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The Impact of Predator-Prey Relationships on the Formation of Harmful Algal Blooms in *Heterosigma Akashiwo*

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THE IMPACT OF PREDATOR-PREY RELATIONSHIPS
ON THE FORMATION OF HARMFUL ALGAL
BLOOMS IN *HETEROSIGMA AKASHIWO*

BY

ELIZABETH L. HARVEY

A DISSERTATION SUBMITTED IN PARTIAL FULFILLMENT OF THE
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DOCTOR OF PHILOSOPHY DISSERTATION
OF
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2013

ABSTRACT

Heterotrophic protists are the primary grazers of phytoplankton in the marine environment, and changes in the rates of consumption will influence phytoplankton abundance, community composition, and ecological function. The objective of this research was to investigate the influence of predators on the formation and promotion of harmful algal blooms (HABs), specifically blooms of the toxic raphidophyte alga, *Heterosigma akashiwo*. To observe microscopic predator-prey interactions and their macroscopic distributions, video and image analysis were used to simultaneously quantify population distributions and individual 3D movements of both protistan predators and prey cells in laboratory tanks with realistic salinity structures. In behavioral experiments, the ciliate predator, *Favella ehrenbergii* did not avoid a layer of *H. akashiwo*, leading to high ciliate mortality (Harvey and Menden-Deuer, 2011). Contrary to our hypotheses, the presence of *Favella* sp. caused *H. akashiwo* to significantly alter its swimming behavior, resulting in avoidance of the predator (Harvey and Menden-Deuer, 2012). This is the first report of predator-induced shifts in the population distribution of a phytoplankton species. Chemical cues were shown to be important in driving predator-prey behavioral shifts. Both predators and prey were shown to shift their movement behavior in response to the presence of only chemical cues, however there was a high level of species-specific responses of both predator and prey (Harvey et al. accepted). Additionally, predator-induced avoidance behaviors in *H. akashiwo* were also observed in these experiments. The results presented here suggests that *H. akashiwo* has multiple mechanisms, including toxicity, predator-induced avoidance behaviors, and the use of chemical cues that will result in

a reduction in grazing pressure, leading to increased survival and potential for accumulation in this HAB alga. These findings highlight the importance of quantifying the mechanistic basis of organism interactions in addition to bulk growth/loss rates in understanding phytoplankton population dynamics and ultimately the fate of material and energy in the marine food web.

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PREFACE

This dissertation is presented in manuscript format and is divided into three chapters. Chapter one observes the behavioral response of a ciliate predator, *Favella ehrenbergii* to a layer of the toxic raphidophyte, *Heterosigma akashiwo*; this is a published manuscript. Chapter two details novel predator avoidance behaviors that were observed when *H. akashiwo* was in the presence of the ciliate predator, leading to shifts in the population distribution of *H. akashiwo* away from the predator; this is a published manuscript. Chapter three investigates the role of infochemicals in influencing the behavior of both heterotrophic protist predators and phytoplankton prey and is a manuscript accepted for publication.

Two appendices follow the main text. Appendix A contains an introduction encompassing the three manuscripts. Appendix B contains a speculative discussion and implications of the results presented in the three manuscripts.

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CHAPTER 1

“Avoidance, movement, and mortality: The interactions between a protistan grazer and *Heterosigma akashiwo*, a harmful algal bloom species”

by

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ABSTRACT

A reduction in predator induced grazing pressure may be a mechanism that facilitates the formation and persistence of harmful algal blooms. Here, the hypothesis was tested that the heterotrophic ciliate *Favella ehrenbergii* would use avoidance behaviors to reduce encounters with the toxic bloom-forming alga, *Heterosigma akashiwo*. Using video and image-analysis, population distributions and three-dimensional movements of *F. ehrenbergii* and *H. akashiwo* were quantified in triplicate, hourly for 11 h, at 9 horizons in a 1 L experimental column. The salinity structure in the column was manipulated to include a halocline, resulting in layer formation by *H. akashiwo*. The ciliate's vertical distributions were restricted to high salinity waters below the halocline, while *H. akashiwo* was broadly halo-tolerant and could occupy the whole water column. When observed together, *F. ehrenbergii* did not avoid layers of *H. akashiwo*. In the presence of *H. akashiwo*, *F. ehrenbergii* mortality rates were higher than in either no prey or beneficial prey controls. Swimming behaviors of *F. ehrenbergii* were erratic, in response to *H. akashiwo*, compared to aggregative movements in response to beneficial prey, indicating either a behavioral response or the effect of *H. akashiwo* toxicity on the ciliate. The inability of *F. ehrenbergii* to avoid *H. akashiwo* enhanced predator mortality and may contribute to the survival of the harmful algal bloom species, ultimately promoting the formation of *H. akashiwo* harmful algal blooms.

INTRODUCTION

Harmful algal blooms (HABs) can be detrimental to marine ecosystems, human health, and fishing economies. Blooms of the toxic raphidophyte, *Heterosigma*

akashwo (Hada), have been found globally in temperate coastal waters (Smayda 1998). These blooms can be ichthyotoxic, causing mortality in both caged and naturally occurring fish populations (Honjo 1993; Khan et al. 1997). Sublethal effects, including the destabilization of cellular defense mechanisms in oysters (Keppler et al. 2005) and altered respiratory activity of mammalian cells (Twiner et al. 2004) have been observed upon exposure to *H. akashiwo*. Although there is no consensus on the mode of toxicity for *H. akashiwo*, possible modes include the production of reactive oxygen species (Twiner et al. 2001), mucus (Nakamura et al. 1998), and neurotoxins (Khan et al. 1997).

Phytoplankton population dynamics are driven by the relative rates of cell growth and loss; formation of an algal bloom can result when population growth rates exceed loss rates. It is unclear why many HAB species are highly successful at frequently forming mono-specific blooms. Hypotheses on the mechanisms of bloom formation include vertical migration (Smayda 2002), allelopathy (Granéli et al. 2008), eutrophication (Anderson et al. 2002), and pelagic-benthic life cycles (McGillicuddy et al. 2003). Another possibility is that HAB species have a greater grazer resistance than other phytoplankton species. Heterotrophic protists are the main consumers of marine phytoplankton biomass, consuming on average 50-60% of phytoplankton production (Calbet and Landry 2004). Low protist grazing pressure could therefore shift the population dynamics from net loss to rates of rapid accumulation. Low grazing pressure has been suggested as a possible mechanism of HAB formation (Strom et al. 2001; Tillmann 2004; Irigoien et al. 2005). Experimental evidence on the grazing response of heterotrophic protists to *H. akashiwo* is varied. Laboratory

research has shown that the heterotrophic dinoflagellates *Oxyrrhis marina*, *Noctiluca scintillans*, and *Stoeckeria algicida* feed readily on *H. akashiwo* (Jeong et al. 2003; Clough and Strom 2005). However, other protists, such as large ciliates die when they ingest *H. akashiwo* cells (Verity and Stoecker 1982; Clough and Strom 2005; Graham and Strom 2010). Thus, while some predators readily consume *H. akashiwo* others die or avoid feeding on this HAB species. In the field, *H. akashiwo* blooms have been associated with low heterotrophic protist grazing rates (Verity and Stoecker 1982; Kamiyama et al. 2000; Menden-Deuer et al. 2010). Moreover, heterotrophic protist grazer abundance was reduced during a *H. akashiwo* bloom (Kamiyama 1995). Therefore, both laboratory and field studies suggest that low grazing pressure may be an important contributor to *H. akashiwo* HAB formation.

The measurable, grazer-induced mortality rate is a community-level average of many individual-level predator-prey interactions; different types of interactions can result in similar population abundances. For example, low grazing pressure may be due to low predator abundance caused by mortality, avoidance behaviors, reduced ingestion, or a combination thereof. Moreover, not all predator-prey interactions result in consumption. Non-consumptive effects on predator-prey population dynamics, including prey selectivity or prey avoidance, can have consequences that may change predator or prey behavior or growth (Lima 1998; Schmitz et al. 2004). Each type of interaction has different consequences for HAB population dynamics. Predator mortality or avoidance of the area reduces overall grazing pressure on all prey species. On the other hand, prey selectivity reduces predation pressure only on the avoided species and possibly reduces resource competition among algae, favoring the avoided

species. Deciphering the nature of these cell-cell interactions provides the opportunity to understand the mechanisms driving average grazing rates and is necessary for predictions of HAB occurrence.

Here we investigated behavioral interactions between *H. akashiwo* and the ciliate predator, *Favella ehrenbergii* (Jørgensen). Specifically, we mimicked the patchy nature of *H. akashiwo* blooms, where bloom patches are bordered by low HAB abundances that could theoretically provide a spatial refuge for the predator. In the laboratory, we created a halocline structure that induced layer formation of *H. akashiwo* and measured *F. ehrenbergii*'s population distribution and swimming behavior using high-resolution video analysis. We found no evidence that *F. ehrenbergii* avoided dense *H. akashiwo* layers, despite increased mortality of the ciliate in the presence of *H. akashiwo*.

METHODS

Culture of microorganisms – A strain of *Heterosigma akashiwo* (CCMP 2809), known to be toxic to some heterotrophic protists (Graham and Strom 2010) was used for all behavior experiments. *Heterocapsa triquetra* (unknown origin) and *Isochrysis galbana* (CCMP 1323) were used to rear ciliate cultures. *Heterocapsa triquetra* were also used as a beneficial control prey in behavior experiments. We will refer to *H. triquetra* as beneficial prey and *H. akashiwo* as toxic prey; however, this does not imply that these are the only prey species that could promote the growth or death of the predator. All phytoplankton cultures were grown in 0.2 μm sterile-filtered autoclaved seawater (FSW), enriched with f/2 nutrients (Guillard 1975). The ciliate predator, *Favella ehrenbergii* (SPMC 133), was cultured in FSW only. All cultures

were maintained on a 12:12 h light:dark cycle, at 15°C, salinity of 30, and a light intensity of 70-80 $\mu\text{mol photon m}^{-2} \text{ s}^{-1}$ for the phytoplankton cultures and 8-15 $\mu\text{mol photon m}^{-2} \text{ s}^{-1}$ for *F. ehrenbergii*. The cultures were not axenic. Phytoplankton cultures were transferred every 4-7 d to maintain exponential growth. Ciliate cultures were fed *H. triquetra* (final concentration of 200 cells mL^{-1}) and *I. galbana* (final concentration of 1500 cells mL^{-1}) twice a week. Throughout all experiments, cell concentrations of both predator and prey cultures were determined by microscope counts using samples fixed in 1% acid Lugol's solution.

Experimental chamber set-up - To quantify population distributions and movement behaviors, a 30 cm tall, 5.5 cm wide, 800 mL octagonal, acrylic observational chamber was used (Fig. 1). The chamber was filled with FSW using a peristaltic pump; this method allowed for the creation of defined salinity structures and eliminated fluid convection in the chamber (Bearon et al. 2006; Menden-Deuer and Grünbaum 2006). A halocline was created in the middle of the chamber between two weakly stratified linear salinity gradients, one from 8-10 (above) and another from 27-30 (below). This halocline was used to induce layer formation of *H. akashiwo* (Bearon et al. 2006). The same source water was used in all experiments and cultures.

Three treatments were used to quantify predator-prey interactions: 1) *F. ehrenbergii* alone, 2) *F. ehrenbergii* and *H. akashiwo* together, and 3) *F. ehrenbergii* and *H. triquetra* together. All treatments were run in triplicate. Using a syringe, organisms were introduced at the bottom of the tank through silicone tubing with an internal diameter of 1 mm. Cells were introduced slowly at a rate of 10 mL min^{-1} to reduce stress to cells as well as disturbance to the water column. Phytoplankton cells

were introduced into the tank first, and allowed to acclimate for 10 min. *Heterosigma akashiwo* was added to the tank for an average final concentration of 180 cells mL⁻¹. In order to observe a final concentration of 50 *F. ehrenbergii* mL⁻¹, 2 L of ciliate culture were gently condensed to 30 mL using a submerged, 20 µm Nitex mesh 15 min before introduction to the bottom of the experimental chamber. Cells were then added to the tank using the same silicone tubing as used for phytoplankton. An equivalent volume of water was added to the control tanks, so that volumetrically the treatments remained the same. To minimize prey cell carryover and to maximize feeding motivation, *F. ehrenbergii* cultures were starved for three days prior to use in the experiment. The residual prey in the concentrated samples were enumerated using microscope counts; the concentration of *H. triquetra* was below the detection limit, and the carry over of *I. galbana* was < 2 cells mL⁻¹, or 5000 pg C of total *I. galbana* biomass in the entire tank, equivalent to the biomass of 1.7 *F. ehrenbergii* cells (Verity and Langdon 1984).

An equal abundance of the 12 µm *H. akashiwo* and 30 µm *H. triquetra* were offered in terms of carbon content, not cell concentration. Carbon content was determined from cell size (Menden-Deuer and Lessard 2000), the calculated carbon content for *H. akashiwo* was 134 pg C cell⁻¹ compared to 796 pg C cell⁻¹ for *H. triquetra*. Offering equivalent carbon abundances ensured that the encounter rates of *F. ehrenbergii* on *H. akashiwo* (2.2 cells day⁻¹ predator⁻¹) and *H. triquetra* (2.4 cells day⁻¹ predator⁻¹) remained theoretically constant (Kiørboe, 2008). Final concentration in the tank was ~ 31 *H. triquetra* cells mL⁻¹, lower than the 200 *H. triquetra* mL⁻¹ that *F. ehrenbergii* were fed in maintenance cultures. Therefore, in *F. ehrenbergii*

experiments with beneficial prey, prey concentrations were an order of magnitude lower than the concentrations used to sustain growth of the predator.

Video capture and analysis - Two infrared sensitive cameras (Pixelink) with Nikon 60-mm Micro Nikkor lenses monitored a two-dimensional (2D) field of view of 1.5 cm x 1.3 cm x 3.3 cm. The cameras were mounted at a 45° angle with maximally overlapping fields of view to enable reconstruction of three-dimensional (3D) movement behaviors. All filming was conducted with no visible light illumination, to eliminate the potential for light-mediated behavioral responses. In order to view the organisms, the chamber was illuminated with dark field infrared (960 nm) light-emitting diodes (LEDs). Filming occurred at nine, initially random, horizons, vertically distributed evenly approximately 2-3 cm apart. This constituted approximately 36% of the viewable volume. To calculate the 3D volume captured, we used the Cartesian positions of a subset of cells and calculated the area of a convex hull to be 3.2 cm³. Thus, the viewing volume was 3.2 mL per horizon. Each horizon was filmed for 1 min every hour for an 11 h period, resulting in a total of 108 one-minute videos per treatment. Video was captured at 30 frames s⁻¹.

To determine the population distribution and swimming behaviors of both the ciliate and phytoplankton species, all videos were analyzed using the same protocol. The 2D position of each individual organism in each frame of the stereo cameras was determined, using automated ImageJ image-processing software to remove stationary background objects. The threshold was determined manually, so that background subtraction could be automated. The same threshold values were used for all videos. In mixed species videos, those organisms that had an area of 20 ± 5 pixels were classified

as *F. ehrenbergii*, and those with an area of 5 ± 3 pixels were classified as *H. akashiwo* or *H. triquetra* depending on the experiment. This size classification was established using the control videos. Abundances were determined by averaging the number of individuals per frame over the 1 min video (1800 frames). Due to the volume of data generated with each of these experiments, only the distributions of organisms at time (t) = 1, 5, and 11 hours elapsed are shown graphically, representing the beginning, middle, and end of the observation period. The results reported here accurately represent the results observed between time points.

Three-dimensional swimming paths were determined by first assembling 2D trajectories from Cartesian coordinates of each organism in each stereo frame and then joining 2D tracks based on matching space-time occurrence in the two 2D segments. Trajectories from all treatments were determined using the exact same video analysis and trajectory assembly parameters; more details are reported in (Menden-Deuer and Grünbaum 2006). Swimming behaviors, including the x, y, and z velocity vectors and turning rates were calculated from 3D paths, subsampled at 0.25 s intervals. Turning rate is a measure of the magnitude of the directional change an organism undergoes over time. The vertical velocity component of the speed indicates the rate of vertical displacement of the cell with negative values indicating downward movement and positive values upward movement. The top and bottom 0.5% of each frequency distribution were discarded before analysis to eliminate extreme outliers. Trajectories from each horizon and replicate were pooled by time point. For *F. ehrenbergii* videos, only time points that had 10 or more individuals in each replicate and cells tracked for a minimum duration of 5 s or longer were used to measure movement behaviors.

Reduced abundance over time limited analysis of *F. ehrenbergii* swimming behavior in the latter half of the experiment, since greater data density was required to quantify *F. ehrenbergii* movement behaviors compared to population distributions. Thus, population distributions are reported for the entirety of the observation period, but movement behaviors could only be analyzed from the first 6 h.

Statistical analysis - The Kolmogorov-Smirnov test (*K-S* test) was used to determine significant differences among the abundance data, as well as among distributions of swimming behaviors. The abundance data are displayed graphically as percent population distribution to allow visual comparisons between time points and treatments. All statistical analyses were done on the absolute abundance data, not the percentages. Mortality rates of *F. ehrenbergii* were determined by subtracting the difference between initial and final cell abundance per time elapsed. A one-way Analysis of Variance (ANOVA) was used to test for significant differences in predator mortality rates among treatments. For all analyses the significance level was $p < 0.05$.

RESULTS

Prey distributions – Both prey species, in the presence of *F. ehrenbergii*, rapidly formed an aggregation at the halocline horizon (Fig. 2). Approximately 36% of the *H. akashiwo* cells and 42% of the *H. triquetra* population were found at the halocline after 1 h. Prey cells were found at all horizons observed. While the abundance of cells of both prey species significantly increased below the halocline over time ($p = < 0.001$), the largest proportion of the population was always found aggregated at the halocline horizon.

***Favella ehrenbergii* distributions** – After 1 h, in the filtered seawater (FSW) control, 47% of the *Favella ehrenbergii* population were aggregated at the halocline (Fig. 3). There were no significant changes in *F. ehrenbergii* distribution over time (min. $p = 0.10$), with at least 40% of *F. ehrenbergii* consistently found at the halocline. All remaining cells were found below the halocline. No *F. ehrenbergii* cells were ever observed above the halocline, at salinities > 15 .

In predator-prey exposure experiments, *F. ehrenbergii* did not avoid layers of *H. akashiwo*. The population distribution of *F. ehrenbergii* did not significantly change when exposed to a layer of *H. akashiwo* ($p = 0.12$) or beneficial prey ($p = 0.82$), in comparison to a FSW control. When *H. akashiwo* were present in the tank, approximately 55% of the *F. ehrenbergii* population was aggregated at the halocline. The population distribution of *F. ehrenbergii* in the presence of *H. akashiwo* did not change over time (min. $p = 0.95$, Fig. 3). Nearly identical to the other two treatments, 52% of *F. ehrenbergii* aggregated to the halocline in the presence of the beneficial prey *H. triquetra* and did not change over time (min. $p = 0.97$).

While there were no significant differences observed in the distributions of *F. ehrenbergii* in the three treatments, we observed significant differences in the abundance of *F. ehrenbergii* among treatments over time ($p = 0.009$, Fig. 4). *Favella ehrenbergii* showed significantly higher mortality rates when *H. akashiwo* were present in the tank (24.4 ± 2.8 *F. ehrenbergii* h^{-1}) vs. control (15.4 ± 2.5 *F. ehrenbergii* h^{-1}) or *H. triquetra* (6.2 ± 1.9 *F. ehrenbergii* h^{-1}) treatments, signifying enhanced mortality of *F. ehrenbergii* in response to *H. akashiwo*.

***Favella ehrenbergii* swimming behaviors** – Swimming speeds of *F.*

ehrenbergii were significantly different among all treatments (all $p = < 0.001$). The magnitude of variability was large, with the coefficient of variation ranging from 10-100%. *Favella ehrenbergii* swam the slowest in response to the beneficial prey and initially the fastest in the FSW control (Fig. 5A). After 1 h of observation, the mean swimming speed of *F. ehrenbergii* cells when with *H. akashiwo* was $737 \pm 460 \mu\text{m s}^{-1}$, 17% faster than *F. ehrenbergii* with *H. triquetra* ($607 \pm 511 \mu\text{m s}^{-1}$) but 12% slower than in the FSW control ($847 \pm 534 \mu\text{m s}^{-1}$; Fig. 5A). For all treatments, swimming speeds of *F. ehrenbergii* decreased over time. On average, the mean swimming speed of *F. ehrenbergii* in the control was 26% slower than the swimming speed in the presence of *H. triquetra*, over the entire observation period. From 2 to 6 h, the mean swimming speed in the FSW control and in presence of *H. triquetra*, while different from each other, remained relatively consistent from hour to hour. However, the mean swimming speed of *F. ehrenbergii* in the presence of *H. akashiwo* changed over time. Mean swimming speeds increased over time until peaking at hour three of observation ($975 \pm 496 \mu\text{m s}^{-1}$). Mean speeds then slowed each hour, until it reached a low at hour six ($444 \pm 331 \mu\text{m s}^{-1}$), which was 38% slower than the mean speed in the control and 4% slower than when *H. triquetra* were present.

Overall, *F. ehrenbergii* consistently had the highest turning rates in response to *H. triquetra* and the slowest turning rates in response to *H. akashiwo* with turning rates in the FSW control intermediate between those measured in response to the two prey types. Despite the high variation of turning rates in all treatments (average coefficient of variation over 100%), distributions of turning rates were significantly

different among the treatments (all $p = < 0.001$). After 1 h of observation, the mean turning rate of *F. ehrenbergii* when *H. akashiwo* were present was 105.1 ± 135.7 degrees s^{-1} (Fig. 5B). This was approximately 20% slower than the mean rate measured in either the control (133.0 ± 160.3 deg s^{-1}) or when *H. triquetra* were present (134.0 ± 162.3 deg s^{-1} , Fig. 5B). From 2 to 6 h, *F. ehrenbergii* turned on average 7% faster in the presence of *H. triquetra* than in the filtered seawater control. In contrast, turning rates of *F. ehrenbergii* in the presence of *H. akashiwo* continued to be slower than in the presence of either the control (12%) or in the presence of *H. triquetra* (18%).

DISCUSSION

The ability of *H. akashiwo* and other HAB species to frequently form dense, mono-specific blooms is puzzling and begs the question, what factors enhance the species survival rate? A decrease in heterotrophic protist grazing pressure may contribute to *H. akashiwo*'s success. Observing the details of predator-prey interactions provides a quantitative understanding of the resultant population abundances. To mimic the patchy conditions found in the ocean, where *H. akashiwo* forms dense surface slicks, the experiments reported here were designed to include spatial structure that would afford the predator refuge from exposure to the toxic alga. *Heterosigma akashiwo* abundances varied by orders of magnitude across the tank, providing areas where *F. ehrenbergii* could have avoided interaction with the majority of the *H. akashiwo* population. Our results show that *F. ehrenbergii* did not exploit a spatial refuge that could have reduced its rapid mortality in the presence of the HAB alga.

Montagnes et al. (2008) depicted six steps in protistan prey capture: searching, contact, capture, processing, ingestion, and digestion. Despite almost certain mortality as a result of *H. akashiwo* exposure, our results suggest the toxicity of *H. akashiwo* does not induce *F. ehrenbergii* to reduce contact with the toxic alga by avoidance. Over the course of the experiment, the vertical distribution of *F. ehrenbergii* did not change significantly, regardless of the presence of *H. akashiwo*, beneficial algal prey, or in the absence of potential prey all together. The inability of *F. ehrenbergii* to avoid layers of *H. akashiwo* suggests that *F. ehrenbergii* does not detect the toxicity of *H. akashiwo* prior to capture. It is known that *Favella* sp. will reject captured *H. akashiwo* cells (Taniguchi and Takeda 1988; Stoecker et al. 1995), and will discriminate against *H. akashiwo* in prey mixtures (Graham and Strom 2010). However, when *H. akashiwo* is ingested by *Favella* sp., mortality of the ciliate rapidly follows (Verity and Stoecker 1982; Clough and Strom 2005; Graham and Strom 2010).

The inability of *F. ehrenbergii* to avoid the toxic *H. akashiwo* has important consequences for our understanding and ability to predict HABs. Regardless of the lethality of *H. akashiwo* to *F. ehrenbergii*, our results show that the herbivore remains in the area occupied by *H. akashiwo*. If feeding were to continue, it is likely that feeding selectivity could remove other algae. Such selectivity would have a two-fold benefit to *H. akashiwo*: First, selective avoidance of *H. akashiwo* would promote further accumulation of *H. akashiwo* cells. Second, since *H. akashiwo* has a relatively high nutrient requirement (Smayda 1998), the removal of co-occurring phytoplankton could decrease nutrient competition, further increasing *H. akashiwo* growth rates.

Furthermore, we observed that the *F. ehrenbergii* population died rapidly, 14.5% per hour in the presence of *H. akashiwo*, which would result in a rapid reduction in grazing pressure over time. If *H. akashiwo* has a similar effect on other predator species, the HAB alga's net survival rate should increase relative to competing algae that do not negatively impact their respective predators. Thus, the toxicity of *H. akashiwo* provides this alga an effective defense against predation even when predators are not deterred from the immediate area. The inability of *F. ehrenbergii* to avoid *H. akashiwo* could therefore benefit HAB formation and persistence, through multiple mechanisms including mortality and feeding selectivity of the predator.

There is evidence that *Favella* sp. will consume low concentrations (< 200 cells mL^{-1}) of other toxic algae such as *Heterocapsa circularisquama* or *Alexandrium tamarense*, without significant changes in growth and survival rate from those observed with non-toxic species (Kamiyama 1997; Kamiyama et al. 2005). However, increases in abundance of *H. circularisquama* increased the mortality of the *Favella* sp. (Kamiyama 1997). This positive correlation in toxic prey abundance and resultant predator mortality suggests that grazing can adversely affect HAB species biomass accumulation when abundances are low. Thus, as long as HAB abundances are below the predator mortality threshold, grazing could prevent HAB formation. The consequences of severe toxicity reported here may only apply to instances where algal abundances are high (> 1000 cells mL^{-1}). Investigating the transition from HAB concentrations that can be tolerated and grazed upon to concentrations that adversely affect protistan predators could provide insight into the mechanisms of *H. akashiwo* toxicity to protistan predators.

Favella ehrenbergii exhibited prey-specific changes in swimming behavior. In response to *H. triquetra*, the ciliate showed more aggregative swimming behaviors (e.g., slower speeds and faster turning rates) compared to the FSW control. Increasing aggregative behaviors is an effective mechanism for remaining in a resource patch (Davis et al. 1991; Visser and Thygesen 2003). Our results agree well with previous work where aggregative swimming was observed in *F. ehrenbergii* in the presence of beneficial prey (Buskey and Stoecker 1988). Despite these modifications in movements, an enhanced aggregation of *F. ehrenbergii* to the beneficial prey layer was not observed, which may have been due to the low abundance of *H. triquetra*, insufficient to sustain *F. ehrenbergii* growth.

The behavioral response to *H. akashiwo* was more complex, undergoing shifts, including dispersive and retentive swimming behaviors. Since *F. ehrenbergii* experienced significant mortality in the presence of *H. akashiwo* we cannot determine if these changes were a behavioral response or a physiological consequence of toxicity. Previous work observed erratic swimming of *Favella* sp. in the presence of the toxic alga *Alexandrium tamarense* (Hansen 1989). The observed changes in *F. ehrenbergii* swimming behavior in response to the HAB species, whether voluntary or induced by toxicity, would ultimately decrease the encounter rate of predator and prey. Given the high mortality of *F. ehrenbergii* it is questionable, though, whether these behavioral modifications were effective. These results support the idea that exposure to toxic phytoplankton species may lead to changes in heterotrophic protist movement behaviors, ultimately altering encounter rates.

The structuring function of salinity was a key aspect to our experiment, where *F. ehrenbergii* distributions were restricted to higher salinity waters below the halocline. In previous experiments, *F. ehrenbergii* was able to cross a halocline with a four-part difference in salinity (Jonsson 1989). Yet, our results showed that with a stronger halocline, *F. ehrenbergii* was excluded from lower salinity regions of the tank. This indicates either intolerance to low salinity water or an inability to cross the salinity density gradient. Given this restricted distribution, *F. ehrenbergii* could not access a large portion of the tank that was significantly lower in abundance of the toxic prey species. This restriction on the *F. ehrenbergii* distribution in the tank coupled with the broad halo-tolerance of the HAB alga, enabled *H. akashiwo* to occupy depths void of the predator. Thus, strong haloclines could prevent *F. ehrenbergii* from co-locating in areas where toxic phytoplankton may exist. This physical barrier would further benefit survival and persistence of the toxic alga, and impact the success of HAB formation and persistence.

Exposure to light is also an important factor in influencing plankton behavior and vertical distribution. These experiments were deliberately conducted in the dark to eliminate light-mediated behaviors. Layers of *H. akashiwo* still form in the light, under similar salinity conditions (Bearon et al. 2006). Our own preliminary experiments showed no significant difference in *H. akashiwo* distribution in the presence or absence of light (data not shown). Therefore, *H. akashiwo* distributions would be unchanged in the light. If the ciliate were positively phototactic, increased aggregation of the ciliate at the halocline would result, further increasing its exposure to *H. akashiwo* and possibly ingestion rates (Strom 2001; Jakobsen and Strom 2004). Our

results indicate that co-location of these two species led to the mortality of *F. ehrenbergii*. Thus, all currently available data suggests that our results would be qualitatively the same, if the presented experiments were done in visible light, and the conclusion that *F. ehrenbergii* was unable to avoid *H. akashiwo* would remain.

Our results highlight the importance of deciphering the underlying predator-prey interactions that mechanistically identify why certain population dynamics arise. Decreases in heterotrophic protist grazing rates on phytoplankton may facilitate the formation or enhance the duration of blooms. There are both lethal (toxicity) and non-lethal (avoidance) mechanisms that can lead to such decreases in grazing pressure. We hypothesized that *F. ehrenbergii* would avoid dense layers of *H. akashiwo* to avoid mortality induced by this toxic prey species. Instead, we found that *F. ehrenbergii* did not exploit a spatial refuge nor show avoidance behaviors of the toxic alga, and died rapidly in the presence of *H. akashiwo*. The inability of *F. ehrenbergii* to utilize an avoidance behavior significantly enhanced its mortality rate. If these laboratory observations apply to conditions in the ocean and other predators and their potential HAB prey, predator-prey interactions may constitute an important factor in deciphering the success of harmful algal blooms.

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FIGURE LEGENDS

Figure 1 – **Photo of the experimental filming set-up.** (A) Two cameras monitor (B) the 30 cm high tank that is illuminated by (C) two light emitting diode (LED) light banks. The entire camera and light bank platform moves vertically through computer control.

Figure 2 – **Distribution of *H. akashiwo* and *H. triquetra*.** Distributions of *H. akashiwo* (circle) and *H. triquetra* (triangle) after 1 h, 5 h, and 11 h in the presence of *F. ehrenbergii*. For both prey species, the majority of the population remained at the halocline at all time points. Dashed line indicates the halocline. Error bars represent one standard error of the mean of triplicate incubations.

Figure 3 - **Population distributions of *F. ehrenbergii*.** Population distributions of *F. ehrenbergii* after 1 h, 5 h, 11 h, with *H. akashiwo* (white triangles), in the FSW control (black circles), and with beneficial prey (gray squares). None of these distributions were significantly different from one another, either among treatments or across time. Dashed line indicates the position of the halocline. Error bars represent one standard error of the mean of triplicate incubations.

Figure 4 – **Mortality rate of *F. ehrenbergii*.** Mortality rate (cells loss h⁻¹) of *F. ehrenbergii* in the total water column under the three different prey conditions, no prey (black), toxic (*H. akashiwo*) prey (white), and beneficial prey (*H. triquetra*) (gray). There was a significantly higher mortality rate of *F. ehrenbergii* in the

presence of *H. akashiwo* than in either the control or beneficial prey treatments. Error bars are one standard error of the mean of triplicate incubations.

Figure 5 – **Movement behaviors *F. ehrenbergii***. (A) Mean turning rate (deg s^{-1}) and (B) swimming speed ($\mu\text{m s}^{-1}$) over time for *F. ehrenbergii* cells in the control (black circle), with *H. akashiwo* (white triangle), and with beneficial prey (gray square).

Turning rate of *F. ehrenbergii* was significantly slower in response to *H. akashiwo* than either of the controls. Swimming speed varied over time only in response to *H. akashiwo*, but decreased steadily in response to the two controls. Error bars represent one standard error of the mean and are largely contained within the symbols.

Movement data are not shown after 6 h, because too few long swimming trajectories were observed due to *F. ehrenbergii* mortality.

Figure 1.

Figure 2.

Figure 3.

Figure 4.

Figure 5.

CHAPTER 2

“Phytoplankton fleeing from predators: a new mechanism for harmful algal bloom formation?”

by

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ABSTRACT

The rates at which planktonic heterotrophs encounter and select phytoplankton prey on microscopic scales has immediate ramifications for the abundance and distribution of planktonic primary producers and ultimately global primary production and biochemical cycling rates. Factors such as size, shape, nutritional value, and presence of chemical deterrents are well known to affect predation pressure exerted, which amounts to removing >50% of daily phytoplankton production in the ocean. Behavioral responses of either predator or prey that could modulate predation pressure, and particularly fleeing behaviors in phytoplankton are thus far unknown. Here, we quantified individual 3D movements, population distributions, and survival rates of the toxic phytoplankton species, *Heterosigma akashiwo* in response to a ciliate predator and predator-derived cues, and observed predator-induced defense behaviors previously unknown for phytoplankton. Modulation of individual algal movements during and after predator exposure resulted in an effective separation of predator and prey species. The strongest avoidance behaviors were observed when *H. akashiwo* co-occurred with an actively grazing predator. Predator-induced changes in algal movements resulted in a reduction in encounter rate and a 3-fold increase in net algal population growth rate. A spatially explicit population model predicted rapid phytoplankton bloom formation only when fleeing behaviors were incorporated. These model predictions reflected field observations of rapid *H. akashiwo* harmful algal bloom (HAB) formation in the coastal ocean. Our results suggest a new mechanism for HAB formation and document a novel behavior in phytoplankton that significantly reduced predation pressure. Phytoplankton behaviors that minimize predatory losses,

maximize resource acquisition, and alter community composition and distribution patterns could have major implications for our understanding and predictive capacity of marine primary production and biochemical cycling rates.

INTRODUCTION

Blooms of toxic phytoplankton can negatively impact coastal ecosystems and economies through poisoning events that induce high mortality rates in fish (1), birds (2;3), and illness in humans (4). HAB events appear to be increasing in frequency, and it is hypothesized that climate change related alterations of the environment will exacerbate the toxicity of HABs (5-7). Therefore, understanding why phytoplankton in general and many HAB species particularly are successful in forming frequent, large-scale, often mono-specific blooms is of ecological, societal, and economic concern (6;8).

The balance between growth- and loss-promoting factors largely determines the abundance and distribution of phytoplankton in the marine environment. Research particularly in HAB species has largely focused on mechanisms that impact algal growth rates. Hypothesized causes of HAB formation include vertical migration (9), allelopathy (10), eutrophication (6), and pelagic-benthic life cycles (11). Increased nutrient availability from land, including agricultural run-off has been considered a chief cause of HABs, but both field and laboratory investigations show diverse HAB nutrient physiology and provide contradictory results between and even within species (12). Less emphasis has been placed on algal mortality, specifically the degree with which reduced predation pressure might enable HAB formation (13-15). Predation by unicellular zooplankton (i.e. heterotrophic protists) is the single largest mortality

factor for phytoplankton in the ocean, with on average over 50% of daily primary production consumed (16). Deterrence of heterotrophic protist predators and reduction in predation pressure could therefore significantly enhance algal survival and thus the potential for harmful algal bloom formation.

The palatability of an algal species to a predator depends on factors such as cell size, structure, shape, chemical composition, and nutritional quality (e.g. 14). Consequently, some phytoplankton species are subject to decreased grazing pressure based on intrinsic characteristics of their morphology or physiology. Known predator-induced algal defense mechanisms include changes in morphology (17-20) and production of chemical deterrents (21-24). Several studies have reported predator-induced changes in movement behaviors involving metazoan plankton (25;26), and some dinoflagellates conducted escape jumps to avoid copepod predators (27). Such predator-induced changes in movements may lead to changes in the population distributions of prey. For instance, the vertical distribution of the freshwater zooplankter, *Daphnia* sp. shifted away from predators or predator exudates (25). Similarly, the marine copepod, *Acartia hudsonica* changed its vertical distribution and migratory behavior depending on the presence or absence of a common fish predator (28). The capacity of phytoplankton species to exhibit similar, predator-induced avoidance behaviors that result in a shift in population distributions has, to our knowledge, not been examined. Such avoidance behaviors would have ramifications for the abundance and distribution of algal populations, and thus for the magnitude of matter and energy flow in marine planktonic food webs.

To examine the role of behavior in planktonic predator-prey interactions, we quantified the microscopic cell-cell interactions, population dispersal, and growth rates of *Heterosigma akashiwo* (Raphidophyceae, Hada) a common HAB alga in the presence of either the heterotrophic protist predator *Favella sp.* or a range of predator-derived cues in vertically structured 1 L tanks imaged with stereo video cameras that captured 3D movement behaviors at multiple horizons every hour for 12 hours (SI 1). We found that the presence of an actively grazing heterotrophic protist predator induced changes in algal movement behaviors, which led to a significant population-level avoidance of the predator, and a 3-fold increase in net algal growth rates. These fleeing behaviors were essential in model predictions reproducing empirical observations of *H. akashiwo* HAB formation in the coastal ocean.

RESULTS AND DISCUSSION

Observed avoidance behaviors

Significant modulation of algal swimming speed and vertical velocity was observed in *H. akashiwo* swimming when exposed to the actively grazing predator, *Favella sp.* ($p < 0.001$; Fig. 1a & b). Swimming speed increased by 38% and upward vertical velocity was 29% faster. These differences were maintained for the entire observation period although mean movements changed over time. The presence of the predator resulted in effective modulations of *H. akashiwo* dispersal rates, approximated by the root mean square distance (RMSD) covered. The most dispersive swimming was observed in response to the presence of the predator (Fig. 1c). Predator-induced changes in individual movement behaviors resulted in a rapid and sustained upward flux of the *H. akashiwo* population (Fig. 2). Vertical migration

behaviors are well known for phytoplankton, (29;30) but can be excluded as a causative mechanism here, as the expected changes in distribution would be the same for all treatments. In the predator-free control, algal population flux was only briefly shifted upwards after 8 h, whereas upward flux commenced immediately in the presence of the predators and after 6 h the entire population was moving upwards.

Increases in algal diffusivity were only observed when algae were directly exposed to the predator, below the halocline. Above the halocline, in a low salinity surface layer (termed refuge) inaccessible to the stenohaline predator, algal fleeing behaviors vanished. Instead, swimming speed and vertical velocity decreased (Fig. 3 a & b). These differences were maintained over the entire observation period. Effective dispersal was more retentive than in the control (Fig. 3c), resulting in increased retention of *H. akashiwo* in this refuge area.

In all treatments, behavioral changes commenced after a lag phase, amounting to 2-4 hours of exposure. Thereafter, predator-induced changes in motility were sustained for the remainder of the observation period.

The ability of *H. akashiwo* to migrate to lower salinity water has been documented in several laboratory experiments (31-33). However, this behavior has not been identified to provide a release from predation. Decreasing co-occurrence with a predator would have a two-fold beneficial effect on *H. akashiwo* survival rates: First, movements away from the predator decrease predator-prey encounter rates and ultimately predation pressure. Second, unlike toxicity that eliminates predators, such avoidance behaviors do not remove predators from the system; so overall predation pressure is shifted and thus increased on other phytoplankton species that do not

display similar anti-predator defenses. This redirected predation pressure would remove competitors from the water column and decrease nutrient competition with other phytoplankton, further increasing the prey species population growth rates (34). The capacity to successfully avoid predators may distinguish this species from phytoplankton that are less successful at bloom formation.

Mechanism of avoidance response

We conducted several control experiments modulating predator-derived cues to probe for possible mechanisms that explain the induction of the algal fleeing behaviors (SI1). First, we exposed *H. akashiwo*, under identical conditions and water column structure to predators that did not feed on this alga (35;36) thus stimulating *H. akashiwo* with the chemical and mechanical cues of swimming predators, but lacking predation pressure. Second, we exposed *H. akashiwo* to a cell-free filtrate from actively grazing predators, thus removing all mechanical cues. Both these treatments elicited qualitatively identical and significant changes in movements and population fluxes but at a quantitatively lower magnitude than responses observed in the presence of actively grazing predators ($p < 0.001$; Fig. 4). Third, we examined the role of the water column structure by removing the low salinity refuge at the top of the tank, forcing a continuous exposure of predator to prey throughout the water column. The presence of the predator in the absence of a refuge elicited a significant increase in swimming speed and vertical velocity in the HAB alga, identical to the fleeing behavior observed in the structured water column; in the absence of a low salinity refuge the difference in vertical velocity from the control was 25% greater in magnitude ($p < 0.001$; Fig 4). It is noteworthy that under these conditions, the

enhanced vertical velocity was in the downward direction, reflecting avoidance of the predator that was aggregated at the top of the tank. Therefore, *Heterosigma akashiwo* is capable of fleeing away from the predator, rather than expressing an inherent reaction of moving to the top of the water column in response to predator presence. Treatment specific characteristic differences in individual motility patterns are shown in Figure 4d. Together, these experiments strongly suggest that algal fleeing behaviors are induced by the presence of predator-derived cues, partially driven by dissolved cues, and quantitatively most significantly driven by the active feeding process.

Both chemical and mechanical stimuli have been reported as effective in eliciting phytoplankton responses (21;27) and exchanges of chemical information within and among trophic levels can directly impact population structure (25;28; 37). Interestingly, we observed that *H. akashiwo* exhibited predator-induced avoidance behaviors for an extended period of time, even when *Favella* sp. abundance was low or distant or occurred several hours prior. Neither a mechanical or chemical cue could be responsible for the sustained modulation of algal movements within the low salinity refuge because the signal could not be effectively transmitted over several cm and hours (38), thus we do not know how this sustained response was elicited. Although we cannot determine the nature of this sustained cue, our observations suggest that either *H. akashiwo* retains information on prior predator exposure for several hours or that cues from conspecifics may have mediated sustained modification of *H. akashiwo* swimming behavior.

Impact of avoidance on population growth

To determine the effectiveness of the fleeing behaviors on net population survival rates, we measured algal growth rates in the different experimental treatments. With the exception of the presence of the feeding predator, none of the other treatments affected algal abundances. Under control conditions the HAB alga grew at a rate of $\mu = 0.81 \pm 0.11 \text{ day}^{-1}$ (Fig 5; mean $\pm$ SEM), equivalent to a population doubling every 21 hours. In the absence of a low salinity refuge, a forced exposure of predator and prey, the ciliate effectively preyed upon *H. akashiwo* cells, resulting in removal of all of the primary production as well as some of the standing stock, yielding a net negative population growth rate ($\mu = -0.67 \pm 0.12 \text{ day}^{-1}$). At this rate the entire algal population would be removed within 28 hours. Fleeing HAB algae, that could access the low salinity refuge, avoided predation with a significantly higher net population growth rate of $\mu = 0.33 \pm 0.06 \text{ day}^{-1}$, or a population doubling within 48 hours ($p = 0.001$). Thus, by invoking the observed fleeing behaviors *H. akashiwo* can avoid population elimination as rapidly as within 1 day and attain considerable population growth rate in the presence of a predator.

The significant increase in fitness imparted by the algal fleeing behavior suggests that there is little cost associated with this defense. Generally, an anti-predator response is associated with a cost; and organisms are subject to a tradeoff between the benefits and costs of exhibiting specific behaviors (39). It is well known that *H. akashiwo* is tremendously halo-tolerant and that some strains can maintain maximal growth rates between 5 and 30 psu (40-42). Yet, the use of low salinity water as a refuge from predation affords the alga with a competitive advantage because

significant positive population growth rates can be maintained in the low salinity surface water due to the halo-tolerance of the alga.

The experimental conditions mimic frequently observed salinity structures in an estuarine setting, including the Fraser River Estuary, British Columbia, Canada (43;44), where *H. akashiwo* blooms have been documented frequently (45). Occurrence of salinity structures that include strong haloclines in the field suggests the defense mechanism we observed in the laboratory may be effective in the coastal ocean. In estuarine systems, grazing rate is positively correlated with salinity, increasing from nearshore to offshore (46). This gradient is established because differential salinity tolerance in populations of heterotrophic protists (47;48). Thus, *H. akashiwo* may be exposed to similar concentration gradients in predator abundance as in the halocline experiments. Given *H. akashiwo*'s broad halo-tolerance, there may be little reduction in growth rate in *H. akashiwo* occupying low salinity waters yet the toxic alga would benefit from reduced predator exposure. Thus, the structuring function of salinity combined with these novel predator induced avoidance behaviors provide a tremendous advantage to *H. akashiwo* and would result in significant increases in fitness to this and by implication other algae that express predator-induced avoidance behaviors.

Model predictions of HAB formation

To quantify the ramifications of the observed individual movement behaviors for ecosystem level processes and the formation of HABs, we formulated a spatially explicit, individual based model that predicted *H. akashiwo* abundance in a 10 m water column with a typical salinity profile for areas with near annual outbreaks of *H.*

akashwo HABs (43;44;49). A below-detection limit seed population of *H. akashiwo* ($100 \text{ cells ml}^{-1}$) that expressed the observed fleeing behaviors attained toxic bloom concentrations of $10^4 \text{ cells ml}^{-1}$ in approximately 2 days (Fig. 6). Identical simulations that omitted fleeing behaviors predicted bloom concentrations only after 6 days. Ephemeral occurrences of HAB blooms, including *H. akashiwo* are well known (34;50) and contribute to their enigma. Although laboratory experiments suggest that *H. akashiwo* can form dense patches at depth through horizontal shear (51), available field data show that *H. akashiwo* blooms occur as surface slicks, where light intensity is highest and salinity lowest (50; 52;53). Our data imply that these surface aggregations could form, at least in part, by *H. akashiwo* movements to avoid predators.

Conclusions

Plankton population dynamics have been difficult to predict and in many cases the species composition has been enigmatic. For example, eutrophication has long been thought to be the driving factor in the formation and persistence of harmful algal blooms (5 and others). However, by linking individual movement behaviors with population distributions and abundances, we show that the consequences of predator-prey interactions in driving localized increases in phytoplankton population abundance may also be important in promoting HAB formation and persistence. Predictions of HAB ecology and management efforts may underestimate population dynamics if they fail to incorporate predator-prey interactions. Moreover, phytoplankton fleeing behaviors may be present in non-HAB phytoplankton and may impact planktonic predator prey interactions in general. These observations highlight the importance of

considering phytoplankton motility and modulation thereof, as well as non-lethal (predator avoidance) predator-prey interactions in influencing plankton spatial distributions, population dynamics, and ultimately carbon cycling in the ocean.

METHODS

Culturing

All experiments were performed on the same strain of the common harmful algal bloom species, the raphidophyte *Heterosigma akashiwo* (CCMP 2809). All phytoplankton cultures were grown in 0.2 μm sterile-filtered autoclaved seawater (FSW), enriched with f/2 nutrients; ciliate predators were grown in FSW only. All cultures were maintained on a 12:12 h light:dark cycle, at 15°C, salinity of 30 psu, and a light intensity of 70-80 $\mu\text{mol photon m}^{-2} \text{ s}^{-1}$ for the phytoplankton cultures and 8-15 $\mu\text{mol photon m}^{-2} \text{ s}^{-1}$ for *Favella* sp. The cultures were not axenic.

Experimental Design

To quantify population distributions, movement behaviors, and grazing rate a 30 cm tall, 5.5 cm wide, 1 L octagonal, acrylic observational tank was used. The tank was filled with 1 L FSW using a peristaltic pump; this method allowed for the creation of defined and stable salinity structures and eliminated fluid convection in the chamber (54). To create a low salinity refuge, the water column was spatially structured to mimic frequently observed estuarine stratification through a halocline with salinities ranging from 8 to 30 psu (33). To force predator-prey encounter throughout the water column, a linear gradient from 27-30 psu was created in the tank. Source water (300 L) from Narragansett Bay, RI was collected prior to the experiments at a salinity of 30 psu. The same source water was used in all experiments and for cultures.

Four treatments were used to quantify the effects of predator presence on *H. akashiwo* population distribution and movement behaviors (SI1): 1) an algal only control, containing *H. akashiwo* alone, 2) a grazing-predator treatment, containing *H. akashiwo* and a strain of *Favella* sp. that actively grazed on the alga, 3) a predator-exposure treatment of the alga to a non-grazing strain of *Favella* sp., 4) a test of the response of *H. akashiwo* to chemical cues derived from the actively grazing *Favella* sp. strain by carefully filtering live predator cultures that were actively feeding on *H. akashiwo* through a 20 μm mesh (SI1). Three separate tanks were used to simultaneously run 1 replicate each of 3 of the 4 treatments and their respective controls. Experiments were repeated until 3 independent replicates of each treatment were acquired. This procedure was repeated for each salinity structure, using predator and prey cultures that were in identical growth conditions and stages.

Using a syringe, organisms were introduced at the bottom of the tank through silicone tubing with an internal diameter of 1 mm. Introduction occurred slowly at a rate of 10 mL min⁻¹ to reduce stress to cells and disturbance to the water column. Phytoplankton cells were introduced to the tank first, and allowed to acclimate for 10 min. *Heterosigma akashiwo* was added to the tank for an average final concentration of 180 cells mL⁻¹. This concentration was chosen to maximize the duration individual cells could be tracked continuously before overlapping with tracks from other cells. Predator cells were then added to the tank using the same silicone tubing as used for phytoplankton. To minimize the transfer volume, 2 L of ciliate culture (final concentration 50 cells mL⁻¹) were gently condensed to 30 mL using a submerged, 20 μm Nitex mesh 15 min before introduction to the bottom of the experimental chamber.

For experiments testing the effect of dissolved cues, the *Favella* sp. culture was condensed prior to filling the tank, and gently filtered through a 0.2 μm syringe-tip filter. Due to the ciliates limited halo-tolerance *Favella* sp. distributions were restricted to salinities >15 psu, that is depth below the halocline (36). To mimic this restricted predator distribution in the dissolved cue treatment *Favella* sp. filtrate was only added to the lower half of the tank. An equivalent volume of water was added to the control tanks, so that volumetrically the treatments remained the same.

To determine *H. akashiwo* growth and predator grazing rates the 1L volume of the tanks were gently homogenized after 24 h incubations and one 30 mL subsample was withdrawn from each tank and preserved with 1% acid Lugol's solution. Ten milliliters of the preserved sample were settled and enumerated using the Utermöhl method (55).

Video capture and analysis

The methods of video capture and analysis followed those presented in Harvey and Menden-Deuer (36). Briefly, three-dimensional movement trajectories were derived from stereo-video images, captured at 30 fps for 1 minute at 6-8 horizons equally spaced along the tank, for a total of 12 hours. Image analysis was automated and identical track assembly and analysis parameters were applied to all videos. Predator and prey were distinguished based on their size difference, which was calibrated with the control videos. Only time points that had ≥ 50 individuals in each replicate, and cells tracked for a minimum of 3 s or longer were used in the movement analysis. All time points were used in abundance analysis.

Statistics

The Kolmogorov-Smirnov test was used to determine significant differences among distributions of swimming behaviors. A Repeated Measures Analysis of Variance (ANOVAR) was used to test for differences in *H. akashiwo* population distributions and swimming behaviors over time. A one-way ANOVA was used to compare growth rates between treatments or salinity structure. Vertical flux was calculated from time- and horizon-specific vertical velocity and cell density measurements. For all analyses the significance level was set to $p < 0.05$.

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FIGURE LEGENDS

Figure 1 – **Below halocline movement behaviors.** Mean (a) swimming speed ($\mu\text{m s}^{-1}$) and (b) mean vertical velocity ($\mu\text{m s}^{-1}$) during the observation period; (c) root mean square distance (RMSD) (mm) of *H. akashiwo* in the absence (white triangles) and presence of the ciliate predator (purple circles) below the halocline. Error bars here and in all figures are one standard error (SE) of the mean. Frequently SE is small and contained within the symbols. Movement behaviors reflect significantly greater upward motility below the halocline in response to the presence of the predator.

Figure 2 – **Below halocline vertical flux.** Vertical flux ($\log \text{ cells } \mu\text{m}^{-2} \text{ sec}^{-1}$) of *H. akashiwo*, below the halocline, in the absence (top) and presence (bottom) of the predator. Warmer colors = upward flux and cooler colors = downward flux of algal cells. After 6h, in the presence of the predator, the vertical flux of *H. akashiwo* was strongly and persistently upward. In contrast, the vertical flux of *H. akashiwo* in the absence of a predator was initially downward and then directionally inconsistent resulting in no effective change in population distribution.

Figure 3 – **Above halocline movement behaviors.** Mean (a) swimming speed ($\mu\text{m s}^{-1}$) and (b) mean vertical velocity ($\mu\text{m s}^{-1}$) during the observation period; (c) root mean square distance (RMSD) (mm) of *H. akashiwo* in the absence (white triangles) and presence of the predator (red circles) above the halocline. Movement behaviors reflect significantly more retentive swimming above the halocline in response to the presence of the predator.

Figure 4 – **Impact of predator-derived stimuli on movement behavior.** Modulation of algal movements as a function of predator-derived cues. Difference in (a) swimming speed ($\mu\text{m s}^{-1}$) and (b) vertical velocity ($\mu\text{m s}^{-1}$), (c) absolute root mean square distance (mm), a proxy of population dispersal rates and (d) characteristic swimming tracks and speeds in the different predator exposure treatments, black circles denote the beginning of a swimming track.

Figure 5 – **Salinity structure impact on *H. akashiwo* growth rate.** Growth rate (μ day⁻¹) of *H. akashiwo* either in a halocline (black) or linear (gray) salinity gradient measured in the algal only control (left) and in the presence of the predator (right). Predator induced fleeing behaviors effectively reduced predator-prey encounter rate and resulted in significant growth of *H. akashiwo* in the presence of the predator.

Figure 6 – **Model results.** Effect of predator-induced fleeing behaviors on HAB formation. Predictions of abundance and distribution of *Heterosigma akashiwo* in a spatially explicit model that does (top) and does not (bottom) include empirically observed algal avoidance of predator inhabited, deeper waters. All parameters, including growth rates were empirically measured and, with the exception of fleeing behaviors, identical in all model runs.

SUPPLEMENTARY MATERIAL

SI1 - Diagram of experimental design and the population distributions of predator and prey in the four treatments. Salinity (psu) is indicated along the height of the tank. The first three treatments included a halocline (8-10 and 27-30 psu) and the last a linear gradient (27-30 psu). From left to right: (1) halocline with grazing *Favella* sp. (red triangle); (2) halocline with non-grazing *Favella* sp. (orange triangle), (3) halocline amended with filtrate (light red fill) from a grazing *Favella* sp. culture added to the bottom half of the tank; and (4) linear salinity gradient with grazing *Favella* sp. strain. Each treatment was observed in triplicate, independent tanks, along with triplicate *H. akashiwo* and *Favella* sp. only controls (not shown). Species distributions reflected

their halo-tolerance: *Favella* sp. is stenohaline and can only persist at salinities > 15psu, in the bottom half of the halocline tank but distributed throughout the water column in the linear salinity gradient, while *H. akashiwo* cells distributed throughout the experimental tank, irrespective of the salinity structure but aggregated to the halocline if present.

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6

SI Figure 1

CHAPTER 3

“Avoidance and attraction: chemical cues influence predator-prey interactions of planktonic protists”

by

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ABSTRACT

Grazing by heterotrophic protist predators mediates phytoplankton abundance and distribution in the ocean, and may be a mechanism that facilitates the formation of large-scale phenomena such as harmful algal blooms (HABs). Here, we investigated the role of chemical cues in influencing the grazing interactions between two heterotrophic protist predators (*Stoeckeria algicida* and *Gyrodiniellum shiwhaense*) and a common HAB alga, *Heterosigma akashiwo*. Using video and image-analysis, population distributions and three-dimensional movement behaviors of the predators in response to a layer of *H. akashiwo* culture filtrate were quantified in triplicate, hourly for 6 h, in a 1-liter experimental column. Experiments were also conducted exposing *H. akashiwo* to a layer of predator culture filtrate. In response to a layer of its preferred prey, *H. akashiwo*, *S. algicida* displayed more retentive swimming behavior by rapidly increasing swimming speed and turning rate. In contrast, *G. shiwhaense*, did not preferentially consume *H. akashiwo*, and exhibited no shifts in behavior in response to the filtrate layer. When exposed to *S. algicida* filtrate, *H. akashiwo* significantly increased vertical velocity in the upward direction, resulting in an increase in abundance in the horizons above the predator filtrate layer. These results suggest that chemical cues influence the grazing interactions between predators and prey, ultimately influencing the population dynamics of both predators and prey, affecting large-scale events and the cycling of material and nutrients.

INTRODUCTION

Grazing by heterotrophic protists is particularly important in mediating phytoplankton dynamics, and ultimately the flow of energy and material throughout

the food web (Sherr and Sherr 1988). Heterotrophic protists are the main consumers of marine phytoplankton biomass, consuming on average greater than 50% of daily phytoplankton production (Calbet and Landry 2004). Thus, small shifts in grazing pressure can play a significant role in influencing changes in phytoplankton population abundance and distributions. However, given the many thousands of species that are simultaneously interacting in the complex, heterogeneous, marine environment, it is difficult to mechanistically understand the factors that mediate grazing processes.

Heterotrophic protist predators are selective feeders; selectivity can impact the abundance, distribution, and composition of the phytoplankton community. Determining the factors that impact selectivity and the mechanisms involved in selection will provide increased understanding on how predators impact phytoplankton population dynamics. The palatability of an algal species to a potential predator is dependent on size, shape, chemical composition, and nutritional quality of the alga (Tillmann 2004 and refs therein). There are many sensory mechanisms that predators can utilize to detect suitable prey; chemical signals are one mechanism by which an individual can gain information about its environment. Chemical signals produced by aquatic organisms have been shown to play important role in deterring grazers, providing cues for foraging, reproduction, and assessing danger (Hay and Kubanek 2002). Information-containing chemical signals that induce a physiological or behavioral response in the receiver are known as infochemicals (Dicke and Sabelis 1988). Infochemicals have been shown to be integral in grazing interactions between heterotrophic protist predators and algal prey (Fenchel and Blackburn 1999; Wolfe 2000). Montagnes et al. (2008) listed six steps: searching, contact, capture, processing,

ingestion, and digestion that are involved in protist feeding. Infochemicals are likely influential in the success of the predator at each of these steps. For example, dissolved chemical cues can increase the success of locating a prey item. Gradients of dimethylsulphiothiopropionate (DMSP) given off by algal species have been shown to both deter and attract grazers (Fredrickson and Strom 2009; Breckels et al. 2010; Seymour et al. 2010). Further, mannose-binding lectins on the surface of the heterotrophic dinoflagellate, *Oxyrrhis marina* have been identified as important in prey recognition and feeding (Wootton et al. 2007). Predator-derived infochemicals can also induce defense mechanisms in phytoplankton such as changes in morphology (Long et al. 2007; Selander et al. 2011), chemical composition (Selander et al. 2006), and avoidance behavior (Harvey and Menden-Deuer, in review) that would decrease the grazing pressure on those algal species exhibiting the defense. Given the importance of these infochemicals in impacting grazing interactions, there is surprisingly little data linking the presence of infochemicals, with shifts in organism behavior, and resultant grazing success. Observing individual behavior in response to chemical cues may provide greater understanding of the grazing process, factors that mediate grazing pressure, and the role of chemical cues in influencing larger-scale phenomena, such as the formation of harmful algal blooms (HABs).

The mechanisms involved in bloom formation, are generally not well known, but factors such as eutrophication (Anderson et al. 2002), allelopathy (Graneli et al. 2008), and benthic-pelagic life cycles (McGillicuddy et al. 2003) have been shown to influence bloom formation. Additionally, it has been suggested that bloom formation results from a reduction in grazing pressure on HAB species, increasing the net

growth rate, leading to bloom formation (Strom et al. 2001; Irigoien et al. 2005). By observing the interaction between heterotrophic protist predators and HAB algae chemical cues, we can begin to understand the role of prey selectivity in the formation, maintenance, and decline of HABs.

Here, using high-resolution video analysis, we quantified the movement behaviors of two similar heterotrophic protist predators, *Stockeria algicida* and *Gyrodiniellum shiwhaenses* when exposed to discrete layers of *Heterosigma akashiwo* culture filtrate. These two predators are similar in size, have the same feeding mechanism, and co-occur in the natural environment but differ in their feeding preference: *H. akashiwo* is the preferred prey of *S. algicida*, whereas the ingestion rate of *G. shiwhaense* on *H. akashiwo* is 1% of the ingestion rate of favorable prey (Jeong et al. 2011).

METHODS

Culturing

Heterosigma akashiwo and *Amphidinium carterae* were cultured in 0.2 μm sterile-filtered autoclaved seawater (FSW), collected from Shiwa Bay, Korea and enriched with f/2 nutrients minus silica (Guillard, 1975). Two heterotrophic protists, *Stockeria algicida* and *Gyrodiniellum shiwhaense* were cultured in FSW only. All cultures were maintained on a 14:10 light:dark cycle at 22°C, salinity of approximately 30 psu, and a light intensity of 20-40 $\mu\text{mol photon m}^{-2} \text{sec}^{-1}$. Cultures were not axenic. Phytoplankton cultures were transferred weekly to maintain exponential growth. Cultures of *S. algicida* were fed *H. akashiwo* and *G. shiwhaense* were fed *A. carterae* every other day, to maintain the predator cultures. All cell

concentrations of both predator and prey cultures were counted in 1% Lugol's solution fixed microscope samples.

Experimental tank set-up

To quantify predator and prey movement behaviors and population distributions, a 30 cm by 5.5 cm, 800 ml octagonal, acrylic observational tank was used. The tank was filled with FSW using a peristaltic pump; this method allowed for the creation of defined salinity structures, while also eliminating convection in the tank (Bearon et al., 2006; Menden-Deuer and Grunbaum, 2006). In all experiments, a linear salinity gradient from approximately 18 to 30 psu was created in the tank, the water used in these experiments was the same as was used to maintain cultures.

The following combinations of organism and culture filtrate were observed in triplicate, individual tanks. *Stoeckeria algicida* was exposed to a layer of the culture filtrate of their preferred prey, *H. akashiwo* as well as *A. carterae*, a prey they do not ingest (Jeong et al. 2005; Table 1). *Gyrodiniellum shiwhaense* was exposed to a layer of *H. akashiwo* culture filtrate. *G. shiwhaense* will ingest *H. akashiwo* at a reduced rate, compared to preferred prey, but the ingestion does not support growth (Jeong et al. 2011; Table 1). Due to logistical constraints, it was not possible to expose *G. shiwhaense* to *A. carterae*. Finally, *H. akashiwo* was exposed to a layer of *S. algicida* culture filtrate. All organisms were also exposed to a layer of FSW, as a control. For all treatments, organisms were slowly introduced first to the bottom of the tank through silicone tubing with an internal diameter of 1 mm. The final average concentration of *H. akashiwo* in the tank was approximately 400 cells ml⁻¹ and 150 cells ml⁻¹ for both *S. algicida*, and *G. shiwhaense*. Immediately after live cell addition, filtrate was added slowly to the tank using the silicone tubing.

Filtrate was created by slowly filtering 10 ml exponentially growing prey culture or 20 ml starved predator culture through a 0.2 μm syringe-tip filter, this is approximately the same volume of live cells that were introduced into the tank. Equivalent volumes of syringe-tip filtered FSW was used as a control. Approximately 500 μl of dionized water was then added to the filtrate to match the salinity at the area of the tank where the filtrate was gently introduced. This process created a stable layer of filtrate in the middle of the tank, approximately 0.3-0.6 cm thick.

Video capture and analysis

The method for video capture and analysis have been detailed in Menden-Deuer and Grünbaum (2006). Two infrared sensitive cameras (Pixelink) with Nikon 60-mm Micro Nikkor lenses monitored approximately 2 ml of water in the center of the tank. The cameras were mounted at a 45° angle with maximally overlapping fields of view to enable reconstruction of three-dimensional (3D) movement behaviors. All filming was conducted in the dark, to eliminate the potential for light-mediated behavioral responses. In order to view organisms, the chamber was illuminated with infrared (960 nm) light-emitting diodes (LED). Filming occurred at 5 horizons, 2 cm apart. Each horizon was filmed for 1 min every hour for a 6 h period. Video was captured at 30 frames s^{-1} .

To determine predator and prey population distributions, each video was analyzed using the same protocol. The 2D position of each individual organism in each frame of the stereo cameras was determined, using ImageJ image-processing software to remove stationary background objects. The threshold was measured manually, so that background subtraction could be automated. The same threshold values were used for all videos. Three-dimensional swimming paths were determined

by first assembling 2D trajectories from Cartesian coordinates of each organism in each stereo frame and then joining 2D tracks based on matching space–time occurrence in the two 2D segments. Trajectories from all treatments were determined using the exact same video analysis and trajectory assembly parameters. The population distributions are based on abundance of tracks at discrete time points and horizons; more details are reported in (Harvey and Menden-Deuer 2011).

Swimming behaviors, including the x, y, and z velocity vectors and turning rates were calculated from 3D paths, subsampled at 0.2-s intervals. The upper and lower 0.5% of each frequency distribution were discarded before analysis to eliminate extreme outliers. For swimming behavior analysis, trajectories from each horizon and replicate were pooled by time point. Only time points that had more than 30 individuals in each replicate and cells tracked for a minimum duration of 3 s or longer were used to measure movement behaviors.

Statistical Analysis

The Kolmogorov-Smirnov test was used to determine significant differences among the distribution and swimming behavior data. For all analyses the significance level was $p < 0.05$.

RESULTS

Predator response to prey filtrate

To test the response of predators to prey filtrate, we first exposed *S. algicida* to filtrate from *H. akashiwo* and *A. carterae*. *Stoeckeria algicida* modified its movement behavior in response to a layer of *H. akashiwo* culture filtrate. Over the 6 h observation period, predator cells significantly increased turning rate by $18 \pm 4\%$ and swimming speed by $24 \pm 5\%$, compared to responses in the control of a layer of FSW

($p < 0.001$; Figure 1). *S. algicida* showed a similar response to a layer of *A. carterae* filtrate, the alga that is not eaten by *S. algicida*. *Stoeckeria algicida* significantly increased turning rate by $17 \pm 2\%$ and swimming speed by $29 \pm 6\%$, compared to the FSW control ($p < 0.001$).

While dramatic shifts in movement behavior of *S. algicida* were observed in response to both *H. akashiwo* and *A. carterae* filtrate, the magnitude of the response to both algal types was similar. When in the presence of *H. akashiwo*, average turning rate and swimming speed were $85 \pm 3 \text{ deg s}^{-1}$ and $315 \pm 32 \text{ } \mu\text{m s}^{-1}$, respectively, compared to $84 \pm 2 \text{ deg s}^{-1}$ and $322 \pm 28 \text{ } \mu\text{m s}^{-1}$ when in the presence of *A. carterae*.

Swimming speed increased significantly over time across all treatments ($p < 0.001$). After hour 2, the rate of increase was the same in all treatments, and the treatment specific differences persisted at all time points. Turning rate remained relatively constant in all treatments across after the initial 2 hours of observation ($p < 0.001$). Likely the differences observed between the first two hours of observation and the rest of the experimental time period was due to cell acclimation to the experimental conditions.

To investigate predator response to a non-preferred prey item, we recorded the movement behavior of *Gyrodiniellum shiwhaense* in response to a layer of *H. akashiwo*. While this predator does consume *H. akashiwo*, ingestion does not support positive growth, and is not a preferred prey item (Table 1; Jeong et al. 2011). Over the 6 h filming period, *G. shiwhaense* did not significantly modify its movement behavior when in a layer of *H. akashiwo* filtrate. Over the course of the experiment, the average turning rate and swimming speed of *G. shiwhaense* in the FSW control were 109 ± 9

deg s⁻¹ and $146 \pm 9 \mu\text{m s}^{-1}$, respectively (Fig. 2). When in a layer of *H. akashiwo* filtrate, average turning rate and swimming speed were $105 \pm 10 \text{ deg s}^{-1}$ and $139 \pm 8 \mu\text{m s}^{-1}$, a decrease in turning rate of 4% and 5% in swimming speed.

Swimming speed increased significantly at the same rate over the observation period for both treatments ($p < 0.001$). Turning rate decreased significantly and similarly over time in both treatments ($p < 0.001$). As was observed with *S. algicida*, movement parameters in the first two hours were dramatically different than those observed during the remainder of the experiment, likely a function of initial acclimation to the tank.

Prey response to predator filtrate

To understand how prey react to predator filtrate, the movement response of *H. akashiwo* to a layer of *S. algicida* culture filtrate was observed. When in a layer of *S. algicida* filtrate, *H. akashiwo* exhibited significant shifts in movement behavior (Fig. 3; $p < 0.001$). When in the predator filtrate layer, average turning rate increased by $18 \pm 5 \text{ deg s}^{-1}$ and swimming speed decreased by $20 \pm 4 \mu\text{m s}^{-1}$, compared to a FSW control. Furthermore, vertical velocity was $33 \pm 5 \mu\text{m s}^{-1}$ when in a layer of predator filtrate, significantly faster upward movement compared to $1.2 \pm 7 \mu\text{m s}^{-1}$ in the FSW control.

The high vertical velocity of *H. akashiwo* in the presence of the predator filtrate layer resulted in a significant increase in *H. akashiwo* cells above the filtrate layer ($p = 0.01$; Fig. 4). While in each treatment *H. akashiwo* was observed at all horizons examined, after 6 hours of observation, when the predator was present in the tank, the largest proportion of the *H. akashiwo* population was found at the top two

horizons ($55 \pm 7\%$), compared to the largest proportion of the population found at the lowest two horizons when cells were exposed to the FSW control ($67 \pm 11\%$; $p = 0.01$).

DISCUSSION

The mechanisms that influence predator grazing selectivity can drive shifts in phytoplankton abundance, species composition, and population distribution. The presence of infochemicals can modulate the behavior of both predator and prey, ultimately shifting encounter rate and predation pressure. Chemical cues from potential food algae provide spatially-directed information on food distribution and quality for potential consumers (Larsson and Dodson 1993). We found that the response to prey-derived filtrate was both predator- and prey-dependent. Furthermore, we observed that predator-derived filtrate stimulated the induction of predator avoidance behaviors, resulting in shifts in prey population distribution. These observed behavioral responses to infochemicals will have significant consequences for both predator and prey population dynamics.

We did not characterize the chemical composition of the culture filtrates used in these experiments; however, they likely contain a mixture of carbohydrates, proteins, and amino acids (Hellebust 1965). The stability of these exuded chemicals varies dependent on environmental conditions. Bacteria are the main consumers of phytoplankton extracellular material in the marine environment, while our cultures were not axenic, the concentration of bacteria in the experimental tank was very low (approximately 1000 cells added), thus the chemical cue likely did not change dramatically over time. In this study, the stimulus that the organisms were exposed to

is unclear. Yet, most of the movement parameters did not shift over time suggesting that the behavioral stimulus was constant over the entirety of the observation period.

It has been well-established that *S. algicida* feeds exclusively on *H. akashiwo* (Jeong et al. 2005; Jeong et al. 2011). Chemosensory attraction to an area of elevated prey density will reduce future searching time, increase the potential for prey contact and feeding efficiency (Montagnes et al. 2008). The less dispersive swimming of *S. algicida* observed in the presence of *H. akashiwo* filtrate would allow the predator to co-occur in areas where *H. akashiwo* is prevalent. However, these benefits for the predator would result in increased grazing pressure on *H. akashiwo*. Field data shows that *S. algicida* can have a significant grazing impact on populations of *H. akashiwo*, and it has been suggested that this predator can contribute to the decline of *H. akashiwo* blooms (Jeong et al. 2005). Thus, the use of infochemicals is a mechanism that this predator can utilize to exploit high-density areas of *H. akashiwo*.

Modifications of movement behavior are not without costs to the predator, therefore, it would be most advantageous for highly selective predators, such as *S. algicida*, to have a highly specified biochemical prey recognition capacity (Montagnes et al. 2008). However, despite its highly specific prey preference, *S. algicida* also exhibited less dispersive swimming behaviors when in the presence of a prey item it does not ingest, *A. carterae*. A selective predator that does not display prey preference specificity during the searching phase of an encounter would need to have post-encounter prey discrimination. For example, the heterotrophic protist, *Oxyrrhis marina*, has been shown to reject certain food types during the prey-processing stage, including those prey that are depleted in nitrogen or have high DMSP lyase activity

(Flynn and Davidson 1993; Wolfe et al. 1997). Likely, the mechanisms driving these post-encounter decisions are either driven by receptor-mediated processes on the cell surface that trigger cell signaling pathways (Roberts et al. 2011), or inability to process prey could be the result of prey escape, which has been documented for predators that commonly feed via a pallium or peduncle (Buskey 1997; Jakobsen 2006). Thus, for predators such as *S. algicida*, the presence of infochemicals can enhance encounter rates with potential prey items, but modifications in movement behavior cannot be used as a reliable estimate for successful predation.

No shift in behavior was exhibited by *G. shiwhaense* in response to the presence of *H. akashiwo*, a non-preferred prey species. While, *G. shiwhaense* has been observed to ingest live *H. akashiwo* cells, ingestion rate was low and did not support growth of the predator (Jeong et al. 2011). However, feeding experiments have shown that *G. shiwhaense* is not strict in its food selection, and will feed and positively grow on a diversity of prey types, including *Amphidinium carterae*, *Isochrysis galbana*, *Prorocentrum minimum*, *Rhodomonas salina*, and *Teleaux* sp. (Jeong et al. 2011). Thus, *G. shiwhaense* is more of a foraging generalist, compared to *S. algicida*. Generalist herbivores often have a singular foraging goal of energy and nutrient maximization (Belovsky 1986). In this case, *H. akashiwo* does not support growth of the predator, thus this particular predator would not expend energy modifying its behavior to encounter this prey item. Thus, for more generalist predators such as *G. shiwhaense*, modulation of movement will likely signal feeding, and shifts in movement could be used to predict predation pressure.

In response to the presence of *S. algicida* filtrate, *H. akashiwo* dramatically increased swimming speed in the vertical direction, resulting in an accumulation of the algae in a predator filtrate free portion of the tank. Altering movement behavior to avoid the predator will benefit *H. akashiwo* survival by decreasing predator-prey encounter rates and predation pressure. Furthermore, these avoidance behaviors will lead to shifts in the spatial aggregation of this toxic alga, that could lead to bloom formation in this species (Harvey and Menden-Deuer, in review; Strom et al. in review). Avoidance as a defense mechanism has been observed in other marine predator-prey systems; many zooplankton undergo diel vertical migration, feeding in surface waters at night when visually foraging predators are less active, a process that is cued by chemical detection of zooplankton predators (De Meester et al. 1999).

Similar avoidance behaviors have been observed in *H. akashiwo* previously (Harvey and Menden-Deuer, in review), however in these experiments the greatest shift in *H. akashiwo* behavior was observed in the presence of actively grazing predators. Avoidance behaviors were observed in response to the culture filtrate of the ciliate predator, *Favella* sp., but the shift was minimal. In comparison, in response to *S. algicida*, *H. akashiwo* exhibited a dramatic increase in vertical velocity. This differential response may be correlated to the predation risk each predator exerts over *H. akashiwo*. In the natural environment, predation risk is not constant, but rather varies both spatially and temporally, thus anti-predator mechanisms should include some sensitivity to current level of predation risk (Lima and Dill 1990). It has been shown that large ciliates often die when they ingest *H. akashiwo* cells (Verity and Stoecker 1982; Clough and Strom 2005; Graham and Strom 2010; Harvey and

Menden-Deuer 2011), thus, in general, the predation risk from *Favella* spp. would be low. However, *S. algicida* is a voracious predator, singularly consuming *H. akashiwo*, therefore a heightened escape response would allow *H. akashiwo* a greater opportunity to escape predation. Thus, infochemicals are a crucial link in the predation risk assessment mechanisms of *H. akashiwo*.

The mechanisms that drive the interactions between heterotrophic protists and phytoplankton are difficult to characterize. However, by creating a link between infochemical presence, movement behavior, and grazing success we can begin to understand the how infochemicals mediate these predator-prey interactions. The population dynamics of both predators and prey may be improperly estimated if they fail to incorporate the population structuring function of infochemicals. Our findings highlight the importance of both predator and prey motility in influencing population abundance, composition, and distribution, ultimately influencing the flow of energy and material through the marine environment.

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FIGURE LEGENDS

Figure 1 – ***Stoeckeria algicida* movement behaviors.** (A) *Stoeckeria algicida* swimming speed ($\mu\text{m s}^{-1}$) and (B) turning rate (deg s^{-1}) measured at hourly intervals in response to a filtered seawater (FSW) control (black), *Heterosigma akashiwo* filtrate (white), and *Amphidinium carterae* filtrate (gray). Error bars represent one standard error of the mean of all movement tracks collected at the target horizon in triplicate experiments and are contained within the symbols. (C, D) Projected swimming tracks collected over 1 min in a volume of 2 mL, showed a significant shift in movement behavior of *S. algicida* (D) in the presence of *H. akashiwo*, compared to the control (C).

Figure 2 – ***Gyrodiniellum shiwhaense* movement behaviors.** (A) *Gyrodiniellum shiwhaense* swimming speed ($\mu\text{m s}^{-1}$) and (B) turning rate (deg s^{-1}) over time in response to a filtered seawater (FSW) control (black) and *H. akashiwo* culture filtrate (white). Error bars represent one standard error of the mean and are contained within the symbols. (C, D) Projected swimming tracks, collected over 1 min in a volume of 2 mL, showed no significant changes in movement behavior in the presence of *H. akashiwo* (D), compared to the control (C).

Figure 3 – ***Heterosigma akashiwo* movement behaviors.** (A) *Heterosigma akashiwo* swimming speed ($\mu\text{m s}^{-1}$), (B) turning rate (deg s^{-1}) and (C) vertical velocity ($\mu\text{m s}^{-1}$)

over time in response to a filtered seawater control (black) and *S. algicida* filtrate (white). Error bars represent one standard error of the mean and are contained within the symbols. In response to the presence of *S. algicida* filtrate, *H. akashiwo* significantly increased turning rate, swimming speed and upward motility.

Figure 4 – ***Heterosigma akashiwo* population distribution.** Population distribution of *H. akashiwo* after 6 h in response to the filtered seawater control (black) and *S. algicida* filtrate (white). The increased upward swimming movement of *H. akashiwo* in the presence of *S. algicida* resulted in significantly more *H. akashiwo* at the top two horizons of the tank when the predator was present. Error bars represent one standard error of the mean of triplicate experiments.

Figure 5 – **Encounter rate model.** Predicted encounter rate ($\text{prey pred}^{-1} \text{d}^{-1}$) for (A) *S. algicida* and (B) *G. shiwhaense*, under control conditions (black circles), in the presence of *H. akashiwo* filtrate (gray triangles), and when *H. akashiwo* (HA) was exhibiting fleeing behaviors in the presence of predator filtrate (white squares). *G. shiwhaense* did not respond to *H. akashiwo* filtrate and had an overall lower predicted encounter rate due to lower swimming speeds than *S. algicida* (SA). Increase in swimming speed by *S. algicida* in response to prey filtrate, after hour 2, resulted in increased encounter rates. Incorporation of *H. akashiwo* fleeing behavior resulted in an initial decrease in predicted encounter rate that was later nullified by predator modulation of movements in response to prey filtrate.

Table 1 – **Maximum growth and ingestion rates for predators.** Maximum growth rates (μ , d⁻¹) and maximum ingestion rates (I, ng C grazer⁻¹ d⁻¹) of the heterotrophic dinoflagellates *Gyrodiniellum shiwhaense* and *Stoeckeria algicida* on prey species used in this study.

	<i>G. shiwhaense</i>		<i>S. algicida</i>		References
	μ	I	μ	I	
<i>Heterosigma akashiwo</i>	-0.2	0	1.6	0.8	1, 2
<i>Amphidinium carterae</i>			did not feed		2

¹Jeong et al. 2005a; ²Jeong et al. 2011

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

APPENDICES

A. INTRODUCTION

Phytoplankton are pelagic autotrophs responsible for approximately 50% of total global primary production (Field et al. 1998), which sustains fisheries production and represents an integral component in the transfer of material in the global carbon cycle. The balance between phytoplankton growth and loss processes largely determines the abundance and distribution of phytoplankton in the marine environment. Understanding the mechanisms that influence phytoplankton growth and loss rates will increase the ability to predict shifts in phytoplankton community dynamics based on biotic and abiotic factors, and ultimately shifts in global nutrient cycling.

One frequent shift in population dynamics of phytoplankton is the formation of harmful algal blooms (HABs). Primarily, these blooms of toxic phytoplankton are mono-specific, and frequency of bloom events has been increasing worldwide (Smayda 1990). The mechanisms that drive the formation, persistence, and decline of these blooms are not well understood, however HABs can have detrimental impacts on coastal marine ecosystems and economies. These blooms are an example of excess net phytoplankton growth. The majority of previous research into the factors that promote HAB formation has focused on eutrophication as being the driving factor (Smayda 1998; Anderson et al. 2002). However, grazing by unicellular heterotrophic protists is the single largest mortality factor for phytoplankton in the ocean, with on average over 50% of daily primary production consumed by these predators (Calbet and Landry 2004). Thus, heterotrophic protist grazing directly impacts phytoplankton population

abundance, and low grazing pressure has been suggested as a possible mechanism of HAB formation (Strom et al. 2001; Tillmann 2004; Irigoien et al. 2005).

To quantitatively examine heterotrophic protist grazing pressure, bulk estimates of grazing rates are commonly measured. However, these rates are the result of multiple individual interactions between predators and prey. Traditionally, consumption is the only interaction between protist grazers and phytoplankton that has been examined, however not all interaction result in consumption. Non-consumptive interactions, such as predator selectivity against a particular algal species, can have consequences that may change predator and prey population dynamics (Lima 1998). Each type of individual interaction will have different consequences for phytoplankton dynamics. For example, predator mortality, reduces overall predator abundance, increasing algal competition for resources. Prey selectivity reduces predation pressure on the avoided species, decreasing resource competition for the avoided species. Understanding the nature of these individual interactions provides the opportunity to understand the mechanisms that drive grazing rates and allow for prediction of HAB formation, persistence, and decline.

The interactions of microscopic organisms with their environment, including potential predators and prey, as well as other stimuli, have been investigated for decades (Ulehla 1911). However, many of these investigations have been conducted with a small sample size, tethered organisms, or only a narrow selection of species. The data presented in this dissertation is the result of the use of novel techniques to observe hundreds of free-swimming individuals across space and over time. These techniques allow for a linkage to be made between individual movement behaviors

and resultant population distributions and abundances. These measurements will ultimately, enhance the understanding of marine primary production and biochemical cycling rates in the ocean.

This research was focused on the interactions that the toxic raphidophyte, *Heterosigma akashiwo* has with potential predators. This HAB species are found globally (Smayda 1998), and can be ichthyotoxic (Honjo 1993; Khan et al. 1997), and cause sub-lethal effects in shellfish and mammals (Twiner et al. 2004; Keppler et al. 2005). Prior experimental evidence showed that while some heterotrophic protist readily feed on *H. akashiwo* (Jeong et al. 2003; Clough and Strom 2005) others, do not ingest the toxic alga (Kang et al. 2011), further other potential predators, such as large ciliates die when they ingest *H. akashiwo* (Verity and Stoecker 1982; Clough and Strom 2005; Graham and Strom 2010). In the field, *H. akashiwo* blooms have been associated with low heterotrophic protist grazing rates (Kamiyama et al. 2000; Menden-Deuer et al. 2010). Thus, there is ample evidence to suggest that low grazing pressure may be an important contributor to HAB formation in this species.

This dissertation presents the completed and published manuscripts from three experiments investigating the interactions between heterotrophic protist predators and *H. akashiwo* to gain a better understanding of how grazing interactions influence the ecology of this species. In the first paper, the behavioral response of a potential predator, a layer of toxic prey is investigated. In the second paper, the behavioral response of *H. akashiwo* to predator presence is examined, and a novel predator avoidance behavior is discussed. The third paper focuses on the role of chemical cues in stimulating shifts in the movement behavior of both predators and prey. These three

papers highlight the importance of increasing the mechanistic understanding of predator-prey interactions to understand the impact of heterotrophic protist grazing on phytoplankton population dynamics.

B. DISCUSSION

Phytoplankton are the base of the marine food web, and as such, are central components in the cycling of energy and nutrients throughout marine ecosystems. Decades of research of measuring grazing rates in the ocean have demonstrated that heterotrophic protist predators directly impact phytoplankton population abundance and distribution. Yet, the mechanisms of predator-prey interactions that produce the often-measured bulk grazing rates are not well understood. This dissertation investigated the mechanisms driving predator-prey interactions by examining microscopic, individual movement behaviors and linking them to macroscopic population abundances and distributions. Quantifying individual movement behaviors can provide a basis for understanding how phytoplankton populations respond to environmental or community composition shifts. These observations can then be the basis for creating hypotheses about the ecological and evolutionary consequences of cell-cell interactions in the ocean, resulting in increased understanding of how predator-prey interactions influence the flow of material and energy throughout the ocean.

The spatial distribution of any organism in the ocean is determined by a combination of the organism's behavior and physical oceanographic processes. Even with their small size, the movement behavior of phytoplankton can have a significant impact on population distributions (McManus and Woodson 2012). Phytoplankton can

shift their behavior in response to a variety of environmental parameters, and can have a range of behavioral capabilities from being passive to reacting to vertical or horizontal gradients. For example, many phytoplankton species conduct diel vertical migrations, shifts in vertical distribution based on light cues (Cullen and MacIntyre 1998). Results within this dissertation, suggest that interactions between heterotrophic protist predators and phytoplankton can significantly drive shifts in the behavior of both predator and prey, leading to shifts in population distribution. This is important, as often phytoplankton are modeled as passive particles, whose abundance and distribution are a consequence of fluid dynamics and nutrient concentration. Biophysical models have difficulties resolving high frequency processes such as organism interactions, and further, species-specific behavior is absent for many phytoplankton species (McManus and Woodson 2012). However, exclusion of predator-prey driven behavior would reduce the accuracy of biophysical models, ultimately decreasing the ability to predict phytoplankton population dynamics.

Similarly, individual movement behaviors and resultant shifts in population distributions can be used to understand phenomena that occur naturally in the marine environment, such as the formation of harmful algal blooms (HABs). While the cause of HAB formation is unknown, traditionally research has focused on increased eutrophication, stimulating algal growth rate, leading to bloom formation (Anderson et al. 2002). However, this dissertation demonstrates the importance of investigating the impact of changes in phytoplankton mortality rates on HAB formation. Through these experiments, I show that even a slight decrease in mortality rate as a result of alga behavioral modifications, can result localized increases in phytoplankton population

abundance. HAB ecology and management of such blooms may misrepresent population dynamics without the incorporation of phytoplankton loss rate dynamics, ultimately decreasing the accuracy of bloom formation prediction.

The importance of grazing as the major source of phytoplankton mortality in the ocean, suggests that there is intense selective pressure for the evolution of defenses against predation (Smetacek 2001). While, it is known that there is a range of morphological and chemical defenses against predation (as referenced in Tillmann 2004), behavioral defenses against predation are less explored. The experiments included in this dissertation demonstrate that, for at least one algal species, phytoplankton can exhibit an array of defense mechanisms in response to predator presence, including toxicity, avoidance, and salinity tolerance. There are two distinct categories of mechanisms that can mediate prey survival: (1) avoidance mechanisms that reduce the probability that a predator and prey will co-occur and (2) anti-predator mechanisms that reduce the probability of successful predation when predators and prey do co-occur. These two mechanisms are under different selective pressures, evolving one mechanism reduces the selective pressure on the other (Brodie et al. 1991). The results presented here suggest that phytoplankton can use both simultaneously. In the complex habitat of the coastal marine environment, having multiple mechanisms to defend against changing predator abundance and diversity would be evolutionarily advantageous for phytoplankton. Further, the capacity to exhibit multiple, environment- or species-specific predator defense mechanisms may distinguish these phytoplankton from other species who are less successful at predator defense, ultimately increasing the abundance of well-defended species.

One defense mechanism in particular that I observed was predator avoidance. In multiple experiments, the HAB alga, *H. akashiwo* shifted its movement behaviors in response to predator presence, resulting in distributions of cells away from areas with high densities of predators (or predator chemical cues) to areas with a decreased predator presence. This was the first observation of a shift in population distribution to avoid a predator by a plant species. Predator-induced defensive responses can help prey avoid being consumed, but often come at a physiological cost. For example, induction of defensive behavior may result in prey fleeing to a less hospitable habitat (Sih 1994; Lima 1998) or risk capture by another predator (Sih et al. 1998), reducing survival. To best benefit survival, the use of avoidance behaviors by phytoplankton should occur when the costs of exhibiting such behaviors are less than the benefits of decreased grazing pressure. In some experiments fleeing behavior was beneficial to *H. akashiwo* growth, as the alga fled to predator-free low salinity water, a refuge, as declines in algal growth rate are only observed at salinities less than 6 psu. Physical refuges from predation are not well documented in the plankton. They have been shown to be important in the rocky intertidal, often being a primary component in structuring many of these communities (Menge and Lubchenco 1981, Pfister and Hay 1988). Less is known about how influential physical refuges from predation are in structuring planktonic communities. They may provide complete protection from predation, but more likely, there are gradients in the efficiency and degree of protection that the refuge provides (Milchunas and Noy-Meir 2002). In terrestrial studies, physical refuges resulted in an increase in plant diversity, and may lead to shifts in biogeography (Milchunas and Noy-Meir 2002). However, *H. akashiwo* also

exhibited avoidance behaviors in the absence of a refuge, suggesting that avoidance is a behavioral default given predator presence, but are only maximally beneficial under environmental conditions and water column structure are prime for net population growth. Further investigations will provide constraints around the exhibition of these behaviors under natural, field conditions.

Future investigations into the impact of heterotrophic protist predators on phytoplankton should include observations of how predators and prey interact with one another. The findings within highlight the importance of considering phytoplankton motility and non-lethal (predator avoidance) predator-prey interactions in influencing the macroscopic plankton spatial distributions and population abundance. To truly represent population dynamics, research needs to move beyond considering primarily those factors that impact growth, and incorporation more focus on those factors that impact loss processes. Ultimately, it is the combination of these factors that will dictate the accurate understanding of carbon and nutrient cycling in the marine environment.