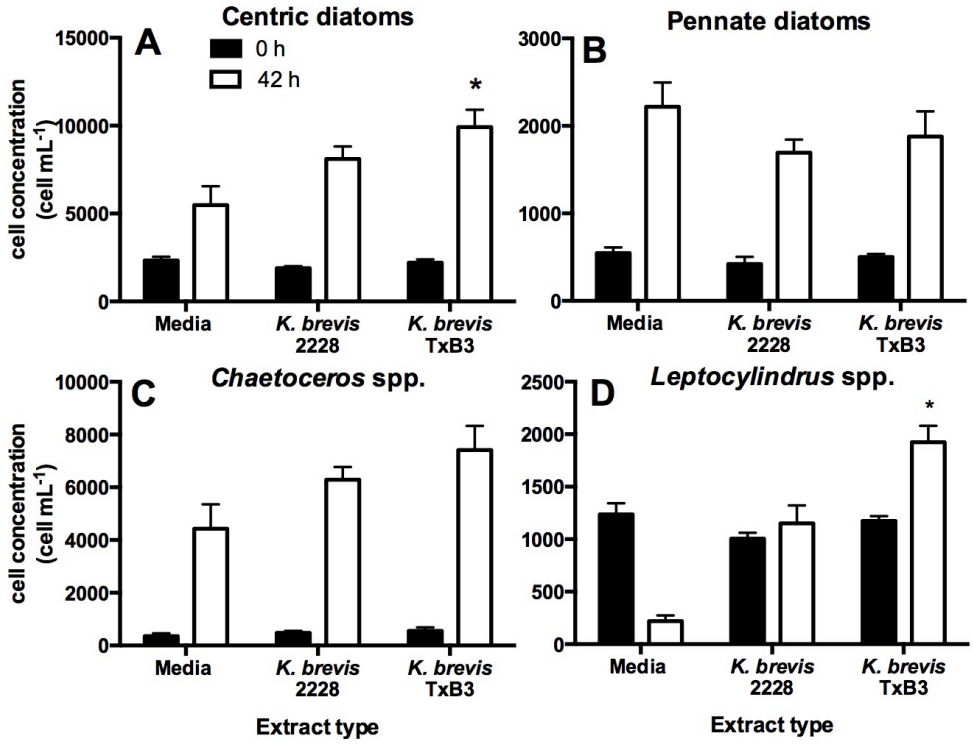

Figure 4.1. Effects of *K. brevis* extracellular extracts on Gulf of Mexico field assemblages of (A) centric diatoms and (B) pennate diatoms after 42 h. The growth of dominant community members (C) *Chaetoceros* spp. and (D) *Leptocylindrus* spp. in response to control extracts of media ("Media"), *K. brevis* strains 2228 ("*K. brevis* 2228") and TxB3 ("*K. brevis* TxB3") is also shown. Asterisks (*) indicate significant differences in growth between treatments and controls (n = 6; 1-way ANOVA with Tukey post test, $p \leq 0.05$). Error bars represent ±1 SEM.

genera within the centric diatom group included *Thalassionema*, *Rhizosolenia*, and *Guinardia.* Pennate diatoms (dominated by *Pseudo-nitzschia* spp. and *Cylindrotheca* spp.) appeared neither stimulated nor inhibited by *K. brevis* compounds ($p = 0.41$; Figure 4.1B), whereas effects on the much less abundant offshore dinoflagellates were more variable (data not shown). In general, there were either neutral or weakly stimulatory effects of *K. brevis* chemical cues on this natural phytoplankton assemblage.

In a second experiment with offshore phytoplankton assemblages, diatoms again dominated the community, in particular *Rhizosolenia* spp., *Chaetoceros* spp., and *Pseudo-nitzschia* spp.. Additionally, *Leptocylindrus* spp., *Guinardia* spp., *Thalassionema* spp., and *Cylindrotheca* spp. were present. In this experiment we exposed field-collected assemblages to chemical cues from live *K. brevis* contained in dialysis tubing, which prevented contact among the two species but allowed exchange of chemical cues throughout 48 h. This also allowed the ongoing production of allelopathic compounds by live *K. brevis*, which was not possible in the first experiment with extracellular extracts. The growth of both centric and pennate diatoms was significantly stimulated by chemical cues exuded by live *K. brevis* cells ($p = 0.014$; $p = 0.012$, respectively; Figure 4.2A, B). When considered separately, however, the growth of dominant centric diatom genera, specifically *Rhizosolenia*, *Cheatoceros*, and *Leptocylindrus* were not significantly affected by *K. brevis* chemical cues although trends towards stimulation were observed ($p = 0.86$, $p = 0.12$, and $p = 0.29$, respectively; Figure 4.2C, D, 2E). Of the pennate diatoms, *Pseudo-nitzschia* spp. dominated the assemblages, significantly stimulated by 42 % by exposure to *K. brevis* relative to controls ($p = 0.0045$). Dinoflagellates, which represented a very small proportion of the phytoplankton community, were not impacted by *K. brevis*

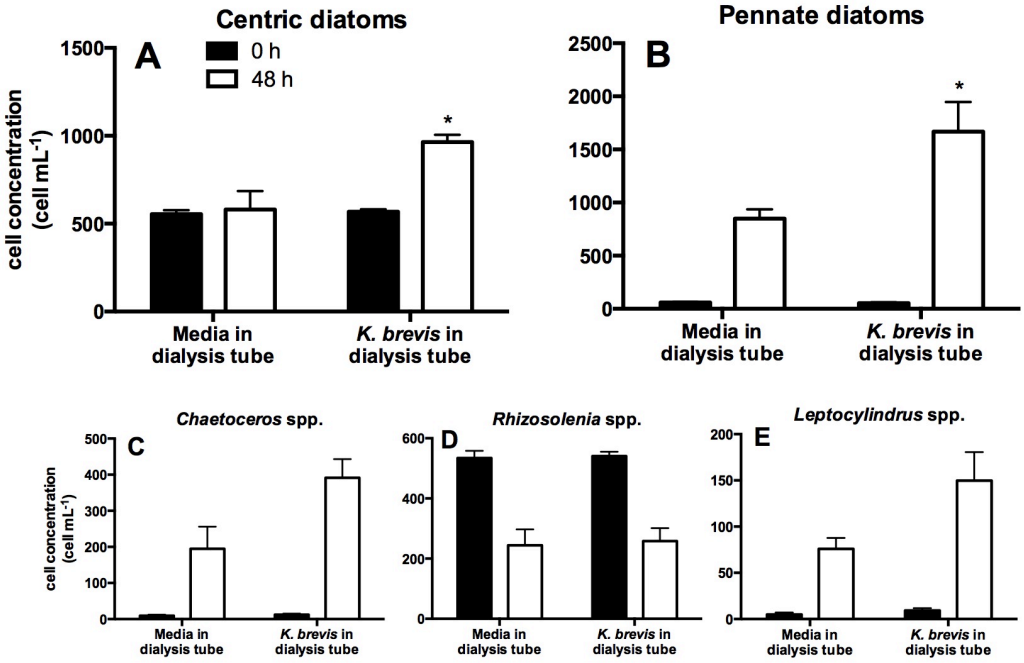

Figure 4.2. Effects of chemical cues from live *Karenia brevis* on Gulf of Mexico field assemblages of (A) centric diatoms and (B) pennate diatoms after 48 h. The growth of dominant community members (C) *Chaetoceros* spp., (D) *Rhizosolenia* spp., and (E) *Leptocylindrus* spp. in response to *K. brevis* chemical cues exuded through dialysis tubing is also shown. Asterisks (*) indicate significant difference in growth between treatments and controls after 48 h (n = 7; unpaired t test, $p \leq 0.05$). Error bars represent ±1 SEM.

chemical cues (data not shown). Overall, exposure to *K. brevis* exudates caused only stimulatory effects on the growth of diatoms, mainly *Pseudo-nitzschia* spp., from this plankton assemblage.

Responses of inshore and offshore competitors to *Karenia brevis* chemical cues in pair-wise lab experiments.

Of four cultured diatoms known to occur offshore in the Gulf of Mexico (three of which were also dominant in our field assemblage experiments), two appeared stimulated by, and two were resistant to *K. brevis* chemical cues. The growth curve generate from *in vivo* fluorescence measurements of *Rhizosolenia setigera* was significantly different when exposed to *K. brevis* chemical cues versus dilute media controls (F test, $p = 0.0037$; Figure 4.3). Additionally, *R. setigera in vivo* fluorescence was significantly enhanced by *K. brevis* after eight days, relative to controls ($p < 0.0027$; Figure 4.3). The growth of *Stephanopyxis turris* was stimulated by exposure to *K. brevis* after several days: *in vivo* fluorescence was enhanced by exposure to *K. brevis* by the end of the 10 day experiment ($p = 0.0007$; Figure 4.3). However, the percentage growth of both species, which was calculated from initial and final cell counts, was not impacted by exposure to *K. brevis* allelopathy after 10 d of growth (unpaired t test, $p = 0.59$ for *R. setigera* and $p = 0.17$ for *S. turris*; Figure 4.3). The calculated exponential growth rate (μ) of *R. setigera* and *S. turris* was also not significantly impacted by *K. brevis*. Neither the exponential growth rates (μ) nor percentage growth of *Leptocylindrus danicus* and *Chaetoceros affinis* were significantly impacted by exposure to *K. brevis* ($p = 0.86$ and $p = 0.35$, respectively), indicating that these competitors are relatively resistant to *K. brevis* compounds. Overall,

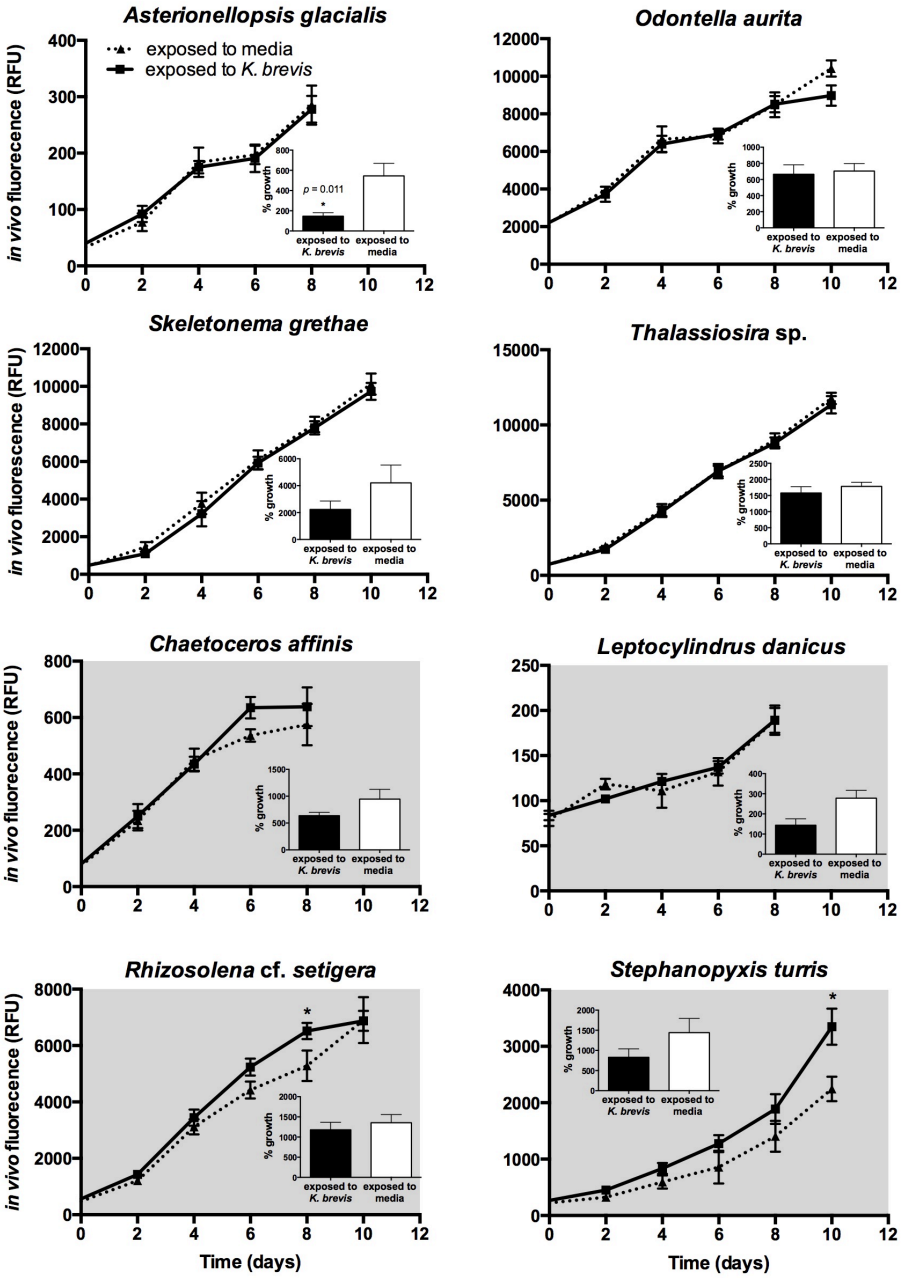

Figure 4.3. Growth of competitors exposed, across 5 µm mesh, to live *Karenia brevis* (solid line) or dilute media controls (dashed line). Phytoplankton known to occur near shore in the Gulf of Mexico are denoted by white background; offshore species by grey background. Asterisks (*) indicate significant differences of in vivo between treatments and controls (n = 3-6; two way repeated measures ANOVA with Sidak multiple comparison, $p \leq 0.05$). Inserts show % growth of each competitor exposed to *K. brevis* (black bars) *vs.* controls (white bars) calculated from cell concentration. Error bars represent ±1 SEM.

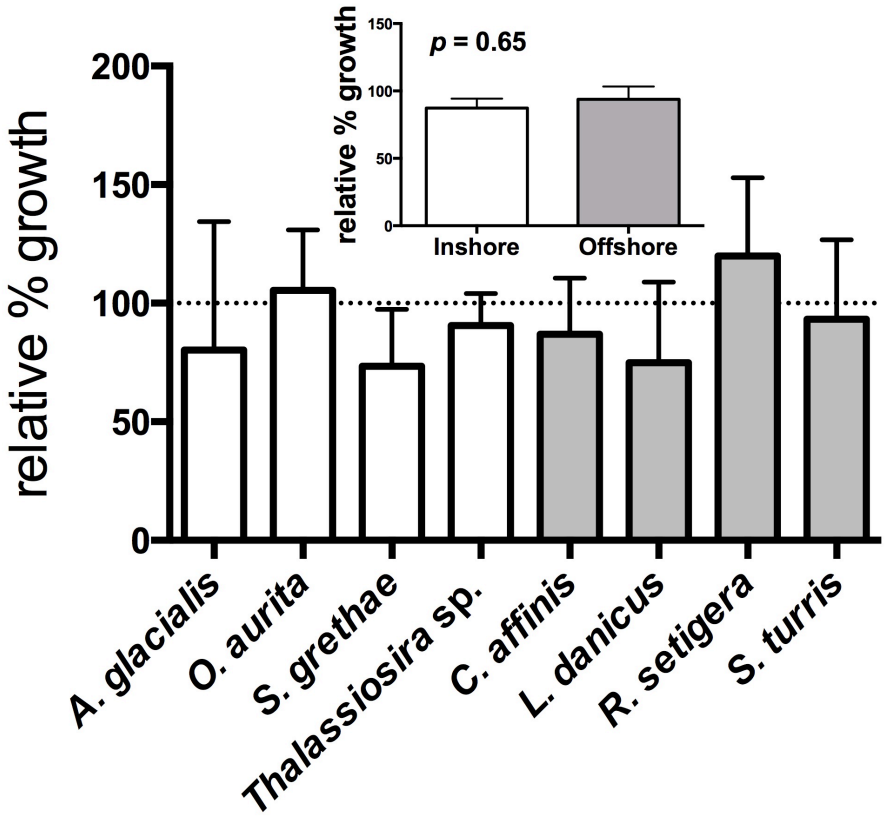

Figure 4.4. Effects of *K. brevis* exposure on inshore (white bars) and offshore (grey bars) competitor growth after co-culture for 8-10 days, separated by 5 μm mesh (n = 3-7). Growth equivalent to controls is indicated by dotted lines at 100%. Insert: mean relative growth of inshore *vs*. offshore competitors (n = 4, unpaired t test, $p = 0.65$). Error bars represent ±1 SEM.

offshore competitors were either resistant or slightly stimulated by exposure to *K. brevis* chemical cues (Figures 4.3-4.4), consistent with observations using field assemblages (Figures 4.1-4.2).

In contrast, the inshore competitors exhibited resistance, with a trend towards growth suppression (but not stimulation) when exposed to *K. brevis* chemical cues (Figures 4.3-4.4). The growth of *Asterionellopsis glacialis*, *Odontella aurita*, *Skeletonema grethae,* and *Thalassiosira* sp. (Figure 4.3) was not significantly impacted by exposure to *K. brevis* versus dilute media controls ($p > 0.5$ for all; Figure 4.3). When considering mean growths for cultured diatoms over the course of 8-10 d, the growth of offshore diatoms was significantly greater than growth of inshore diatoms exposed to *K. brevis* cues ($p = 0.030$; Figure 4.4), indicating that offshore diatom are more resistant to *K. brevis* allelopathy. The mean growth of inshore competitors was reduced 11 % while growth of offshore competitors were stimulated by 23 % relative to controls (Figure 4.4). Offshore competitors were also more variable in their response to *K. brevis* chemical cues, ranging from 92-139 % growth relative to their own controls over 8-10 d, whereas inshore competitors grew consistently 87-94 % relative to their own controls (Figure 4.4).

Discussion

Chemical cues from *Karenia brevis* differentially impact offshore and inshore diatoms

Our field and lab results suggest that offshore diatoms are either resistant to or stimulated by *K. brevis* chemical cues, whereas inshore diatoms are often resistant or slightly inhibited (Figure 4.4). The stimulatory effects of *K. brevis* are evident for

multiple diatom species from two different offshore communities separated by hundreds of kilometers in the Gulf of Mexico and separated temporally across two different years (Figures 4.1-4.2), suggesting relative robustness in offshore diatoms' responses to *K. brevis* allelopathy. When testing the effects of *K. brevis* cues on offshore diatoms in the lab, we found that two of the four species were significantly stimulated by *K. brevis* (*R. setigera* and *S. turris*), and *C. affinis* appeared marginally stimulated by the end of the experiment, whereas *L. danicus* was resistant but not stimulated when comparing *in vivo* fluorescence measurements (Figure 4.3). However, there were no impacts of *K. brevis* allelopathy on these species when comparing the percentage growth over course of the entire experiment. Because *K. brevis* allelopathic compounds reduce photosynthetic efficiency of sensitive competitors (Prince et al. 2008), it is possible that the enhanced *in vivo* fluorescence per cell of *R. setigera* and *S. turris* resulted from reduced photosynthetic efficiency due to exposure to sublethal doses of *K. brevis* compounds. One of these species, *R. setigera,* has also been previously demonstrated to be sensitive to *K. brevis* in lab-based experiments (Kubanek et al. 2005). Additionally, we do not see a negative impact on *A. glacialis* when calculating growth based on initial and final *in vivo* fluorescence (data not shown) but an obvious reduction of growth based upon the cell count data (Figure 4.3), suggesting the *in vivo* fluorescence of *A. glacialis* is also enhanced by exposure to *K. brevis*. Thus, it is likely that *K. brevis* allelopathy was impacting the photosynthetic efficiency of the competitors *A. glacialis*, *R. setigera*, and *S. turris* in our lab-based experiments.

Observations from the lab-based experiments of the current study indicate that allelopathic compounds produced by *K. brevis* are potentially antagonistic towards

competitors commonly found in both near shore and offshore habitats in the Gulf of Mexico (Figures 4.4). All four of the inshore diatom species exposed to *K. brevis* cues in the lab were resistant with trends towards weak suppression by *K. brevis* cues (Figures 4.3-4.4). In contrast to the responses of the offshore competitors, none of the inshore diatoms tested in the current study were stimulated by *K. brevis*. Although offshore species are less likely to experience concentrated blooms, concentrated blooms can from on midshelf fronts (Tester & Steidinger 1997), exposing phytoplankton found more commonly offshore to *K. brevis* allelopathy. However, since the growth of some offshore competitors was stimulated by *K. brevis* chemical cues in our field experiments, it is unlikely that allelopathy is unlikely to aid in the formation of *K. brevis* blooms, as is may only able to impact competitors during concentrated bloom events, as previously predicted (e.g., Jonsson *et al*. 2009).

The discrepancy between the sensitivity of various competitors to *K. brevis* allelopathy in lab and field-based studies (see Poulson *et al*. 2010) may be a result of reduced selective pressure on cultured diatoms to maintain resistance mechanisms to *K. brevis* allelopathy. Previous studies have suggested that shared history leads to evolved resistance to chemical defenses and allelopathy. For instance, some invasive plants (Callaway & Ridenour 2004) and even phytoplankton (Figueredo *et al.* 2007) are particularly allelopathic towards naïve competitors, whereas members of communities where invasive species have been established have evolved resistance (Callaway *et al.* 2005; Lankau *et al.* 2009). This suggests that naïve competitors can be more sensitive to allelopathic compounds than competitors with a history of exposure. Similar patterns have been observed for interactions of grazers and chemically defended algae, such that

allopatric grazers are more sensitive to toxins (Colin & Dam 2002) and feeding deterrents (Kubanek *et al.* 2007) than sympatric grazers that have evolved resistance. Thus we might also predict that species in culture, removed from a host of normal ecological interactions for generations, may experience reduced selective pressure to maintain resistance against *K. brevis* allelopathy, however this requires further investigation.

K. brevis produces a mixture of unidentified allelopathic compounds (Prince *et al.* 2010), which may be directly responsible for growth stimulation of offshore competitors. Alternatively, the *K. brevis* cues responsible for stimulating the growth of offshore diatoms may differ from those causing inhibitory effects on other competitors. If additional compounds cause the stimulatory effects on offshore species, that would indicate that these competitors resist the effects of allelopathic compounds while responding to additional stimulatory compounds. Suikkanen *et al.* (2005) found that exudates of the cyanobacterium *Nodulara spumigena* stimulated the growth of plankton in natural assemblages, whereas exudates were inhibitory towards competitors in lab based studies, suggesting that numerous interactions influence the impact of allelopathy in more complex systems. *K. brevis* may also stimulate competitor growth directly by providing organic nutrients or compounds readily metabolized by offshore phytoplankton. *K. brevis* could also enhance competitors' ability to take up nutrients from the environment by the production of trace metal chelators (Trick *et al.* 1983; Amin *et al.* 2009) or alkaline phosphatases (Vargo *et al.* 2008). The specific mechanisms involved in the observed growth stimulation of offshore competitors are unknown.

In addition to direct stimulation of growth, increases in competitor population density could arise from reduced mortality. Chemical cues from *K. brevis* could release

phytoplankton from grazing or competitive pressures, provide a defense against pathogens, or inhibit death related metabolic pathways. In our experiments we did not observe any reductions in growth of the major genera present (Figures 4.1-4.2), suggesting offshore diatoms were not stimulated by the death of a competitive dominant species. However, we did not assess the bacterial community, which can be greatly impacted by allelopathy among eukaryotic plankton (Uronen *et al.* 2007; Weissbach *et al.* 2010) and could play a role in these interactions.

How might it benefit *K. brevis* to stimulate the growth of potential competitors, if *K. brevis* does indeed benefit? *K. brevis* could benefit by stimulating growth of certain offshore diatoms, particularly if they harbor nitrogen fixing symbionts, as *Rhizosolenia* spp. do (Villareal 1991). *K. brevis* could also benefit from fixed nitrogen released from *Trichodesmium* blooms preceding *K. brevis* blooms on the West Florida Shelf, fertilizing *K. brevis* (Walsh & Steidinger 2001, Lenes *et al.* 2001). Stimulating these diazotrophs may be a mechanism by which *K. brevis* increases its access to fixed nitrogen. This hypothesis remains to be directly tested, however. Alternatively, there may be no ecological or physiological benefit to *K. brevis* when the growth of other planktonic organisms is stimulated. Our results may simply reflect the lack of selective pressure to produce allelopathic compounds targeted towards offshore diatoms, allowing these competitors to utilize beneficial metabolites produced by *K. brevis*. As the prevalence of offshore, mid-shelf blooms increases (Brand and Compton 2007), antagonistic interactions between *K. brevis* its offshore competitors may intensify.

Karenia brevis allelopathy is subtle, with species-specific impacts

Karenia brevis allelopathy is subtle and inconsistent, displaying intraspecific variability in potency and operating in a species-specific manner. In the current study, diatoms from field-collected offshore assemblages were either stimulated or not impacted by compounds exuded from two Gulf of Mexico strains of *K. brevis* (Figures 4.1-4.2). Similarly, in lab-based pair-wise experiments some offshore competitors (i.e., *R. setigera* and *S. turris*) were likely subtly impacted by *K. brevis* chemical cues and others (i.e., *C. affinis, L. danicus*) were resistant (Figure 4.3). Previous studies have also demonstrated species-specificity of allelopathic outcomes involving *K. brevis* and competitors (Kubanek *et al.* 2005). Species-specific effects are a common pattern in studies of allelopathy in the marine plankton. *Alexandrium tamarense* exudates were shown to impact natural plankton communities in a species-specific manner: some groups of competitors were killed, while others, such as *Leptocylindrus* spp., were only moderately suppressed (Fistarol *et al.* 2004). In some cases, other organisms were stimulated by these same *A. tamarense* exudates (Fistarol *et al.* 2004). Weissbach *et al.* (2010) observed variable effects of two *A. tamarense* strains on plankton communities, with negative impacts on competitor abundances observed only with high concentrations of allelopathic exudates. Some species, including *Chaetoceros* spp. were enhanced by addition of non-lytic *A. tamarense* supernatant (Weissbach *et al.* 2010). Filtrates of *Alexandrium fundyense* strongly suppressed growth of many competitors in natural assemblages, while having a positive effect on the growth of other dinoflagellates (Hattenrath-Lehmann & Gobler 2011).

Because of the mild effects that *K. brevis* has on competitors, it is most likely that allelopathy benefits *K. brevis* primarily during its dense blooms, maximizing waterborne concentrations of allelopathic compounds when competition is likely to be especially fierce and when *K. brevis* cells can reap the rewards of reduced competitor growth (Lewis 1986; Jonsson *et al.* 2009). The subtle effects of *K. brevis* allelopathy are in stark contrast to those of other allelopathic phytoplankton including *Alexandrium* spp., which are known to cause rapid, dramatic effects on competitor species including cell lysis, in lab and field-based studies (Tillmann *et al.* 2007; Hattenrath-Lehmann & Gobler 2011). In a mixed assemblage, the effects of these types of cues may also be confounded with exploitation competition among other phytoplankton, suggesting allelopathy could indirectly alter community composition through mechanisms such as apparent competition. Minor influences of variable nutrient concentrations and other ecological interactions (e.g., grazing, parasitism) may overshadow the mild effects of *K. brevis* allelopathy in natural systems (Poulson *et al.* 2010).

Conclusions

The current study highlights the need to investigate the role of allelopathy in more natural settings consistent with field conditions. In ecologically rich contexts, allelopathic species could have unexpected consequences on competitors. Our preliminary results suggest that competitors common to near shore and offshore environments are potentially sensitive to allelopathy, but likely only under conditions where competitors experience dense blooms of *K. brevis* and are expected to encounter high concentrations of allelopathic compounds. These findings support the hypothesis that allelopathy is not

likely to be used by *K. brevis* to support initiation of its blooms, but may play a role in bloom maintenance. It is becoming increasingly clear that microbial interactions in marine systems are complicated, and that only "bottom-up" and "top-down" frameworks are not sufficient to explain patterns of diversity in plankton communities. Investigating the role of chemically mediated interactions in the marine plankton will shed light on additional mechanisms that structure these communities.

CHAPTER 5

CONCLUSIONS AND FUTURE DIRECTIONS

The role of interspecfic interactions between planktonic organisms in mediating large scale ecosystem and biological phenomena is becoming increasingly apparent (Strom 2008). These ecological interactions are often mediated by chemical cues (Hay & Kubanek 2002; Pohnert *et al.* 2007; Ianora *et al.* 2011). This body of work highlights the potential for chemically mediated interspecific interactions to structure plankton communities, and demonstrates how these interactions can affect competitors in a sublethal manner. In addition, the studies herein provide insights into when and where the use of allelopathic compounds may benefit the bloom forming organism *K. brevis*. However, due to its subtlety, the ecological consequences of allelopathy in bloom dynamics is still undetermined, as isolating the impacts of allelopathy from a whole host of other interactions is difficult.

Although we were successful in investigating the physiological effects of *K. brevis* allelopathy on competitors, the isolation and identification of each allelopathic compound, however, is critical for understanding several important factors of allelopathy. Without the ability to test the effects of each compound individually at ecologically appropriate concentrations, knowledge of how multiple compounds act additively or synergistically on competitor metabolism remains unknown. Structure elucidation of allelopathic compounds will aid in understanding the biosynthetic pathways involved their production and the relative influence of abiotic and biotic factors in compound

production. Finally, determining the selective pressures that contribute to the production and maintenance of allelopathic compounds requires the identification of the allelopathic compounds.

Despite great effort, the full molecular structures of allelopathic compounds produced by *K. brevis* remain unknown. This is due to the fact that these cues are labile, polar, and produced in low concentrations. These characteristics impede their isolation and purification from seawater (Prince *et al*. 2010). Because of these issues, novel methodologies are needed to fully elucidate their structures (Pohnert 2010). Fortunately, the use of systems biology based tools such as metabolomics does not rely on the identification of the allelopathic compounds in order to understand how the suite of cues impacts global competitor metabolism. In Chapter 3 of this dissertation, we utilized metabolomics to understand the impact of *K. brevis* allelopathy on phytoplankton physiology. Compounds produced by *K. brevis* alter cellular nitrogen cycling through enhancement of protein degradation, may inhibit enzymatic carbon fixation, and alter energy stores within the cell. Ultimately, these changes likely lead to programmed cell death in competitors that are extremely sensitive to *K. brevis* allelopathy. The next step is to demonstrate how these physiological effects alter large scale ecosystem phenomena including nutrient cycling, primary productivity, flux of material and energy through pelagic food webs and out of the water column (Strom 2008).

While much attention over the last decade has been devoted to investigating the impact of *K. brevis* blooms on other competitors (Kubanek *et al.* 2005, Prince *et al*. 2008a,b), we still know virtually nothing about how other members of the plankton community influence *K. brevis*. There is mounting evidence demonstrating the

preponderance of reciprocal interactions between phytoplankton species, and that many of these interactions are indeed mediated through chemical cues or signals (Sieg *et al.* 2011). It stands to reason that not only does *K. brevis* produce cues impacting the physiology of other species, but that these species also impart their own influences on *K. brevis*. Since extensive work has been devoted to establishing the physiological impacts of *K. brevis* allelopathy on other species (e.g., Prince *et al*. 2008a, this thesis), a natural extension of this is to demonstrate both the fitness benefits of allelopathy, and the effects that other community members may have on *K. brevis* physiology.

Chapters 4 and 5 delve into the variability associated with *K. brevis* allelopathy. We established that extreme variability in *K. brevis* allelopathic potency exists – both among strains and within batch cultures of the same strain. Because all strains investigated were allelopathic, it is likely that the ability to produce allelopathic compounds is genetically fixed within *K. brevis* populations. This appears to be similar to other allelopathic phytoplankton, such as members of the genus *Alexandrium* (Tillmann *et al.* 2009). Additionally, *K. brevis* produces a suite of allelopathic compounds each of which exerts differential potency towards competing phytoplankton. It remains unknown, however, whether the differences in allelopathic potency among *K. brevis* strains are related to the relative proportions of each compound produced, or overall differences in the concentration of the whole suite. This variation may provide a benefit to *K. brevis*, by minimizing the constraints on co-evolution of allelopathy to competitor resistance (Schmitt *et al.* 1995). Since the structures of each compound remain unknown, metabolomic based approaches to rapidly characterize novel compounds from mixtures with minimal bioassay guided fractionation is a valuable tool for researchers seeking to

identify labile natural products (Gillard *et al.* 2013). These techniques also have an additional advantage in that entire suites of compounds can be characterized and used to chemically profile the natural products produced by organisms (Schroeder *et al.* 2007). For *K. brevis*, the methods could be used to determine and compare the allelopathic compound profile of multiple *K. brevis* strains. This may also allow future researchers to predict the allelopathic potency of natural *K. brevis* populations, leading to testable hypotheses about competitive outcomes in the Gulf of Mexico.

In addition to differences in allelopathic potency within *K. brevis* populations, there is also a great deal of variation in the responses of competitors to *K. brevis* allelopathy. Through a series of investigations (Chapter 4-5), we conclude that this variation appears to operate on three levels. First, within each community type (i.e., offshore *vs*. inshore), the impacts of *K. brevis* allelopathy are species-specific with some phytoplankton exhibiting more resistance than others. Second, these species-specific effects are modulated by the a) physiological state and b) population density of each competitor. This intense variability masks the role of allelopathy in natural systems, and highlights the dynamic and potentially chaotic nature of planktonic systems (Beninca *et al.* 2008).

Finally, a characteristic of *K. brevis* allelopathy is that it is often sublethal in nature, which provides a potential model system to investigate the relative importance of sublethal interactions in the plankton, in contrast to more traditional views of density mediated ecological interactions. These sublethal allelopathic effects may benefit *K. brevis* by ensuring sensitive competitor genotypes remain in the environment, minimizing the evolution of resistant competitors via lethal selection pressure. The potential benefit

of sublethal deterrence, as opposed to acute toxicity has been previously hypothesized for copepod-plankton interactions (Flynn & Irigoien 2009), and may operate similarly among competing phytoplankton. Given that genetic differentiation is much greater within phytoplankton communities than previously thought (Rynearson *et al.* 2006; Henrichs *et al.* 2013), this warrants further research. However, investigating these types of interactions require patience, innovative methods (e.g., systems biology approaches), and substantial replication for reproducible results. Once these challenges are overcome, investigating sublethal interactions in the plankton will shed light on the relative importance of trait mediated versus density mediated effects in pelagic communities (Strom 2008).

Beyond the *K. brevis* study system, the work presented in this dissertation could be used as a foundation to investigate how phytoplankton respond to contrasting cues. Are they able to integrate different suites of cues to modulate their response to competitors, grazers, and bacterial attack at once? How important is the identity of cue producers in competitive interactions? It is clear that phytoplankton can recognize and respond differentially to grazers which is important in modulating the resistance of phytoplankton (Long *et al.* 2007; Bergkvist *et al.* 2008) yet little is known about how this plays out in competitive or other antagonistic interactions.

Several other planktonic organisms, including cyanobacteria, diatoms, haptophytes, and other dinoflagellates, produce allelopathic compounds, and contrasting these systems with *K. brevis* will yield valuable information about the prevalence of allelopathy in plankton communities. Is allelopathy most prevalent in harmful algae species? What is the prevalence of allelopathy in other bloom forming phytoplankton,

such as classical spring diatom blooms and coccolithophores, organisms whose blooms are critical in global cycling of carbon and other nutrients throughout the water column. To what extent are these interactions sublethal versus lethal? Answering these questions will allow us to gain new appreciation for the impacts of sublethal interspecific interactions on biogeochemical cycles and other ecosystem processes.

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