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Diatom Frustules as a Mechanical Defense Against Predation by Heterotrophic Dinoflagellates

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**DIATOM FRUSTULES AS A MECHANICAL DEFENSE AGAINST
PREDATION BY HETEROTROPHIC DINOFLAGELLATES**

By
Tyler Spillane

Accepted in Partial Completion
Of the Requirements for the Degree
Master of Science

Kathleen L. Kitto, Dean of the Graduate School

ADVISORY COMMITTEE

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MASTER'S THESIS

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A Thesis
Presented to
The Faculty of
Western Washington University

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Of the Requirements for the Degree
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February, 2016

ABSTRACT

Diatoms contribute up to 40% of the total primary production in the ocean and heavily influence the cycling of carbon and silica. Much of their success results from their silica frustule, which may provide a mechanical defense against grazers. In this study, I sought to determine the possible defense mechanism of the diatom's frustule in the presence of one of their dominant grazers, heterotrophic dinoflagellates. I grew two species of diatoms, *Thalassiosira rotula* and *Coscinodiscus radiatus*, in semicontinuous culture with 80 μM or 20 μM silicic acid. Based on a 2-fold higher BSi cell⁻¹, BSi:C, and BSi:N, this culture method successfully resulted in diatoms of both species with thick and thin frustules. I performed three predation experiments to determine if frustule thickness would affect predator ingestion, digestion or growth rate. I first fed thick and thin *T. rotula* to *Gyrodinium spirale* and measured ingestion and growth rate over 48 hr. I found no difference in ingestion rate between the thick and thin treatments, however *G. spirale* grew significantly slower on the thick-frustuled diatoms. I then fed thick and thin *C. radiatus* to *Noctiluca scintillans*, measuring ingestion rate in one experiment and digestion rate in a second. I found no difference in predator ingestion rate at the end of the 4 hr experiment. However, I did observe a significantly lower predator digestion rate when feeding on the thick-frustuled diatoms. The results strongly suggest that the frustule is providing a defense to the diatoms by slowing the predator's digestion rate, which then decreases their population growth rate. This is the first reported evidence in favor of diatom frustules as a means of defense against microzooplankton. As such, this proposed mechanism has important implications for diatom bloom dynamics as well as global carbon and silica cycling.

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INTRODUCTION

Diatoms are a major source of productivity in the world's oceans, with estimates of up to 40% of the total primary production derived from these silicified organisms (Nelson et al. 1995). An estimated 2150 Tmol C yr⁻¹ of organic carbon production is attributed to diatoms (Nelson et al. 1995). Moreover, diatoms possess a mineralized frustule, making them denser compared to other phytoplankton groups. As a result diatoms are key players in the 'biologic pump' of carbon to the deep ocean (Goldman 1993, Brzezinski et al. 1998, Dugdale and Wilkerson 1998). Diatoms also heavily influence global silica cycling, comprising the majority of biogenic silica production in the oceans and contributing to almost half of silicic acid removal from the euphotic zone (Tréguer et al. 1995, Assmy et al. 2013).

Much of the dominance of diatoms in the oceans can be attributed to the production of a glass frustule composed of amorphous silica (Raven 1983, Martin-Jézéquel et al. 2000, Raven and Waite 2004). Furthermore, the success of diatoms is clearly linked to environmental silica concentrations, which often act as a limiting nutrient in many ecosystems (Paasche 1973, 1980, Gensemer 1990, Dugdale and Wilkerson 1998). While their success is clearly linked to this frustule, the ecological advantage it provides is poorly understood. Hypotheses on the benefit of the frustule include providing an increased nutrient uptake, protection against photoinhibition, control of sinking rate, or acting as a mechanical defense against grazers (Martin-Jézéquel et al. 2000, Hamm et al. 2003, Raven and Waite 2004, Finkel and Kotrc 2010).

The amount of diatom-produced carbon and silica that is exported to the deep ocean is heavily dependent on the properties of the silica frustule. Frustules come with the cost of an increased cell density, and, unless countered by adjustments to buoyancy, an increase in sinking rate, as high as 100 m day⁻¹ (Kiorboe et al. 1996, Raven and Waite 2004). There is a

2-fold increase in sinking rates with thicker, more silicified, frustules compared to thinner frustules in *Odontella* sp. (Durkin et al. 2013). This added density from the frustule results in approximately 40-48% of the biogenic silica sinking out of the euphotic zone while the other 52-60% is remineralized in the upper layer to be used by other diatoms (Tréguer et al. 1995, Ragueneau et al. 2000). In addition to the dense frustule, diatoms possess sugar-containing exudates on their cell wall that greatly increase their chance of attaching to other diatoms or particulate organic carbon (Waite et al. 1995). This 'stickiness', combined with the high density of the frustule, leads to a significant role for diatoms in carbon export out of the euphotic zone and eventual burial in the sediments (Smetacek 1985, 1999, Kiorboe et al. 1996).

For a diatom bloom to form, the amount of growth must exceed the amount of loss from grazing, sinking, and other processes (Tillmann 2004). Therefore, the accumulation of diatom biomass, and thus the level of carbon and silica export, is heavily controlled by predation, which could be limited by mechanical defenses in diatoms. Moreover, there is often a large disconnect between diatom growth and loss from predation, such that diatoms tend to form longer-lasting blooms than other phytoplankton (Kiorboe et al. 1996, Smetacek 1999, Irigoien et al. 2005). This disconnect could be a result of diatom defenses that lead to lower predation pressure from planktonic grazers and thus higher diatom biomass (Strom 2002, Tillmann 2004). A mechanical defense resulting from the frustule could help explain this decoupling, leading to my central hypothesis: that the diatom frustule provides a mechanical defense against microzooplankton grazers.

Factors Affecting Frustule Thickness

Diatom silica frustules often vary in thickness depending on the environmental conditions or particular species. Thickness within a species is predominately determined by the in situ silicic acid (H_4SiO_4) concentrations during growth. Limiting concentrations in a laboratory setting have ranged from 10 μM to 20 μM Si (Davis et al. 1973, Paasche 1973, Harrison et al. 1977, Durkin et al. 2013) while concentrations in the central Salish Sea range from 20 to 60 μM Si (Water quality database, SPMC), and can be considerably lower in enclosed bays such as East Sound, WA (Paul 2010). Under silicic acid-limiting conditions, biogenic silica per cell (and therefore frustule thickness) is considerably reduced (Paasche 1973). During conditions of moderately low silicic acid (~ 20 μM Si) but high nitrogen and phosphorus, silica deposition and cell growth can become decoupled, resulting in constant growth rate but decreased silicification (Paasche 1973, Martin-Jézéquel et al. 2000). Biogenic silica also varies amongst different species of diatoms, ranging from 0.02 – 200 pmol Si cell^{-1} depending on cell size (Martin-Jézéquel et al. 2000).

Silicic acid limitation results in shorter length chains and fewer spines in diatoms (Paasche 1973, Harrison et al. 1977). As a result of decreased chain length, diatoms could have lower nutrient uptake efficiency or altered predation pressure. Chain formation can be vital in increasing nutrient uptake. Elongated diatoms appear to be more efficient at taking up nutrients compared to cylindrical species due to the increased surface area to volume ratio (Pahlow et al. 1997). Others have suggested that chain length can determine ingestion rate for certain grazers; copepods prefer longer-chained diatoms compared to shorter chains (Estep et al. 1990, Bergkvist et al. 2012). Diatoms also have plasticity in chain length when exposed to copepods, which is possibly a mechanism to reduce predation (Bergkvist et al. 2012). Frustule thickness may also increase when diatoms are exposed to grazers as

a potential defense response (Pondaven et al. 2007). Exposing *T. weissflogii* to copepods resulted in a 2-fold increase in BSi per cell compared to diatoms in a control chamber.

A myriad of other environmental conditions can also affect the degree of silicification in diatoms. During non-silica limiting conditions, higher or lower ambient temperature as well as light intensity can decrease the amount of silica per cell (Paasche 1980, Martin-Jézéquel et al. 2000). In the freshwater diatom *Asterionella ralfsii*, pH and aluminum also affected silicification rates (Gensemer 1990, Gensemer et al. 1993). Furthermore, the marine diatom *Thalassiosira weissflogii* grown under zinc and iron limitation had 40-60% more Si per cell and an increased uptake rate of silicic acid (De La Rocha et al. 2000).

Ecological Benefits of Diatom Frustule

While much is known about the formation of the silica frustule, the ecological benefits associated with this external structure in diatoms are less clear. Despite this uncertainty, we do know that there is a high energy cost associated with frustule formation (Raven 1983). Silicic acid concentrations in much of the surface ocean are below saturating levels for diatom uptake (Sarmiento and Gruber 2006). To compensate, much of the silicic acid uptake is through active transport, utilizing ATP as the energy source (Finkel and Kotrc 2010). Conversely, once silicic acid is taken up, only ~2% of the organism's total energy is required to deposit the silica. The higher energetic costs for uptake come from creating the materials required for transport, including transporters, polysaccharides, and vesicles; these costs can be twenty times that of deposition (Raven 1983).

Diatom frustules affect the uptake of light energy and nutrients. Models examining light scattering as a function of frustule thickness in diatoms showed that an increase in frustule thickness results in an increase in light scatter off the cell; this suggests some

protection from photoinhibition (Kitchen and Zaneveld 1992). The ultrastructure of the frustule may also assist in nutrient uptake by increasing the residence time of colloidal nutrient particles around the cell wall, allowing an increase in nutrient diffusion rates (Hale and Mitchell 2001, Musielak et al. 2009, Finkel and Kotrc 2010).

Theoretically, silica frustule formation increases diatom sinking rate as a result of the increased density of biogenic silica compared with that of seawater (2.2 – 2.6 g/cm³ versus 1.02-1.03 g/cm³) (Finkel and Kotrc 2010). However, silica spines and chain formation increase form resistance (Φ) in seawater; combined with a large vacuole that allows for buoyancy adjustments this slows diatom sinking rates (Padisák et al. 2003). While it seems detrimental for diatoms to sink out of the euphotic zone, there may be some benefits involved. Sinking may be advantageous to escape grazers or when switching from a vegetative stage to a resting stage, a major part of some species' life cycles (Smetacek 1985). Furthermore, sinking could be beneficial when surface irradiance is high and has the potential to inhibit photosynthesis or damage the cell (Raven and Waite 2004).

The third possible ecological benefit, which will be directly tested in this project, is that frustules provide a mechanical defense against predation. While many studies have presented the diatom's frustule as a potential defense against predation, few studies have provided reliable evidence to support this (Martin-Jézéquel et al. 2000, Raven and Waite 2004, Finkel and Kotrc 2010, Durkin et al. 2013). A biophysical study looked at the possibility of the frustule as an effective mechanical defense (Hamm et al. 2003). A series of tensile strength tests were performed using glass microneedles to apply increasing levels of pressure on the girdle band and central valve of various diatom species. The authors determined that the frustule had a remarkably high tensile strength, comparable to cortical bone or dental composites. This was expanded upon by applying these strength tests to more diatom species and then observing copepod ingestion rates on species with different

measured frustule strengths (Friedrichs et al. 2013). Copepod ingestion rates were significantly reduced in diatoms with stronger, thick frustules, compared to smaller species with thin frustules. However, because the authors used different diatom species, with a range of sizes, to compare ingestion rates among the copepods this confounds the results found in this study. Cell size and prey type has a large effect on ingestion rate by any planktonic predator (Frost 1972). Rather, a more effective experimental design would be to manipulate the frustule thickness of a single diatom species and then feed them to the predators. Furthermore, most of the arguments in favor of mechanical defense have considered predation only from copepods with no attention to microzooplankton grazers.

Diatom Predation by Microzooplankton

Classically, mesozooplankton (>200 μm), typically copepods and euphausiids, were considered the main grazers of diatoms in the oceans (Fleming 1939). Recently, studies have shown that the dominant grazers of marine diatoms are smaller (<200 μm) microzooplankton, primarily protists (Lessard 1991, Calbet 2001, Strom et al. 2001, Sherr and Sherr 2002, 2007, Calbet and Landry 2004). Microzooplankton grazers, mainly consisting of ciliates and heterotrophic dinoflagellates, represent, on average, 65% of the grazing on bloom-forming diatoms (Strom et al. 2001) and often dominate all of the diatom predation (Sherr and Sherr 2007), while mesozooplankton grazing only consists, on average, of 22% or less of the total predation on diatoms (Calbet 2001, Strom et al. 2001).

During diatom blooms heterotrophic dinoflagellates often constitute a major proportion of the microzooplankton grazer community (Tillmann and Hesse 1998, Sherr and Sherr 2007). Dinoflagellates constitute an evolutionarily broad group of species with a variety of sizes and feeding behaviors (Taylor 1989, Kiorboe and Titelman 1998, Hansen

and Caladob 1999). Feeding behavior of dinoflagellates directly relates to the mechanical defense of diatom frustules. Virtually all frustule defense studies have focused on copepod feeding mechanics, which involve crushing with the mandibles (Hamm et al. 2003, Friedrichs et al. 2013). Copepod-based studies do not adequately address questions about the defenses of diatoms against dinoflagellates because dinoflagellate predation involves directly engulfing prey, tube feeding, or use of a pallium (Strom and Buskey 1993, Hansen and Caladob 1999).

To investigate whether diatom frustules provide a defense against heterotrophic dinoflagellates, I performed predation experiments utilizing 2 species of diatoms, each grown with thick and thin frustules. As opposed to similar studies, I manipulated frustule thickness within a diatom species so as not to add confounding factors such as prey size and species differences. In this study, I considered that diatom frustules were a means of defense if predator ingestion, digestion or growth rate was significantly lower when feeding on thick-frustuled diatoms. To investigate this I asked two questions: 1) Does the diatom frustule provide a means of defense against heterotrophic dinoflagellates? 2) Through what mechanism do frustules protect diatoms from predation?

METHODS

Culture Methods

For the grazing experiments, I used two species of diatoms. Small (8 μm cell diameter), chain-forming, *Thalassiosira rotula* strain CCMP 1647 was isolated from the Mediterranean Sea in 1993. The larger (50 μm cell diameter), solitary, *Coscinodiscus radiatus* strain CCMP 312 was isolated from the Gulf of California in 1973.

I grew *T. rotula* and *C. radiatus* in semi-continuous culture with an artificial seawater (ESAW) medium base (Harrison et al. 1980). ESAW media contained f/2 nutrients, except the silicic acid concentration was adjusted downward to create two separate Si treatments of 20 μM and 80 μM $\text{Na}_2\text{Si}(\text{OH})_4$ (Paasche 1973, Conway et al. 1976, Durkin et al. 2013). To avoid silicic acid contamination cultures were maintained in polycarbonate bottles (Paasche 1973). *T. rotula* was grown on a light table with 285 $\mu\text{mol photons sec}^{-1} \text{ m}^{-2}$ irradiance on a 12:12 light:dark cycle at 15°C. *C. radiatus* was grown on a plankton wheel (1 rpm) with 425 $\mu\text{mol photons sec}^{-1} \text{ m}^{-2}$ irradiance on a 12:12 light:dark cycle at 15°C. Every 24 hr 33% of *T. rotula* and 15% of *C. radiatus* media was replenished for each silicic acid treatment to maintain a constant growth rate.

At least 25 live cell diameters were measured using Image J v1.48 for each species in both silicic acid treatments. Cell volume and surface area were calculated assuming cylindrical cell shape for both *T. rotula* and *C. radiatus* ($V=\pi r^2 h$; $A=2\pi r h+2\pi r^2$). Samples of *T. rotula* and *C. radiatus* from both silicic acid treatments were sent to the University of California, Davis stable isotope facility for cell carbon and nitrogen analysis using a PDZ Europa ANCA-GSL elemental analyzer interfaced to a PDZ Europa 20-20 isotope ratio mass spectrometer.

I used two dinoflagellates as grazers for my experiments. *Gyrodinium spirale* was isolated from East Sound near Orcas Island, WA in July of 2014. I maintained *G. spirale* in ESAW media with f/2 nutrients on a diet of *T. rotula*. *Noctiluca scintillans* strain SPMC 136 was isolated from Burrows Bay, WA in January of 2009. Because *N. scintillans* grows poorly in ESAW, I maintained them in autoclaved filtered seawater (AFSW) with a co-culture of *T. rotula*. Both dinoflagellates were maintained on a plankton wheel (0.5 rpm) with 35 $\mu\text{mol photons sec}^{-1} \text{ m}^{-2}$ irradiance on a 12:12 light:dark cycle at 15°C and were transferred into fresh media every 2-3 weeks. Predator cultures used in experiments were first separated from their maintenance prey by settling out the diatoms and pipetting off the top 80% of the media and transferring the predators into fresh ESAW or AFSW. The predators were then held without food for 12 hr prior to the predation experiments.

Frustule Thickness Verification

To determine frustule thickness for each treatment I used two methods, biogenic silica analysis and Scanning Electron Microscopy (SEM) imaging. Biogenic silica analysis involves a two-step process in which I first dissolved the frustule using the sodium hydrolysis method and then measured the resulting silicic acid colorimetrically. Diatom culture (5-10 ml) was filtered onto 0.6 μm pore size, 25 mm diameter, polycarbonate filters and dried for 6 hr at 60°C. Filters were then placed into 4 ml of 0.2 M NaOH and put into a 95°C water bath for 1 hr. Samples were then cooled to room temperature and 1 ml of 1 M HCl was added (Paasche 1980, Ragueneau and Tréguer 1994, Durkin et al. 2013). Biogenic silica (BSi) was measured colorimetrically for each digested sample (Strickland and Parsons 1972) and BSi per cell was calculated based on cell counts. Frustule thickness was determined based on differences in BSi, including BSi cell^{-1} to surface area ($\text{mol}:\mu\text{m}^2$), BSi:C ($\text{mol}:\text{mol}$), and BSi:N ($\text{mol}:\text{mol}$), between the two silicic acid treatments. Biogenic silicate analysis was

performed before each experiment to ensure differences in frustule thickness. I used a one-tailed t-test to determine significant differences in each parameter between the two silicic acid treatments. BSi:C from and the measured BSi cell⁻¹ in each experiment were used to calculate the carbon content of the diatoms in order to estimate predator ingestion and digestion rates in amount of carbon consumed.

Predation Experiment 1: *G. spirale* growth and ingestion on *T. rotula*

To determine if thicker frustules in *T. rotula* affect the ingestion or growth rate of *G. spirale* I performed a 48 hr grazing experiment using the prey disappearance method (Frost 1972, Heinbokel 1978, Jakobsen and Strom 2004). I took samples of the diatoms from 20 μM (“thin”) and 80 μM (“high”) treatments from the semicontinuous cultures. Cell concentrations were determined using a Sedgwick-Rafter chamber. I combined diatoms with high or low silicic acid treatments in beakers with *G. spirale* to make a final mix of 55% diatom culture and 45% predator culture by volume ($\sim 3 \times 10^4$ cells ml⁻¹ of *T. rotula* and 30 cells ml⁻¹ of *G. spirale*). To account for prey growth in calculating clearance and ingestion rates, I combined each treatment of *T. rotula* in a beaker with the same proportion of ESAW media. Feeding and control beakers for each diatom treatment were separated into five 40-ml polycarbonate culture flasks for a total of 20 flasks. Culture flasks were randomly assigned a location on the plankton wheel (0.5 rpm) with an average of 35 $\mu\text{mol photons sec}^{-1} \text{ m}^{-2}$ irradiance with a 12:12 light:dark cycle at 15°C.

I measured chlorophyll at the beginning and end of the experiment for all 4 treatments. A subsample (2 ml) from each replicate was filtered onto glass fiber filters (GFF), placed in 6 ml of 90% acetone, and kept in a -20°C freezer for 24 hr. Chlorophyll was analyzed fluorometrically with a Turner 10-AU fluorometer using the acidification method (Yentsch and Menzel 1963). Diatom cell counts were performed at the beginning

and end of the experiment for the control flasks. Control subsamples (5 ml) were fixed with 4% (final concentration) acid Lugol's solution and diatom cells were enumerated with a Sedgwick-Rafter Chamber. Predator cell counts were performed from the for each predation flask at the beginning and end of the experiment. Subsamples (10 ml) were fixed with 4% (final concentration) acid Lugol's solution and settled for 12 hours in 6-well plates. The entire settled sample was counted using a ZEISS IM 35 inverted microscope. Diatom cell concentrations in the predation chambers at the beginning (C_1^* , cells ml⁻¹) and end (C_2^*) of the experiment were estimated by dividing the measured chlorophyll (pg chl ml⁻¹) estimated from each treatment by the average chlorophyll per cell (pg chl cell⁻¹) in the control treatments.

To calculate predation rate of *G. spirale* I used a method adapted from Frost (1972) by Heinbokel (1978). The growth coefficient (k , d⁻¹) of *T. rotula* was calculated based on cell concentrations in prey control treatments at the beginning (C_1 , cells ml⁻¹) and end (C_2) of the incubation time (t):

$$k = \frac{1}{t} * \ln \frac{C_2}{C_1}$$

To find average cell concentration ($\langle C \rangle$, cells ml⁻¹) I calculated the predation coefficient (g , d⁻¹) from cell densities in the predation treatments (C_1^* and C_2^*).

$$g = k - \left[\frac{1}{t} * \ln \frac{C_2^*}{C_1^*} \right]$$

$$\langle C \rangle = \frac{C_1^* [e^{(k-g)*t} - 1]}{t * (k - g)}$$

I estimated predator population growth rate (μ , d⁻¹) from the concentration of *G. spirale* before (N_1 , predators ml⁻¹) and after (N_2) the experiment.

$$\mu = \frac{\ln(N_2 - N_1)}{t}$$

Based on time-averaged predator concentrations (\bar{N}) (cells ml⁻¹) and the clearance rate (F, ml predator⁻¹ hr⁻¹) of the predators I calculated ingestion rate (I, cells predator⁻¹ d⁻¹) (Frost 1972, Heinbokel 1978).

$$\bar{N} = \frac{N_2 - N_1}{\ln(N_2 - N_1)}$$

$$F = \frac{g}{\bar{N}}$$

$$I = < C > * F$$

I used a standard one-way ANOVA to test for significant differences in *G. spirale* ingestion rate (in diatoms grazer⁻¹ and carbon grazer⁻¹) and growth rate between high and low frustuled *T. rotula*.

Predator Filtrate Experiment

To ensure that the growth rate of the control *T. rotula* was applicable to the growth rate of the diatoms in the predator chambers, I performed a 48 hr experiment comparing *T. rotula* growth rate in predator filtrate to growth rate in AFSW. Furthermore, to ensure that bacteria associated with *G. spirale* had no effect on diatom growth rate, I prepared filtrate with and without bacteria. Predator filtrate was taken from active *G. spirale* cultures in co-culture with *T. rotula*. Filtrate was prepared by prescreening 200 ml of predator culture through 100 µm nitex mesh to remove any grazers and large particulates. To produce the bacteria free filtrate I then filtered 100 ml of the prescreened culture through a 0.2 µm pore

size, 47 mm diameter polycarbonate filter. Filtrate with bacteria was prepared by filtering the remaining 100 ml culture through a 2.0 μm pore size, 47 mm diameter polycarbonate filter. The experiment contained four replicate filtrate flasks for each type of filtrate as well as four control flasks containing AFSW, for a total of 12 flasks. Diatoms were taken from stock cultures grown in full f/2 + Si ESAW and distributed into 40 ml flasks with a ratio of 50% diatoms to 50% filtrate or AFSW for an approximate cell density of 1.8×10^4 cells ml^{-1} . All flasks were randomly placed on the same plankton wheel, with the same speed, light levels and cycle, used in the first predation experiment.

To calculate growth rate, I performed triplicate cell counts on all replicates at the beginning and end of the experiment using the same methods stated in the first experiment. I then used the initial (C_1) and final (C_2) cell count data to find the growth coefficient (k , day^{-1}) using the same equation stated above.

I used a standard one-way ANOVA to test for a significant difference in growth rate as a result of predator filtrate and followed with a Tukey HSD test to determine differences between treatment pairs.

Predation Experiment 2: *N. scintillans* ingestion of *C. radiatus*

To determine if the frustule thickness in *C. radiatus* affects the ingestion rate of *N. scintillans*, I conducted a short-term (4 hr) predation experiment to measure accumulation rate of diatoms in the food vacuole of the predators (Strom et al. 2003). I took samples of the diatom cultures from each treatment in semicontinuous culture and measured cell concentrations. I combined *C. radiatus* with *N. scintillans*, generating a mix of 50% diatoms in ESAW and 50% predators in AFSW for each silicic acid treatment. I distributed each treatment into eight 40-ml polycarbonate culture flasks for a total of 16 flasks and kept 30 ml of each treatment for initial predator cell counts. Culture flasks were randomly placed on the

middle of the plankton wheel (0.5 rpm) with an average of 35 $\mu\text{mol photons sec}^{-1} \text{ m}^{-2}$ irradiance at 15°C.

I initially counted the cells of predators by fixing three 10-ml samples from each treatment with 1.5% (final concentration) acid Lugol's solution. Each 10 ml sample was settled and counted using a ZEISS IM 35 inverted microscope. I collected and fixed (1.5% acid Lugol's solution) the entire 40 ml sample at 120 min and 240 min time points, collecting four replicates from each treatment at each time point. Fixed samples were sieved through 100 μm Nitex mesh to collect *N. scintillans* cells, which were resuspended in 5-10 ml of AFSW. Resuspended samples were then settled in 10 ml 6-well plates. I then counted all the predator cells in each settled sample and counted the number of *C. radiatus* cells inside each *N. scintillans*.

To determine the ingestion rate of *N. scintillans* I calculated the population vacuole content (PVC) from the average number of *C. radiatus* cells per predator for each replicate and found the average PVC of the 4 replicates at each time point. Assuming zero *C. radiatus* per predator at the start of the experiment, I determined the slope of the average PVC versus time (hr). The slope of this line represents the ingestion rate of *N. scintillans* (*C. radiatus N. scintillans*⁻¹ hr⁻¹) (Strom et al. 2003).

A Welch's one tailed t-test was used to compare ingestion rate (in diatoms grazer⁻¹ and carbon grazer⁻¹) of *N. scintillans* feeding on high vs. low silicic acid treatment diatoms; rates were determined and compared for two time intervals (0-120 min; 120-240 min).

Predation Experiment 3: *N. scintillans* digestion of *C. radiatus*

To determine if the frustule thickness in *C. radiatus* affects the digestion rate of *N. scintillans* I conducted an 8 hr experiment to measure the rate of disappearance of diatoms from the food vacuole of *N. scintillans* over time. I set up the experiment the same way as

with predation experiment #2, described above. Feeding lasted 4 hr, after which I separated the predators from the prey by sieving the solution through 100 μm Nitex mesh. The *N. scintillans* were then resuspended in AFSW for a total volume of 640 ml per treatment. The resuspended predators were distributed into sixteen 40-ml polycarbonate flasks. At 0, 8, and 16 hr, four flasks per treatment were sampled in their entirety. Fixed samples were sieved, settled and enumerated in the same manner as for predation experiment 2.

Using the PVC method described above I found the average *C. radiatus* per *N. scintillans* at each time point for the high and low silicic acid treatments. I determined the slope of the average number of diatoms per predator versus time. This slope represents the digestion rate of *N. scintillans* (average *C. radiatus* *N. scintillans*⁻¹ hr⁻¹).

A Welch's one tailed t-test was used to compare digestion rate (in diatoms grazer⁻¹ and carbon grazer⁻¹) of *N. scintillans* feeding on high vs. low silicic acid treatment diatoms; rates were determined and compared for two time intervals (0-8 hr; 8-16 hr).

RESULTS

Cell Characterization

In general, the solitary *Coscinodiscus radiatus* was larger, more silicified, and slower growing than the chain-forming *Thalassiosira rotula*. Growth rates of *C. radiatus* were significantly lower (~3.0-fold, $p < 0.01$) than those of *T. rotula* (Table 1). Average cell diameter was approximately ten times higher in *C. radiatus* compared to *T. rotula*; similarly, cellular carbon and nitrogen were 5 - 10-fold higher (Table 1). In addition to their larger size and elemental content, *C. radiatus* was much more silicified than *T. rotula*, with a 40X higher cellular biogenic silica content, a 10-fold higher BSi:C (mol:mol), and a 10 - 15-fold higher BSi:N (Fig. 1). Similarly, *T. rotula* had a significantly lower ($p < 0.05$) C:N compared to *C. radiatus* (Fig. 1). Interestingly, *T. rotula* had an order of magnitude higher carbon-to-volume ratio ($\text{pmol}:\mu\text{m}^3$) compared to *C. radiatus* (Table 1).

Within each species, the 80 μM (high) silicic acid cultures appeared to be more silicified than the 20 μM (low) cultures. *T. rotula* and *C. radiatus* had 3 and 1.8-fold significantly higher ($p < 0.01$) biogenic silica content ($\text{pmol BSi cell}^{-1}$) when grown in 80 μM compared to 20 μM silicic acid (Fig. 1A, Table 1). Similarly, there was a 2.3 and 4.3-fold higher BSi:C in *T. rotula* and *C. radiatus*, respectively, when grown in high silicic acid treatments compared to low (Fig. 1B). BSi:N exhibited a similar trend with 3 and 5.5-fold higher ratios in *T. rotula* and *C. radiatus*, respectively, when grown in high silicic acid (Fig. 1C). The ratio of BSi cell^{-1} to surface area ($\text{pmol}:\mu\text{m}^2$) was 3 and 2-fold higher in the 80 μM silicic acid treatment for *T. rotula* and *C. radiatus*, respectively, compared to the 20 μM treatment (Table 1). These results indicate that the cells in the 80 μM silicic acid treatment, for both species, were more silicified than those in the 20 μM treatment. Therefore, I will

now refer to the 80 μM and 20 μM silicic acid treatment diatoms as “thick” and “thin”, respectively.

In addition to affecting frustule thickness, silicic acid concentration also had an effect on the carbon and nitrogen content of both diatoms. There was a significantly higher ($p < 0.01$) cell carbon (pmol C cell^{-1}) in the thick cells compared to the thin for both *T. rotula* and *C. radiatus* (Table 1). *T. rotula* showed no significant difference in cell nitrogen (pmol N cell^{-1}) between the two treatments, while nitrogen content of thick *C. radiatus* cells was significantly higher ($p < 0.05$) than that of the thin (Table 1). C:N was not significantly different between treatments for either species; however thick *C. radiatus* cells had a slightly higher C:N compared to the thin (Fig. 1D). Carbon to volume ratios were almost identical in the two thickness treatments for *T. rotula*; however *C. radiatus* had a higher ratio in the thin cultures (Table 1). Neither cell diameter nor growth rate differed between treatments, regardless of species (Table 1).

Table 1. Growth rates (μ), elemental composition, and cell size for two diatoms, *Thalassiosira rotula* and *Coscinodiscus radiatus*, grown in semicontinuous culture with artificial seawater (ESAW) at low (20 μM) and high (80 μM) silicic acid concentrations. Averages for triplicate cultures (5 replicates for C & N, 25 cell replicates for size) are reported with ± 1 SD. Asterisks indicate significant differences between Si treatments, $p < 0.05$.

Diatom Species	Silicic acid (μM - Si)	μ (d^{-1})	BSi cell^{-1} (pmol)	C cell^{-1} (pmol)	N cell^{-1} (pmol)	Cell Diameter (μm)	BSi:SA (pmol: μm^2)	C:Vol (pmol: μm^3)
<i>Thalassiosira rotula</i>	80	0.52 \pm 0.03	* 1.2 \pm 0.19	* 12 \pm 0.24	2.5 \pm 0.49	5.4 \pm 0.82	0.0046	0.042
	20	0.54 \pm 0.01	0.43 \pm 0.02	14 \pm 0.28	* 2.8 \pm 0.05	6.0 \pm 0.89	0.0015	0.044
<i>Coscinodiscus radiatus</i>	80	0.19 \pm 0.02	* 45 \pm 3.4	* 55 \pm 4.0	6.9 \pm 0.68	49 \pm 3.6	0.0066	0.0015
	20	0.13 \pm 0.01	25 \pm 11	130 \pm 1.2	* 23 \pm 4.1	51 \pm 3.5	0.0034	0.0031

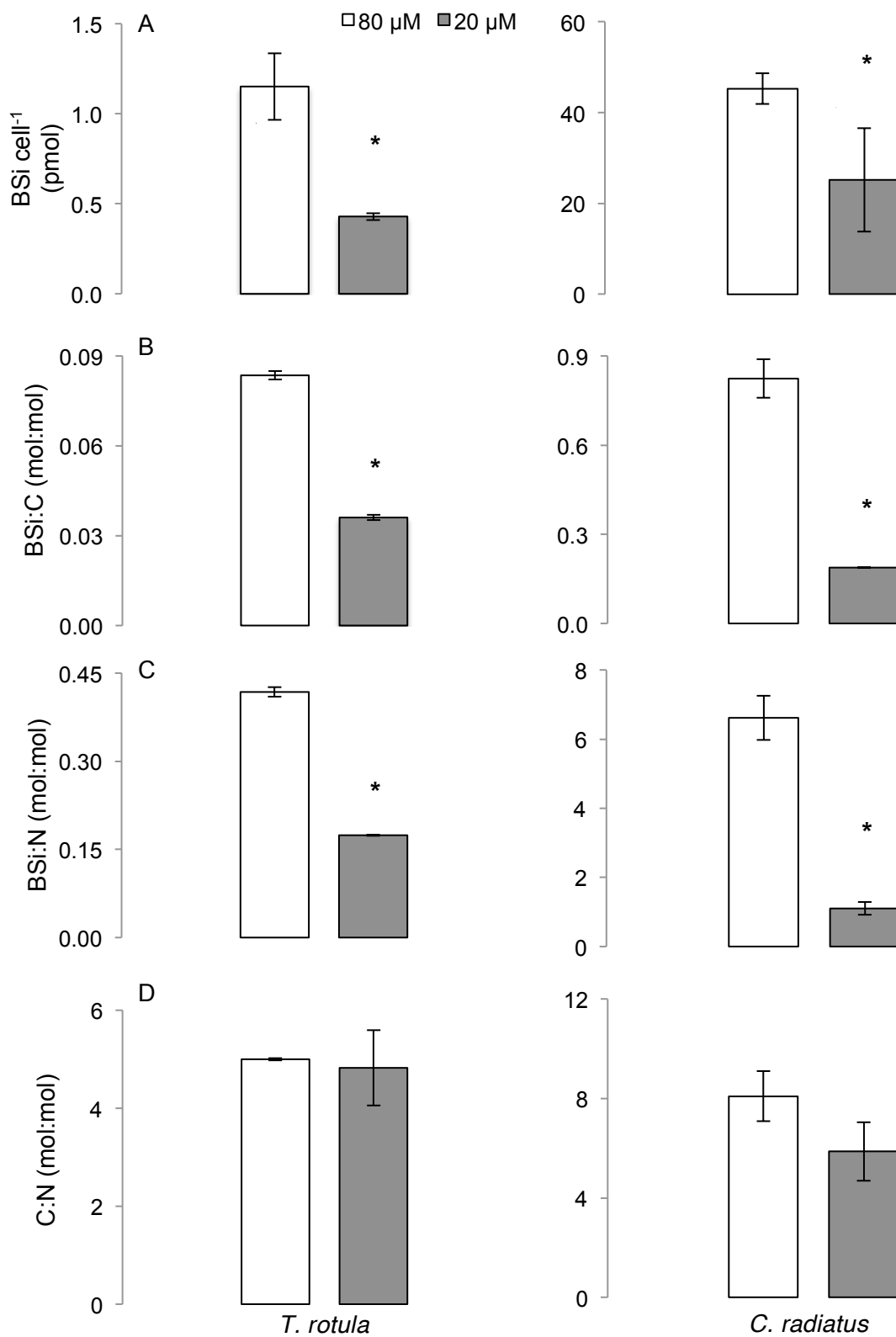


Figure 1. Effect of high (80 μM) versus low (20 μM) silicic acid concentrations on the average biogenic silica cell⁻¹ (A), BSi:C (B), BSi:N (C), and C:N (D) of the diatoms *Thalassiosira rotula* and *Coscinodiscus radiatus* grown in semicontinuous culture. Averages obtained from 5 replicate cultures for BSi:C, BSi:N, and C:N (triplicate for BSi cell⁻¹). Error bars display ± 1 SD. Asterisk indicates a significant difference between the silicic acid treatments, $p < 0.05$. Note y-axis scale differences between species.

Predation Experiment 1: *G. spirale* growth and ingestion on *T. rotula*

Frustule thickness in *T. rotula* had no significant affect on *G. spirale* ingestion rate, however *G. spirale* grew slower when feeding on thick diatoms compared to thin. At the start of the experiment biogenic silica cell⁻¹ was significantly higher ($p < 0.05$) in the thick treatment compared to the thin, with 0.60 and 0.31 pmol-Si cell⁻¹, respectively. Average growth rates of *T. rotula* in the control treatments (k , d⁻¹) were very low; however rates were significantly lower ($p < 0.05$) in the thick versus the thin treatments (Fig. 2). *G. spirale* growth rate ranged from 0.15 to 0.41 d⁻¹, and averaged significantly lower ($p < 0.05$) when feeding on the thick diatoms compared to the thin (Fig. 3). Furthermore, average clearance rates (F) of the dinoflagellate grazers were also significantly lower ($p < 0.01$) when feeding on the thick diatoms, at 3.3 compared to 5.2 $\mu\text{l grazer}^{-1} \text{ day}^{-1}$. However, as a result of a significantly lower ($p < 0.01$) time-averaged cell concentration ($\langle C \rangle$) in the grazing treatments containing thin diatoms, the average ingestion rate of 107 cells grazer⁻¹ d⁻¹ was not significantly different for *G. spirale* feeding on thin versus thick frustuled diatoms (Fig. 4a). When expressed in amount of carbon consumed, ingestion rate was significantly higher ($p < 0.01$) in dinoflagellates feeding on thin diatoms (Fig. 4b).

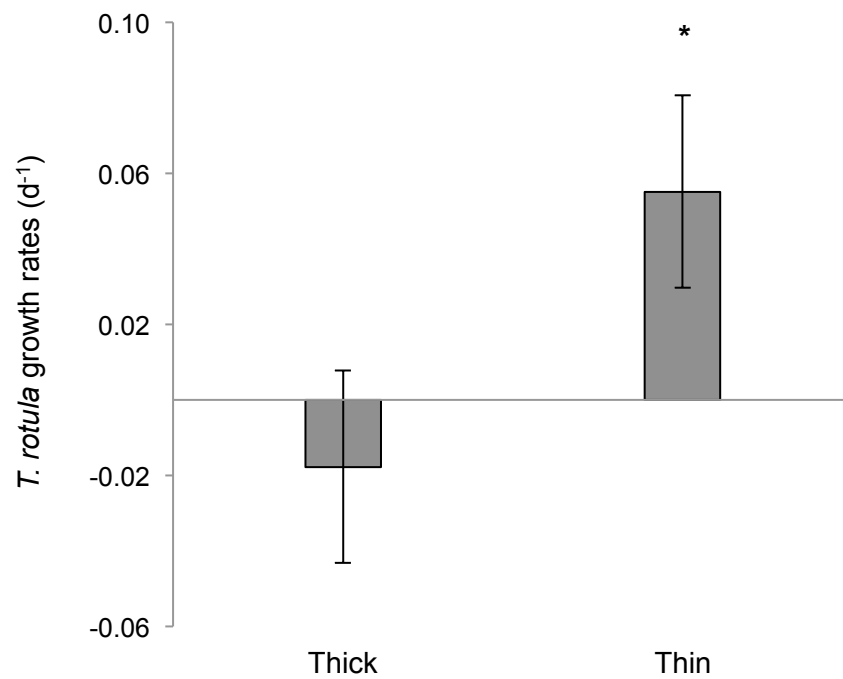


Figure 2. Average (n=5) growth rates (k , d^{-1}) of the diatom *Thalassiosira rotula* in control treatments (thick versus thin frustules). Error bars display ± 1 SD. Asterisk indicates a significant difference between Si treatments, $p < 0.05$.

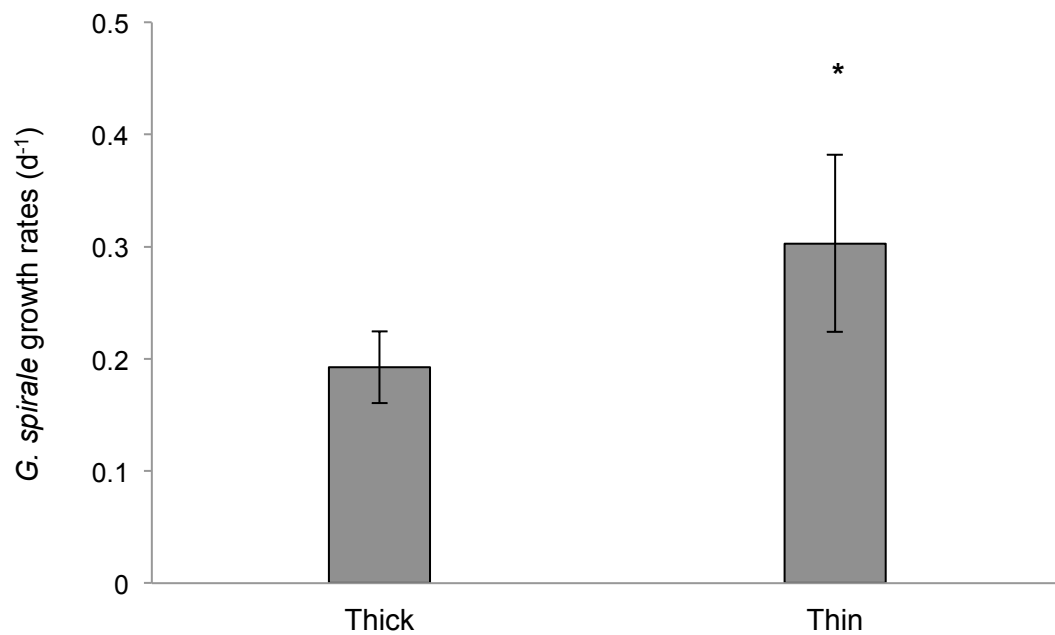


Figure 3. Average (n=5) growth rates (μ , day⁻¹) of dinoflagellate predator *Gyrodinium spirale* feeding on the thick versus thin diatom *Thalassiosira rotula*. Error bars display ± 1 SD. Asterisks indicate significant difference between the two thickness treatments, $p < 0.05$.

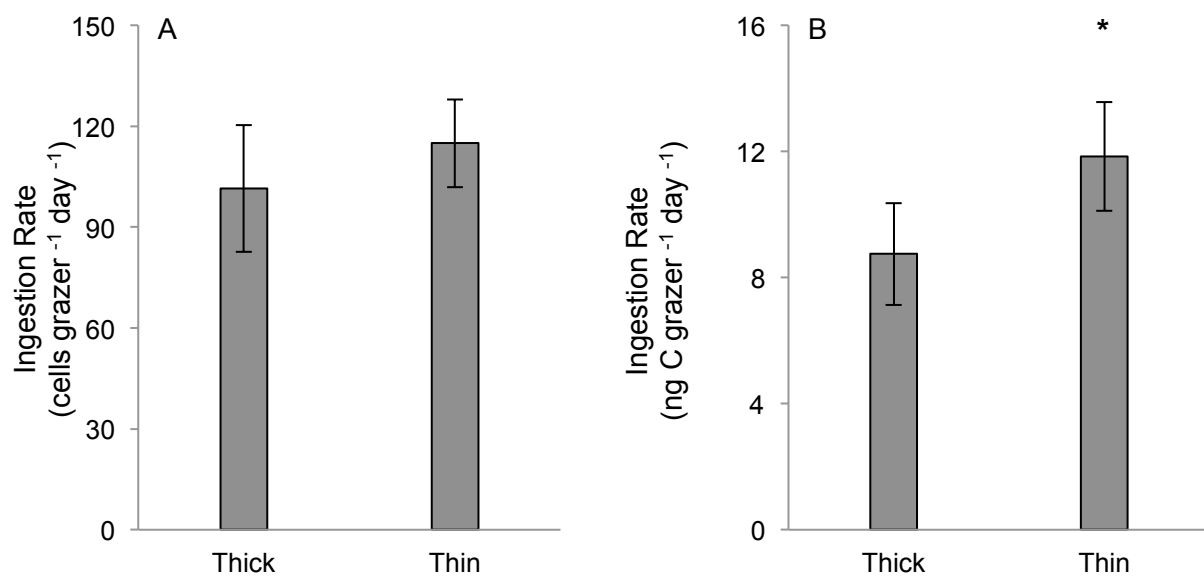


Figure 4. Average ingestion rates of *Gyrodinium spirale* feeding on thick versus thin *Thalassiosira rotula* in predation experiment 1. Rates are expressed in cells (A, cells grazer⁻¹ day⁻¹) and carbon (B, ng C grazer⁻¹ day⁻¹) consumed. Error bars display ± 1 SD. Asterisks indicates significance differences between the two treatments, $p < 0.01$.

Predator Filtrate Experiment

Growth rate (k , d^{-1}) of the diatom *T. rotula* was significantly affected ($p < 0.05$) by filtrate from the dinoflagellate predator *G. spirale* (Fig. 5). When comparing the filtrate treatments separately to the AFSW control, only filtrate with bacteria affected growth, leading to a 2-fold decrease (Tukey HSD, $p < 0.05$). There was no significant diatom growth rate difference in filtrate without bacteria as compared to filtrate with bacteria or to the control.

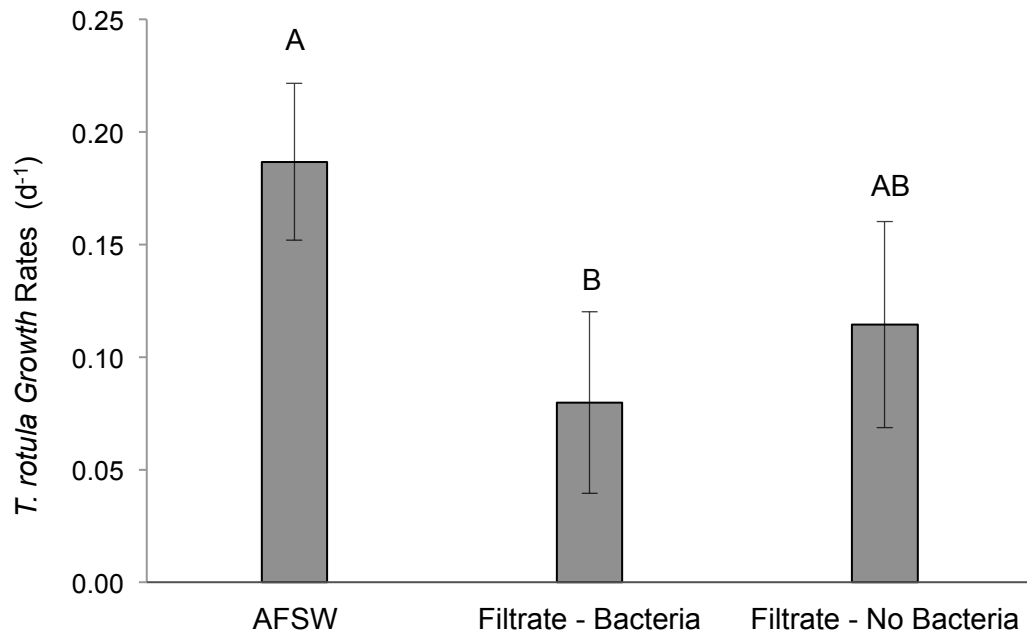


Figure 5. Average growth rates (k , d^{-1}) of *Thalassiosira rotula* grown in *Gyrodinium spirale* culture filtrate with and without bacteria compared to the control treatment in autoclaved filtered seawater (AFSW). Error bars display ± 1 SD. Letters show homogenous subsets ($p < 0.05$, Tukey HSD).

Predation Experiment 2: *N. scintillans* ingestion of *C. radiatus*

Frustule thickness in *C. radiatus* had no effect on the short-term ingestion rate of *N. scintillans*. At the start of the experiment, biogenic silica cell⁻¹ was significantly higher ($p < 0.05$) in thick *C. radiatus* treatments compared to thin, with 25 and 18 pmol-Si cell⁻¹, respectively. There was no significant difference during either time interval (0-120 min and 120 – 240 min) in *N. scintillans* ingestion rate (cells grazer⁻¹ hr⁻¹) when feeding on the thick versus thin *C. radiatus* (Fig. 6a). Similarly, grazer clearance rates were not significantly different between thickness treatments, with an average of 11 μl grazer⁻¹ day⁻¹. However, there was a significant decrease ($p < 0.05$) in ingestion rate during the 120 – 240 min interval compared to the 0 – 120 min in the thick treatment; there was no significant difference in ingestion rate between time intervals in the thin treatment grazers. No significant change in predator density over the course of the experiment was observed for either treatment with an average 1.25 ± 0.2 *N. scintillans* ml⁻¹. Due to the lower C content of thick *C. radiatus* cells, ingestion rate expressed on a carbon basis (pg C grazer⁻¹ hr⁻¹) was 3.2-fold lower ($p < 0.01$) when *N. scintillans* feed on the thick diatoms for both time intervals (Fig. 6b).

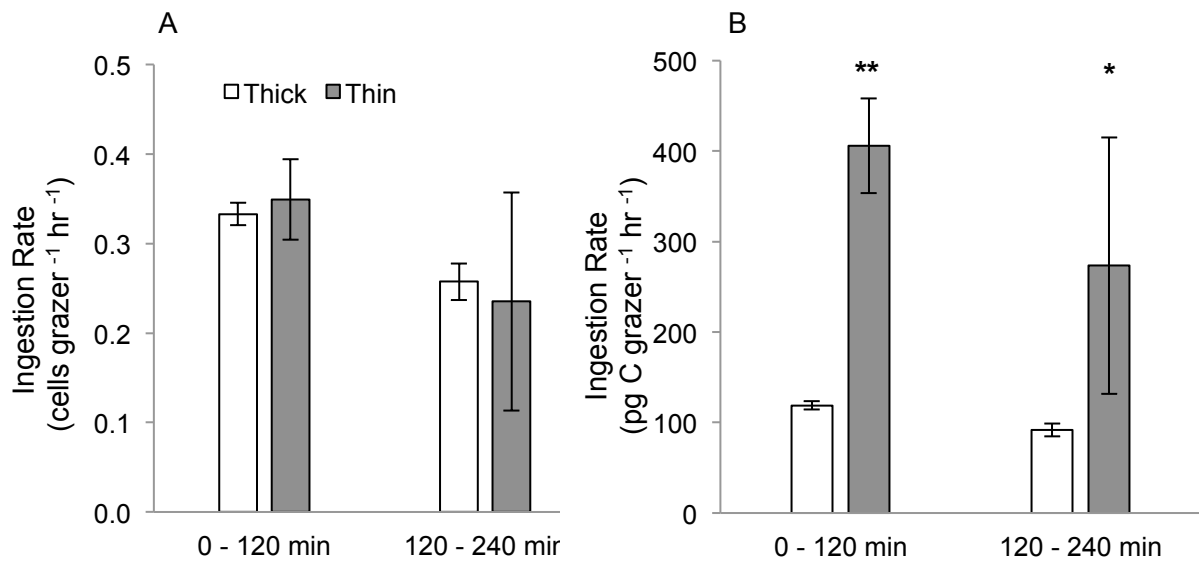


Figure 6. Average ingestion rates of *Noctiluca scintillans* feeding on thick versus thin *Coscinodiscus radiatus* during two time intervals (0-120 min, 120 – 240 min) in predation experiment 2. Rates are expressed in cells (A, cells grazer⁻¹ min⁻¹) and carbon (B, pg C grazer⁻¹ min⁻¹). Error bars display ± 1 SD. Asterisks represent a significant difference between treatments. Single = $p < 0.05$. Double = $p < 0.01$.

Predation Experiment 3: *N. scintillans* digestion of *C. radiatus*

N. scintillans digestion rate was significantly reduced by thick-frustuled *C. radiatus* when compared to thin. At the beginning of the experiment, thick treatment *C. radiatus* had a significantly higher ($p < 0.05$) biogenic silica cell⁻¹ than in the thin treatment, with 23 pmol-Si cell⁻¹ compared to 14, respectively. *N. scintillans* digestion rate (cells grazer⁻¹ hr⁻¹) was reduced by thick *C. radiatus* frustules over the course of the 16-hr experiment (Fig. 7). During the initial 0 - 8 hr period the grazers had a 4.1-fold lower digestion rate ($p < 0.05$) when feeding on the thick-frustuled diatoms compared to the thin (Fig. 8a). Conversely, during the 8 - 16 hr period, grazers feeding on the thick diatoms had a significantly higher digestion rate ($p < 0.01$) compared to the thin. Overall the grazers feeding on thick diatoms had a significantly lower digestion rate ($p < 0.01$) and higher average number of cells in their vacuole by the end of the experiment (Fig. 7). For the thin diatom treatments there was a 3.2 - fold decrease ($p < 0.01$) in grazer digestion rate during the later relative to the earlier time period; there were no significant differences between the time periods for the thick treatment. In terms of carbon digested (pg C grazer⁻¹ hr⁻¹) there was also a significantly lower ($p < 0.01$) digestion rate of the grazers feeding on thick diatoms (Fig. 8b). Contrary to the cell-based rates, higher C-based digestion rates of the thin diatoms were observed during both the 0-8 hr and the 8 - 16 hr intervals. As with the cell-based rates, there was a similar significant increase in digestion rate between the time intervals for the thick treatment.

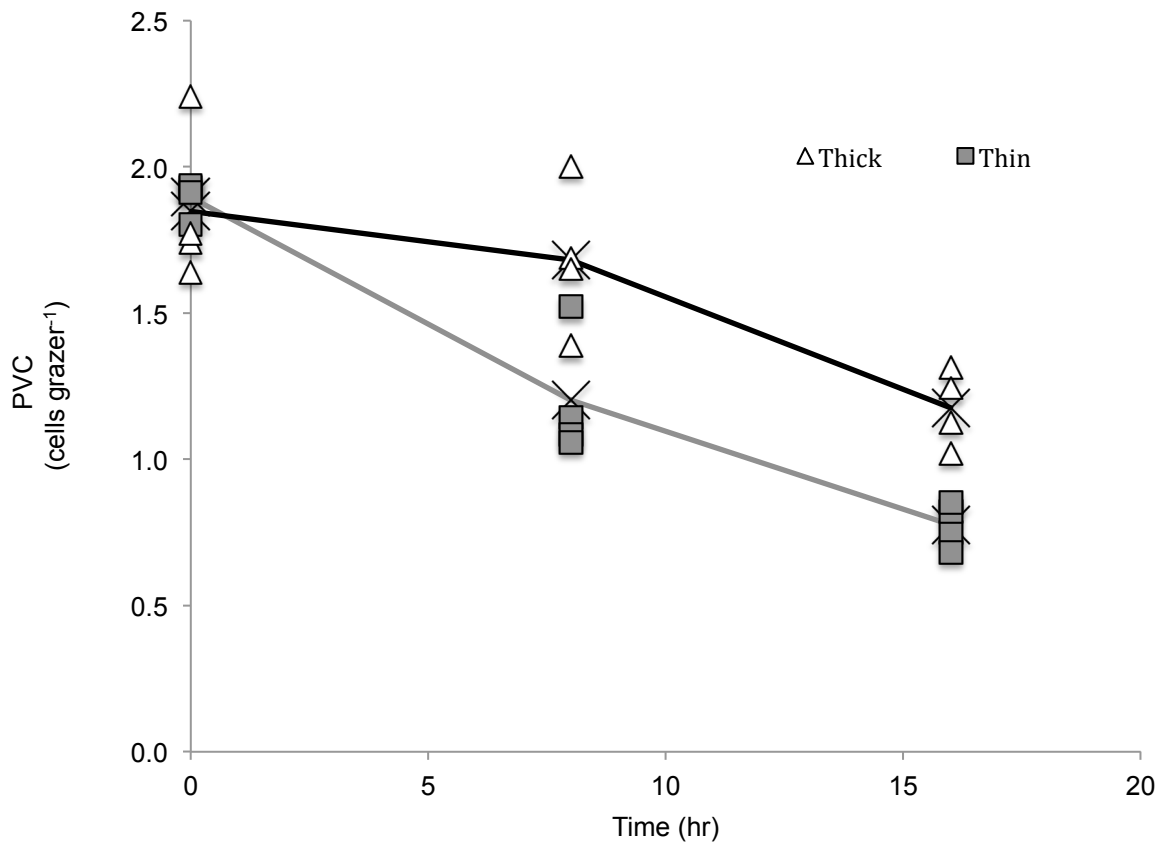


Figure 7. Population vacuole content (PVC, cells grazer⁻¹) over time (hr) of *Noctiluca scintillans* feeding on thick versus thin *Coscinodiscus radiatus* in predation experiment 3. All 4 replicates are shown for each treatment with X indicating the treatment mean PVC. The slope of the lines indicate average digestion rate (cells grazer⁻¹ hr⁻¹). Black line = Thick. Gray line = Thin.

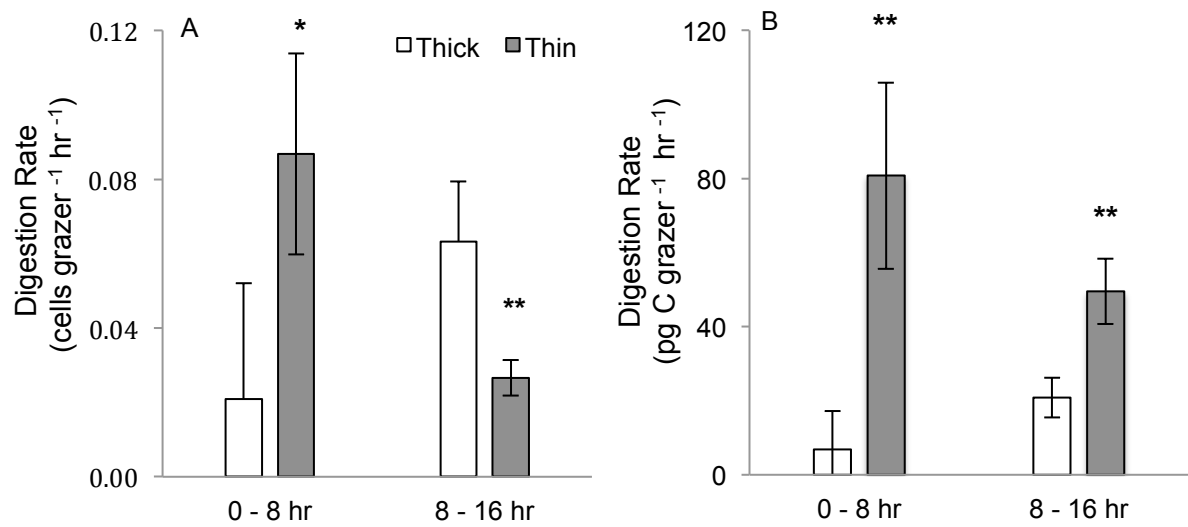


Figure 8. Average digestion rates of *Noctiluca scintillans* feeding on thick versus thin *Coscinodiscus radiatus* during two time intervals (0-8 hr, 8-16 hr) in predation experiment 3. Rates are expressed in cells (A, cells grazer⁻¹ hr⁻¹) and carbon (B, pg C grazer⁻¹ hr⁻¹). Error bars display ± 1 SD. Asterisks represent a significant difference between Si treatments. Single = $p < 0.05$. Double = $p < 0.01$.

DISCUSSION

I found strong evidence in favor of diatom frustules as a mechanical defense against heterotrophic dinoflagellates. The mechanism works by reducing the digestion rate of the predator, resulting in a slowing of their population growth rates. While not observed in this study, this would presumably feed back into lower grazing pressure on the diatom population. The commonly observed decoupling of microzooplankton grazing and diatom growth in the field is likely a result, in part, of this mechanism. This defense mechanism further elucidates the importance of the frustule in the success of this widespread phytoplankton group.

Cell Characterization

Following prolonged exposure to high silicic acid concentrations in semicontinuous culture, there is strong evidence to suggest that both diatom species had thicker frustules in comparison to low silicic acid treatments. The increases in BSi:SA, BSi:C, and BSi:N seen here are all effective proxies for frustule thickness (Paasche 1973, Harrison et al. 1977, Durkin et al. 2013). These findings are in close agreement with other studies that successfully cultured diatoms with thin and thick frustules. Durkin et al. (2013) found 2 - 3-fold higher BSi cell⁻¹, BSi:C, and BSi:N in three species of diatoms acclimated to 80 μM versus 20 μM silicic acid (Durkin et al. 2013). Similarly, three species of diatoms grown in a chemostat system with silicic acid-limiting conditions ($\sim 10 \mu\text{M}$) consistently had 2 - 3-fold less BSi cell⁻¹, BSi:C, and BSi:N than those grown in non-limiting conditions (Harrison et al. 1977).

In addition to affecting the frustule thickness, silicic acid concentration also altered the chemical composition of both diatom species. There were marked increases in cellular

carbon and nitrogen content in the low silicic acid cultures, especially in *C. radiatus*. Similarly, *C. radiatus* showed a higher C:N in the high silicic acid treatment. Silicic acid-dependent differences in diatom elemental composition have been reported previously with instances of a 2-fold increase in carbon and nitrogen in low or limiting silicic acid concentrations (Harrison et al. 1977, Jungandreas et al. 2012, Durkin et al. 2013). However, other species exhibited a halving of their carbon content when grown in low (20 μM) silicic acid conditions (Durkin et al. 2013). Interestingly, while C:N was higher in the high silicic acid treatment for *C. radiatus*, C:N in other studies is typically lower in high silicic acid-acclimated, or non-limited diatoms (Harrison et al. 1977, Jungandreas et al. 2012, Durkin et al. 2013). The dissimilarities in response could be a function of species-specific reactions to varying silicic acid concentrations. Regardless, differences in cellular chemical content, and cell stoichiometry (C:N) indicate that the nutritional quality of these diatoms as prey is influenced by the silicic acid concentrations present during growth. Nutritional quality could, in turn, have affected the predator's growth rates in these experiments.

Predation and Growth Rates

The filtrate experiments were executed to determine if the filtrate of *G. spirale*'s influenced the growth rate of *T. rotula*, which could have affected my estimate of ingestion rate in predation experiment 1. While there was no difference in growth rate of *T. rotula* between the filtrate without bacteria and the AFSW control, there was a significantly lower growth rate in the filtrate + bacteria compared to the control. This suggests that the bacteria associated with *G. spirale* were somehow depressing the growth rate of *T. rotula*. Consequently, if the lower diatom growth rate in the predator chamber were also occurring in predation experiment 1, I would have overestimated the overall ingestion rate of *G. spirale* for both treatments. In experiment 1, if the difference in growth rates were 2-fold lower than

the control, as seen in the filtrate + bacteria treatment, then there would be a similar 2-fold overestimate of ingestion rate. However, this would presumably have occurred in both thickness treatments and would not change the overall conclusion from the experiment.

In comparison to other studies, the ingestion and growth rates of *G. spirale* measured here are comparable, while those of *N. scintillans* are less so. The growth rates of *G. spirale* in predation experiment 1 were lower than the maximum growth rates observed in other studies, which ranged from 0.55 d^{-1} on *Heterocapsa triquetra* (Hansen 1992) to 0.76 d^{-1} on *Prorocentrum minimum* (Kim and Jeong 2004) compared to our observed average growth rates of 0.31 d^{-1} (thin) and 0.19 d^{-1} (thick). Our growth rates are more comparable to those measured at subsaturating prey concentrations (Hansen 1992, Kim and Jeong 2004), but the differences could also be a result of dissimilar prey between studies. In contrast to growth rates, *G. spirale* clearance rates were similar to those recorded in other studies. We observed an average clearance rate on thick and thin *T. rotula* of 3.3 and $5.2 \mu\text{l grazer}^{-1} \text{ d}^{-1}$, respectively; these rates are comparable to the $4.8 \mu\text{l grazer}^{-1} \text{ d}^{-1}$ measured at near-saturating prey concentrations (Hansen 1992). *G. spirale* ingestion rates were less similar to other studies. Our predator's average ingestion rate was three times higher than the maximum ingestion rate when feeding on *Heterocapsa triquetra* (Hansen 1992), and seven times lower than the maximum ingestion rate when feeding on *Prorocentrum minimum*. This is likely a result of differences in prey type, including cell size and food quality.

N. scintillans ingestion and clearance rates were much lower in comparison to other studies. Some studies reported clearance rates 10-fold higher than seen in this study (Nakamura 1998, Frangópulos et al. 2011). Similarly, ingestion rates when feeding on *Prorocentrum micans* were two orders of magnitude higher than in our study when expressed in carbon consumed (Frangópulos et al. 2011). The only reported clearance rate on diatoms was with *Thalassiosira weissflogii*, in which the clearance rate was 10 times

higher than that observed in this study (Kiorboe and Titelman 1998). However, *C. radiatus* is much larger than all of the prey types in these earlier studies, which could explain the lower clearance and ingestion rates that I observed. Furthermore, all three studies had warmer experimental conditions compared to this study (18°C compared to 15°C), which could contribute to the higher feeding and clearance rate of *N. scintillans* that they reported.

Evidence for Frustules as a Defense

Predation experiment 1 gave indirect evidence of frustule thickness affecting predation rate. While I saw no effect of *T. rotula* frustule thickness on *G. spirale* ingestion rate, I did see a significant decrease in predator growth rate when feeding on thicker frustule diatoms. Additionally, when expressed in amount of carbon ingested, I saw a significant increase in ingestion of thin diatoms. However, since cell size was not significantly different between diatom treatments, the C-based results do not change the overall conclusion that ingestion of cells per unit time remained unaffected by thickness. These results suggests two, non-mutually exclusive, possibilities: 1) the thick frustule itself somehow reduced *G. spirale* growth rates, possibly by slowing digestion and thus the supply of energy for growth; and/or 2) the lower cellular carbon content of the thick-frustuled *T. rotula* reduced the amount of energy provided to *G. spirale*, even though they consumed cells at the same rate as with the thin frustuled treatment. In either scenario, this would suggest that over time, lower growth rate of grazers would eventually lead to fewer grazers overall, which would likely result in fewer diatoms being consumed in the population. Perhaps in this experiment 48 hr was not long enough to observe the slower growth rate of *G. spirale* feedback to a significantly lower ingestion rate per grazer.

Predation experiment 2 provided little evidence in favor of frustules as a means of defense, with no observed differences in *N. scintillans* ingestion rate between the thickness treatments. I did observe a notable increase in carbon-based ingestion rates in the thin treatment compared to thick. However, since cell size wasn't significantly different between the thickness treatments this does not change the overall conclusion that *N. scintillans* ingestion rate was unaffected by frustule thickness in this experiment.

The strongest evidence in favor of frustules as a mechanical defense came from predation experiment 3. Here we directly observed a substantially lower digestion rate for *N. scintillans* fed thick-frustuled *C. radiatus* compared to thin. While there was an overall lower digestion rate in the thick treatment we did see a slight increase after 8 hr, combined with a more dramatic decrease in digestion rate for the thin. Perhaps the grazers in the thin treatments slowed their digestion rate after 8 hr in order to preserve their food supply in the absence of an adequate prey source. Regardless, the digestion results provide initial evidence for a possible mechanism by which the diatom frustule may provide a defense diatoms dinoflagellates.

Mechanism of Defense

My results suggest that the mechanism by which the frustule provides a defense is by increasing digestion times in dinoflagellate grazers. Slower digestion rates would eventually lead to two non-exclusive outcomes: 1) lower turnover of diatoms in the digestive vacuole, leading to reduced per-capita grazing rates on the diatom population; and 2) reduced overall energy uptake by the predators, leading to slower population growth rates and thus, over time, fewer predators in the environment. The latter may have occurred in predation experiment 1, which showed a decrease in *G. spirale* growth rate when fed thick

T. rotula. Decreased digestion rates in the thick treatment may have been the underlying cause for the difference in predator growth rate.

Slowing a predator's digestion rather than its ingestion rate is a logical defense mechanism in this system because of the varied feeding strategies of heterotrophic dinoflagellates. The predators used in this experiment, *N. scintillans* and *G. spirale*, both use direct engulfment to consume their prey, a feeding mechanism which does not require the predator to break the prey cell wall (Hansen 1992, Kiorboe and Titelman 1998, Hansen and Caladob 1999). Therefore, a thick frustule would be unlikely to prevent, or slow, the engulfment of the diatom by either of these dinoflagellates. However, other dinoflagellate predators utilize feeding strategies that may be directly inhibited by a thick diatom frustule. The peduncle is a tube-feeding apparatus employed by many species; it extends from the cell and pierces the prey, after which the intercellular material of the pierced organism flows into the food vacuole of the predator (Hansen and Caladob 1999). With this strategy a thick frustule may act as a barrier against the peduncle and slow the predator's ingestion rate, but would unlikely affect its digestion rate. In the pallium feeding strategy, the dinoflagellate surrounds its prey with a veil-like structure into which digestive enzymes are released; the organic matter of the diatom is digested within the pallium (Hansen and Caladob 1999). This mechanism might be affected by a thick frustule similarly to the direct engulfment method, slowing the digestion rate of the predator, albeit externally, but probably not slowing the initial prey capture or ingestion rate. Many have postulated that copepod mandibles have evolved specifically to break the thick diatom frustule (Hamm et al. 2003, Michels and Schnack-Schiel 2005, Friedrichs et al. 2013). Copepods that prey on diatoms possess short, crooked, mandibles composed of opal, assumed to have evolved for breaking silicic acid frustules (Sullivan et al. 1975, Michels and Schnack-Schiel 2005, Friedrichs et al. 2013). In some instances copepods have been observed to feed heavily on diatoms (e.g.

Assmy et al. 2013). However, as mentioned earlier, microzooplankton rather than diatoms often are the dominate prey source of copepods, while microzooplankton generally have the greatest grazing impact on diatoms (Sherr and Sherr 2007). Perhaps the mandibles evolved in response to mechanical defenses from microzooplankton, such as the thecal plates in many dinoflagellates.

Evidence in support of frustules as a mechanical defense in diatoms has been seen in other predators. The abalone *Haliotis diversicolor* showed variability in digestion rate when feeding on different species of benthic diatoms (Onitsuka et al. 2007). Digestion in post-larval abalone was half as efficient when feeding on the heavily silicified *Navicula ramosissima* as compared to three less silicified species; this resulted in a 2-fold decrease in *H. diversicolor* growth rate when compared to the three other food sources. When comparing growth rates of two coastal tintinnids feeding on various phytoplankton, including diatoms, there was a marked decrease in growth and increase in mortality when feeding on spinier diatoms; tintinnids only grew when feeding on diatom species with few or no silica spines (Verity and Villareal 1986). Spine formation is heavily dependent on frustule thickness, with thinly silicified diatoms having shorter and fewer spines than thicker diatoms (Harrison et al. 1977).

Decreased digestibility as a means of defense has been observed in other algal species. Phosphate-limited green algae *Selenastrum capricornutum* and *Scenedesmus subspicatus* have been shown to reduce grazing pressure from *Daphnia pulex* and *Daphnia magna* (Donk and Hessen 1994). Under P limitation the cells increased in size and changed their cell wall morphology. This resulted in decreased digestibility of the algae and lower grazing rates in *D. pulex* and *D. magna*. Similarly, phosphate and nitrate-limited *Chlamydomonas reinhardtii* were less digestible by *D. magna*, resulting in lower grazer clearance rates (Van Donk et al. 1997). However, when cell wall-deficient mutants were fed

to *D. magna* there was no difference in clearance rate and digestibility between the N & P-limited and non-limited cells.

Decreasing a grazer's growth and grazing rates as a means of defense has been observed in other bloom-forming algal species. Brown algal pelagophytes, most notably *Aureococcus anophagefferens* and *Aureoumbra lagunensis*, often cause massive, long-lasting blooms that result in severe alteration or degradation of the local ecosystem and as such are labeled as ecosystem disruptive algal blooms (EDABs) (Sunda et al. 2006, Gobler and Sunda 2012). EDABs usually result because the species associated with them largely evade predation by microzooplankton and bivalves (Deonaraine et al. 2006). Microzooplankton grazing on *A. anophagefferens* can be as much 70% lower compared to other competing algae during EBABs (Caron et al. 2004). This decreased grazing pressure has been attributed to these species' unpalatability and toxicity. As such, these characteristics are effective defense mechanisms the population uses to generate a bloom and persist longer than other competing algae (Gobler and Sunda 2012). The diatom frustule seems to be acting similarly, making the diatom less digestible, or palatable, to microzooplankton grazers, a phenomenon which may contribute to formation of the large, persistent diatom blooms frequently observed in coastal systems.

Instances of preferential grazing by microzooplankton on other phytoplankton, regardless of a large diatom biomass, provide indirect evidence in support of frustules as a defense. In the Equatorial Pacific microzooplankton grazers only consumed approximately half of the total diatom biomass, whereas there was a tighter coupling between microzooplankton predators and small dinoflagellates and prymnesiophytes as prey (Verity et al. 1996). Similarly, microzooplankton grazers in the Celtic Sea showed a preference towards small dinoflagellates, cryptophytes, chlorophytes and prasinophytes and a strong selection against diatoms even though the latter represented the bulk of the algal standing

stock (Burkill et al. 1987). Furthermore, the ratio of microzooplankton grazing to phytoplankton growth (g:μ) in the Gulf of Alaska was consistently lower in the larger (> 8 μm), diatom dominated, size fractions compared to smaller non-diatom phytoplankton (Strom et al. 2001), indicating a decoupling of microzooplankton grazing and diatom growth. These examples support the hypothesis that there is a defense mechanism in diatoms leading to decoupling of microzooplankton grazing and diatom growth, as well as preferential feeding on other, non-frustuled, phytoplankton.

Evolutionary Implications

One problem with slowing digestion as a defense mechanism is that it relies on the death of an individual to reduce the predation pressure on the entire population. Thus, the mechanism does not allow the cell to pass on its genes, which encode for said defense, and cannot be selected for evolutionarily. However, the defense could provide an evolutionary benefit if the sacrifice benefited a nearby clone or genetically similar individual, a phenomenon termed kin selection (Hamilton 1964, Pohnert et al. 2007). For kin selection to favor a thick frustule it must satisfy Hamilton's rule ($rB > C$, where r = genetic relatedness of the population, B = additional reproductive success of the recipients of the altruistic act and C = cost of the defense for the individual) (Hamilton 1964, Flynn and Irigoien 2009). In regards to the proposed defense, B would be expected to increase if the grazer's growth rates are being suppressed by the poorly digestible frustule. However, genetic relatedness (r) may be low in most diatom blooms, where high intraspecific genetic diversity has been observed (Rynearson and Armbrust 2000, Evans et al. 2005, Rynearson et al. 2006). While there is some energetic cost (C) of the diatom frustule, the expenditure for synthesis is markedly less than that of a traditional cell wall (Raven 1983, Finkel and Kotrc 2010). Thus, while it is not possible to quantitatively estimate whether the system would satisfy Hamilton's

rule, the increased benefit provided by the defense (B) and comparatively low cost (C) may indicate that kin selection is acting on this defense.

Even if thick frustules were favored by kin selection, microzooplankton generally do not graze on one species at a time (Sherr and Sherr 2007). Thus the potential benefit a given diatom species receives from this defense (reduced predation pressure) would also be imparted to competing algae (Flynn and Irigoien 2009). Yet, this could still be beneficial if the diatom had a faster intrinsic growth rate compared to the competing phytoplankton, which is often the case. Alternatively, this defense mechanism may be a by-product of natural selection acting on one or more of the other functions provided by a thick frustule (Raven and Waite 2004). This has been seen in the polyunsaturated aldehyde (PUA) defense mechanism in diatoms, which is said to reduce the fecundity of copepods. Flynn and Irigoien (2009), however, concluded that this was not a viable defense mechanism in diatoms and could not be favored for by natural selection. Based on a model of this defense, the increased benefit the surrounding diatoms received (B) from the PUA mechanism was low, and often negative (PUAs allowed for increased predation from microzooplankton), and regardless of the cost (C) PUAs at a defense would unlikely satisfy Hamilton's rule as a selectable trait. They determined that PUAs are likely a metabolite that serves another purpose but also happened to reduce the egg production of some copepods. While it is not clear that the frustule defense mechanism is behaving similarly to the PUA mechanism, more research will be needed to determine the evolutionary driver of the frustule.

Food Quality

An alternate theory for the decreased growth and digestion rates of microzooplankton feeding on thick diatoms is the effect of food quality. Nutritional quality,

including nutrient content, stoichiometry (C:N:P), and lipid content of prey, can have a large impact on predators, especially on growth rate (Mitra and Flynn 2005). We observed marked decreases in carbon and nitrogen content between the high and low silicic acid treatments. When expressing ingestion rates in carbon consumed for experiments 1 and 2, both predators consumed notably higher quantities of carbon in the thin diatom treatment. Additionally, thin *C. radiatus* had a noticeably, albeit not significantly, lower C:N, hinting at a higher food quality in comparison to the thick diatoms. Furthermore, silicic acid limitation can have an effect on fatty acid production. The diatom *Cyclotella meneghiniana* grown in high silicic acid conditions had a 2-fold lower lipid content as percent dry weight (Jungandreas et al. 2012). While less is known about lipid content of prey as it affects microzooplankton success, fatty acid content of diatoms and dinoflagellates has a large effect on copepod fitness. Studies found that egg production and viability in copepods were significantly lower when feeding on prey with less carbon and lipid content per cell (Jónasdóttir 1994, Shin et al. 2003). While food quality could explain the growth rate differences I observed for *G. spirale*, it is unlikely to have produced the lower digestion rate observed in *N. scintillans* feeding on thick diatoms.

Ecological and Biogeochemical Implications

The diatom frustule as a mechanical defense has implications for bloom formation and sustainability. The successful formation of a bloom will only result if the rate of phytoplankton growth exceeds the rate of losses due to predation, sinking and other causes (Tillmann 2004). In coastal systems diatoms are arguably the most successful, and common, bloom-forming phytoplankton. Therefore, a defense mechanism (or multiple mechanisms) is likely present to prevent the population from being consumed before a bloom can form. I propose that the frustule is a key mechanism in protecting diatoms from

predation during early bloom formation. Before blooms form, nutrient levels, including silicic acid, are at their highest. As a result, biogenic silica, and thus frustule thickness, is highest earlier in a bloom (Brzezinski et al. 1998). According to the lower digestion rate mechanism, or even the reduced carbon and nitrogen under high silicic acid, this would cause reduced microzooplankton growth rates and thus lower grazing pressure by the microzooplankton community and would promote an increase in the diatom standing stock, eventually leading to a bloom. As the bloom increases, silicic acid and other nutrients are taken up by diatoms and decrease in the environment. This will eventually lead to a stock of diatoms that have a lower biogenic silica content (Brzezinski et al. 1998). A population of less silicified diatoms would result in higher microzooplankton population growth, thus higher predation pressure by microzooplankton and which would contribute to the termination of the bloom.

The results of this study also have large implications for carbon and silica cycling. If heavily silicified diatoms are more protected from predation than weakly silicified diatoms or other non-silicified phytoplankton, they might be grazed at lower rates and thus tend to sink out of the water column, taking carbon and silica out of the euphotic zone. However, considering that this study and others have shown that increases in silicification result in decreased carbon content, perhaps the more thinly silicified diatoms are larger drivers of carbon sinking than the thick diatoms. In fact, a study during an iron fertilization experiment in the Southern Ocean found that thinner frustuled diatoms were larger contributors to carbon sinking than thick species (Assmy et al. 2013). The thinner species died much earlier than the thicker species and as a result sank out of the upper water column with the bulk of their carbon still intact. The thicker species persisted longer and sank with most of their carbon already remineralized, contributing more to silica sequestration than to carbon sequestration (Assmy et al. 2013). In systems with high levels of silicic acid there would likely be more thick-frustuled diatoms, as a result of increased silicic acid uptake, which are

more defended from predation. This would result in a disconnect between silica and carbon sequestration with regions of high silicic acid deposition compared to relatively low carbon sequestration (Assmy et al. 2013). There is evidence for this decoupling in the silicic acid budget in the Southern Ocean, a region with relatively high silicic acid (Tréguer et al. 1995) and diatoms with BSi:C an order of magnitude higher than species in other ecosystems (Brzezinski 1985). As a result, the Southern Ocean, which produces 20% of the global biogenic silica, contributes up to 40% of the global BSi sequestration but sequesters comparatively low amounts of carbon (Tréguer et al. 1995, Ragueneau et al. 2000, Pondaven et al. 2000).

Conclusions

I have presented the first recorded evidence in support of diatom frustules as a mechanical defense against their primary predators, the heterotrophic dinoflagellates. The proposed defense mechanism targets the digestion and growth rate of the predators, allowing for overall decreased grazing on the diatom population. The frustule defense in diatoms has large implications for food web dynamics in marine and freshwater systems, as well as for global carbon and silica cycling. Therefore, further research is required to fully understand this intricate system. Diatoms are a diverse group of organisms and possess a variety of morphologies; additional studies with more diatoms will be essential in determining whether this defense is present across all diatoms or is species-specific. Microzooplankton are similarly diverse, with many different feeding strategies. Further studies will have to be conducted to determine if these findings are transferable to other microzooplankton, especially ciliates, an abundant and occasionally important grazer of diatoms. We also need to address the evolutionary implications posed by this potential defense mechanism. Is kin selection acting to give rise to this defense mechanism or is it an “accidental” trait

resulting from another known, or unknown, benefit that the frustule provides? Fully understanding how diatoms use their frustule to defend against predators may explain, in part, why diatoms dominate the primary production in coastal ecosystems.

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