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Grazing on *Synechococcus spp.* by the Red-Tide Dinoflagellate *Karenia brevis*: Implications for Bloom Dynamics in the Gulf of Mexico

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**GRAZING ON *SYNECHOCOCCUS* SPP. BY THE RED-TIDE
DINOFLAGELLATE *KARENIA BREVIS*: IMPLICATIONS FOR BLOOM
DYNAMICS IN THE GULF OF MEXICO**

by

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B.S. December 2005, University of Saint Francis (IN)

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ABSTRACT

GRAZING ON *SYNECHOCOCCUS* SPP. BY THE RED-TIDE DINOFLAGELLATE *KARENIA BREVIS*: IMPLICATIONS FOR BLOOM DYNAMICS IN THE GULF OF MEXICO

Leo Austin Procise
Old Dominion University, 2012
Director: Dr. Margaret R. Mulholland

Karenia brevis, the toxic dinoflagellate responsible for massive red tides in the Gulf of Mexico (GOM), causes fish kills, shellfish poisoning, and acute respiratory irritation in humans. Bloom initiation and maintenance have been linked to the physical environment as well as various nutrient input mechanisms. To date, efforts to quantify nitrogen (N) sources fueling *K. brevis* blooms in the GOM have not included mixotrophic grazing although many dinoflagellates, including *K. brevis*, are known to be capable of mixotrophy. This dissertation reports field and laboratory results demonstrating that natural bloom populations and *K. brevis* isolates from the West Florida Shelf (WFS) can ingest a WFS *Synechococcus* isolate. Maximum *K. brevis* ingestion rates were measured within the first 2 to 6 hours in laboratory incubations augmented with *Synechococcus* prey and rates ranged from 7.2 to 48.0 *Synechococcus K. brevis*⁻¹ hr⁻¹. I calculated a lower feeding threshold of 1.86×10^4 *Synechococcus* ml⁻¹, which is the prey concentration necessary for *K. brevis* to ingest this prey organism.

To determine whether dissolved N or light affected ingestion rates for *Karenia brevis* on *Synechococcus*, grazing was measured in N-replete and -deplete cultures and during the day and night when incubation lights were on or off, respectively. Ingestion rates ranged from 2.7 to 7.2 *Synechococcus K. brevis*⁻¹ hr⁻¹ and there were no significant differences in ingestion rates between treatments. I calculate that the N-specific uptake

rates from *Synechococcus* prey were on the order of 10^{-2} to $10^1 \mu\text{mol N l}^{-1} \text{ hr}^{-1}$. I also demonstrate for the first time that *K. brevis* is able to ingest *Prochlorococcus* (27.3 ± 8.3 *Prochlorococcus K. brevis*⁻¹ hr⁻¹) and heterotrophic bacteria ($0.1 - 3.1$ bacteria *K. brevis*⁻¹ hr⁻¹), although the latter are likely underestimates as I tried to minimize contamination by heterotrophic bacteria in *K. brevis* cultures.

Karenia brevis ingestion rates on live and heat-killed *Synechococcus* were not statistically different, 23.4 ± 18.1 and 21.38 ± 12.6 *Synechococcus K. brevis*⁻¹ hr⁻¹, respectively. This allowed me to examine prey uptake versus photosynthetic or amino acid C uptake in the same incubation bottles where grazing was measured. C-specific uptake from *Synechococcus* ingestion ranged from 11.2 to 38.8 pmol C *K. brevis*⁻¹ hr⁻¹, which was 7.5 to 22.4 times greater than photosynthetic C uptake in parallel incubations.

Ingestion rates by *Karenia brevis* on *Synechococcus* measured during cruises to the WFS during three blooms were 0.04 to 15.5 *Synechococcus K. brevis*⁻¹ hr⁻¹, which falls within the range found in laboratory studies. The highest ingestion rates by *K. brevis* on the WFS were measured in 2009 despite low ambient concentrations of *Synechococcus*. N-specific uptake from *Synechococcus* ranged from 0.05 to 13.86 $\mu\text{mol N l}^{-1} \text{ hr}^{-1}$ during laboratory and field experiments. Grazing on *Synechococcus*, as well as other possible picoplanktonic prey, can contribute substantially to the N budget for *K. brevis* growth in the GOM, which has been reported between 0.056 to 0.267 $\mu\text{mol N l}^{-1} \text{ d}^{-1}$ for moderately sized (10^5 cells l⁻¹) blooms growing autotrophically.

This dissertation is dedicated to my mother Rebecca and sister Leslie.

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CHAPTER I

INTRODUCTION

Karenia brevis is an athecate, mixotrophic dinoflagellate that is common throughout the Gulf of Mexico (GOM). Formerly known as *Gymnodinium breve*, *K. brevis* produces a potent brevetoxin and can form massive blooms that negatively affect coastal ecosystems and Florida's Gulf Coast economy. Fish kills, dissolved oxygen depletion, and marine mammal and avian mortality are just a few of the ecological impacts that result from *K. brevis* blooms. *K. brevis* is responsible for neurotoxic shellfish poisoning (NSP), which limits fisheries intake and poses a direct threat to humans who ingest infected shellfish. In its aerosol form, brevetoxins affect mucus membranes in humans causing a persistent cough, sneezing, and is an eye and skin irritant. All of these negative human and ecological impacts impinge on tourism (Kirkpatrick et al. 2004, Hoagland et al. 2009), a major economic driver in Florida.

Karenia brevis blooms on the West Florida Shelf (WFS) form in late spring and fall and can last weeks to months. This region is thought to be nitrogen (N) limited because ample phosphorus (P) is delivered to the system from phosphate mining and farming (Walsh et al. 2006). Initiation of these blooms is thought to occur offshore on the WFS and nearshore blooms have been linked to onshore transport of biomass, diel vertical migration, and physical properties of the water column (Tester & Steidinger 1997, Walsh et al. 2006). Allelopathic inhibition of growth of other taxa (Kubaneck et al. 2005) and the lack of predation on *K. brevis* (Kubaneck et al. 2007) are thought to be other important factors during bloom initiation and accumulation.

Although a variety of sources of bioavailable N have been identified and quantified on the WFS, these N contributions are insufficient to account for the observed biomass accumulation during blooms (Steidinger et al. 1999, Walsh & Steidinger 2001, Vargo et al. 2004). Vargo et al. (2004) estimated that populations of 10^5 cells l^{-1} would need 0.056 to 0.267 $\mu\text{mol N } l^{-1} d^{-1}$ to grow at a rate of $0.2 d^{-1}$. Along the WFS, new N enters the system from estuarine sources (Vargo et al. 2004); total estuarine N inputs can be as high as 0.062 $\mu\text{mol N } l^{-1} d^{-1}$ (Vargo et al. 2008). Atmospheric deposition (Pribble & Janicki 1999), upwelling (Heil et al. 2001, Walsh et al. 2003), hurricanes, and ground water (Hu et al. 2006) are other sources of new N available to *Karenia brevis* on the WFS. However, none of these new N sources appear to be sufficient to generate the large blooms often observed on the WFS. For example, only 5 to 20% of the N requirement for a moderately sized *K. brevis* bloom (10^5 *K. brevis* cells l^{-1} dividing at a rate of $0.2 d^{-1}$) could be met by estuarine inputs (Vargo et al. 2008).

Marine dinitrogen (N_2) fixation is also thought to be an important source of new N fueling bloom initiation in the eastern GOM on the WFS (Lenes et al. 2001, Walsh & Steidinger 2001, Mulholland et al. 2006). Eolian transport of iron-rich Saharan Desert dust stimulates blooms of the diazotrophic cyanobacteria *Trichodesmium* spp. (Walsh & Steidinger 2001) that fix N_2 and release this recently fixed N into the surrounding water (Capone et al. 1994, Glibert & Bronk 1994, Mulholland et al. 2004b), where it is bioavailable to co-occurring algae including *Karenia brevis* (Mulholland et al. 2004b, 2006, Submitted). It has been estimated that the amount of N released from a moderately sized population of *Trichodesmium* (20 colonies l^{-1}) could support a moderately sized *K.*

brevis bloom (10^5 cells l^{-1}) if all of the N released was taken up by *K. brevis* (Mulholland et al. 2006, Vargo et al. 2008).

Internal recycling of N is thought to be important in maintaining the high biomass observed during large annual *Karenia brevis* blooms. Processes that rapidly regenerate bioavailable N within the water column include zooplankton excretion and the decay of dead and dying micro- and macrofauna. It was calculated that zooplankton excrete ammonium at rates ranging from 0.01 to 6.8 $\mu\text{mol N } l^{-1} \text{ d}^{-1}$ and this could support the maintenance of blooms (Lester 2005, Vargo et al. 2008). Together, new and recycled N sources, respectively, could potentially provide enough N to produce and maintain *K. brevis* cell densities of about 10^5 cells l^{-1} on the WFS (Vargo et al. 2008).

In addition to the nutrient sources identified above, it was recently discovered that *Karenia brevis* are capable of grazing on the ubiquitous cyanobacteria, *Synechococcus* spp. Multiple isolates of *K. brevis* from the Provasoli-Guillard National Center for Culture of Marine Phytoplankton (Provasoli-Guillard National Center for Culture of Marine Phytoplankton (CCMP) 2228, CCMP2229, and an unidentified CCMP strain) have been shown to graze on *Synechococcus* isolates (Genbank Accession Number DQ023295 from the East China Sea and CCMP1768 from the GOM) in the laboratory (Jeong et al. 2005b, Glibert et al. 2009). If grazing also occurs in nature, this could provide *K. brevis* with another nutrient source that is unavailable to co-occurring phytoplankton that are strictly autotrophic. This dissertation was aimed at determining the capacity of *K. brevis* isolates from the WFS to graze on picoplanktonic organisms, and quantifying grazing by natural populations of *K. brevis* on the WFS. Environmental controls on grazing were also examined (i.e. light and nutrient supply).

Picocyanobacteria, such as *Synechococcus*, are among the most abundant organisms on earth and their ingestion may provide carbon (C) and N to *Karenia brevis* that could stimulate *K. brevis* bloom initiation and foster their growth on the WFS. Grazing may also contribute to the maintenance of 'seed' populations of *K. brevis* in offshore waters below the euphotic zone (35 - 50 m) or in other areas where nutrients or light are scarce (Stumpf et al. 2003, Walsh et al. 2009, Grabowski 2010). Because both *K. brevis* and *Synechococcus* are more abundant in coastal waters than in offshore waters, high prey abundance might stimulate grazing nearshore (Jeong et al. 2005b, Glibert et al. 2009). Numerous blooms of *Synechococcus* have been documented from Florida coastal waters (Butler et al. 1995, Philips et al. 1999, Glibert et al. 2004). While heterotrophic nanoflagellates are often considered the primary grazers of *Synechococcus* in marine systems (Guillou et al. 2001, Worden & Binder 2003, An-Yi et al. 2007), *K. brevis* may also contribute to grazing of *Synechococcus* on the WFS.

The idea that many dinoflagellate species can switch between autotrophic and heterotrophic metabolisms may be fundamental to their success particularly in eutrophic systems, where dinoflagellate mixotrophs abound (Stoecker 1998), many of which are potentially harmful (Burkholder et al. 2008). Jeong et al. (2005b) reported that 18 species of red tide forming dinoflagellates were able to ingest *Synechococcus*, including 5 dinoflagellate species not previously known to be mixotrophic, including *Karenia brevis*. The contribution of grazing to the N demand of blooms of *K. brevis* has not been quantified outside of laboratory experiments (Jeong et al. 2005b, Glibert et al. 2009) and this may provide an important missing link necessary to reconcile the N budget for this system.

Mixotrophy among dinoflagellates has important ecological ramifications. Mixotrophic protists have been shown to grow better in the presence of algal prey than without prey growing autotrophically (Jeong et al. 2005a) and this can stabilize community productivity (Hammer & Pitchford 2005). Through top-down control, mixotrophic dinoflagellates may also limit excessive picoplankton growth (Jeong et al. 2005b, Glibert et al. 2009). Mixotrophic dinoflagellates, including *Karenia brevis* (Breier & Buskey 2007, Cohen et al. 2007, Kubanek et al. 2007), can themselves be grazed by zooplankton (Teegarden & Cembella 1996). Many large zooplankton have size thresholds for prey ingestion and so cannot ingest picoplankton directly (Berggreen et al. 1988, Jeong 1995). Therefore, grazing by *K. brevis* on picoplankton, such as *Synechococcus* may facilitate the trophic transfer of dissolved organic material through the microbial food web (Azam 1983) to much larger zooplankton.

The objective of this study was to determine rates of mixotrophic grazing by *Karenia brevis* on various planktonic organisms under laboratory conditions (Chapter 2, 3) and on the WFS during blooms (Chapter 4), and to examine environmental controls on mixotrophic grazing by *K. brevis* (Chapters 2, 3, and 4). The broad questions that I wanted to answer in this study were: Are cultured isolates and natural populations of *K. brevis* from the WFS able to ingest *Synechococcus*? If so, what are some of the environmental factors that control grazing by *K. brevis*? For the latter, the availability of nutrients, light, and prey, all have been shown to affect mixotrophic grazing by other dinoflagellates and so these were the factors examined here. In particular, I hypothesized that:

- Natural populations of *K. brevis* and *K. brevis* isolates from the WFS can graze on *Synechococcus* and other co-occurring plankton.
- *K. brevis* graze at higher rates when growing under nutrient-deplete conditions.
- *K. brevis* graze at higher rates in the dark when it cannot photosynthesize.
- *K. brevis* obtains cellular C and N from grazing on *Synechococcus* that can contribute to its growth.

In Chapter 2, after establishing that WFS *Karenia brevis* isolates are capable of grazing, a functional response relationship between *K. brevis* grazing and *Synechococcus* abundance was constructed to determine the lower feeding threshold for grazing on this organism. I also determined whether *K. brevis* could ingest other planktonic organisms, such as heterotrophic bacteria, a small haptophyte, and three other unicellular cyanobacteria, as has been observed for other dinoflagellates. I compared grazing coefficients for *K. brevis* on *Synechococcus* under nutrient-replete and -deplete conditions and in day and nighttime incubations. In Chapter 3, I estimated cellular N and C quotas for cultured *Synechococcus* CCFWC 502, isolated from the WFS, and then estimated rates of C and N acquisition by *K. brevis* from grazing. I compared C uptake from grazing with photosynthetic C fixation, and uptake of C from urea and an amino acid mixture. In Chapter 4, I estimated grazing by *K. brevis* during 3 blooms along the WFS. I compared results from grazing experiments on the WFS with laboratory results as well as physical conditions and nutrient concentrations observed along the WFS during cruises.

CHAPTER II

GRAZING BY THE DINOFLAGELLATE *KARENIA BREVIS*: DIVERSITY OF PREY AND CONTROLS ON MIXOTROPHY

Introduction

Mixotrophy, the ability of organisms to employ both auto- and heterotrophic metabolisms, has been examined in recent years in order to understand its role in the initiation and persistence of harmful algal blooms (Stoecker et al. 2006; 2008, Burkholder et al. 2008, Jeong et al. 2010, Yoo et al. 2010). Many common bloom-forming dinoflagellates are mixotrophic and while capable of photosynthesis, they also acquire cellular carbon (C) via osmotrophy or phagotrophic grazing (Stoecker et al. 1997, Glibert & Legrand 2006). Mixotrophy has many potential benefits (reviewed by Burkholder et al. 2008) and in addition to C, it can supply many other macro- and micronutrients and trace elements necessary for growth. Nutritional flexibility may allow mixotrophs to out-compete strictly autotrophic and heterotrophic organisms by offering them more nutritional choices or by allowing them to ingest their competitors (Bockstahler & Coats 1993a, b, Thingstad et al. 1996, Rothhaupt 1996, Li et al. 1999, Tittel et al. 2003). Additionally, mixotrophs may acquire C during both the dark and light periods and where light is limiting for photosynthesis, e.g., near the bottom of the euphotic zone or in coastal waters (Legrand et al. 1998). For example, under low light, some mixotrophs graze at higher rates and so may be able to out-compete co-occurring autotrophs (Caron et al. 1993, Jones et al. 1993, 1995).

For most mixotrophs, grazing is dependent on prey concentration and there is usually some threshold of prey abundance necessary for grazing to commence, and above this threshold, ingestion rates increase with prey concentration until some maximum clearance rate is reached (Legrand et al. 1996, Jeong et al. 2004, Jeong et al. 2005b).

Karenia brevis is highly toxic and blooms in the Gulf of Mexico (GOM) on the West Florida Shelf (WFS) annually. These blooms are thought to be fueled by a variety of nutrient sources, none of which can individually satisfy nitrogen (N) demand during blooms (Vargo et al. 2008); it is likely therefore that multiple nutrient sources contribute to bloom initiation and maintenance. Recently, multiple strains of *K. brevis* culture isolates (an unidentified CCMP strain, CCMP 2228 and CCMP 2229) were discovered to be capable of mixotrophic grazing on the marine cyanobacterium, *Synechococcus*, but ingestion rates were highly variable between studies ranging from 0.96 - 83.8 *Synechococcus K. brevis*⁻¹ hr⁻¹ (Jeong et al. 2005b, Glibert et al. 2009). If natural *K. brevis* populations graze at similar rates this could provide limiting nutrients and substantially enhance cellular growth rates within blooms (Glibert et al. 2009). However, to date, grazing has not been examined in natural populations of *K. brevis* and previous culture studies (Jeong et al. 2005b, Glibert et al. 2009) only examined grazing by *K. brevis* under nutrient-replete conditions in light bottles.

The diversity of prey available to *Karenia brevis* is also unknown. Other dinoflagellate mixotrophs consume a variety of picoplankton including heterotrophic bacteria (Jeong et al. 2008), cyanobacteria (Landry et al. 1995a, b, Jeong et al. 2009), picoeukaryotes (Lee 2006), and heterotrophic nanoflagellates (Jeong et al. 2007). Some mixotrophic dinoflagellates can also ingest larger prey such as haptophytes (Berge et al.

2008), chlorophytes (Strom & Buskey 1993), ciliates (Smalley and Coats 2002, Park et al. 2006), diatoms (Jacobson & Anderson 1986, Menden-Deuer et al. 2005, Yoo et al. 2009), and other dinoflagellates (Tillman 2004, Adolf et al. 2007). The diversity of ingested particles extends further to include blood cells (Burkholder & Glasgow 1997, Jeong 2006) and fluorescent beads (Nygaard et al. 1988). Here, I report ingestion rates of *Synechococcus* by multiple *K. brevis* isolates from the WFS in the GOM grown under N-replete and -deplete media conditions. Grazing was also measured in light and dark bottles and during day and nighttime incubations. Experiments were conducted to estimate maximum ingestion rates and the lower feeding threshold for *K. brevis* grazing on *Synechococcus*. In addition, five other planktonic species were tested as possible prey for *K. brevis*: *Isochrysis* sp. (CCFWC 363) and *Synechocystis* sp. (CCFWC 493), also isolated from the WFS; *Prochlorococcus marinus* (CCMP 1986) and *Crocospaera watsonii* (WH8501), isolated from other oceanic marine systems; and heterotrophic bacteria that co-occur in cultures.

Methods

Cultures. *Karenia brevis* cultures were obtained from Florida Wildlife Research Institute (FWRI) and were isolated from Florida coastal waters near Jacksonville, *K. brevis* CCFWC 251 (JC4), Sarasota Bay, *K. brevis* CCFWC 254 (SB3), and Charlotte Harbor, *K. brevis* CCFWC 257 (CH2). Coastal *Synechococcus* (CCFWC 502), *Synechocystis* sp. (CCFWC 493) and *Isochrysis* sp. (CCFWC 363), also isolated from the WFS, were also obtained from FWRI. Culture isolates from FWRI were maintained on GP media, which includes nitrate, phosphate, vitamin and metal solutions, and soil

extract in full strength autoclaved artificial seawater (Loeblich & Smith 1968).

Prochlorococcus marinus (CCMP 1986) isolated from surface waters of the Mediterranean Sea was obtained from The Provasoli-Guillard National Center for Culture of Marine Phytoplankton. *Crocospaera watsonii* (WH8501) was isolated from the tropical South Atlantic Ocean and obtained from Woods Hole Oceanographic Institution (WHOI). At Old Dominion University cultures were acclimated from their original growth media to grow on 0.2 μm filtered artificial seawater (ASW) at a salinity of approximately 31 with f/2 nutrients and vitamins or modified f/2 without nitrate (N) and phosphate (P) (Guillard & Ryther 1962). They were maintained between 22 and 26 $^{\circ}\text{C}$ on a 12:12 light:dark cycle and supplied with 70 -100 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ using 20W “cool white” fluorescent light bulbs, which provide enough light in the range of 400 - 470 nm for photosynthesis. Cultures were not axenic, but bacterial contamination was kept at a minimum by transferring cultures in exponential phase growth in a laminar airflow hood (NuAire; downflow 70 ft min^{-1} , inflow 105 ft min^{-1}) using aseptic techniques. Algal and cyanobacterial growth was monitored using flow cytometry (BD FACSCalibur 15 mW 488nm air cooled argon-ion laser).

Each *Karenia brevis* isolate used was in exponential growth phase upon initiation of grazing experiments with target *K. brevis* and *Synechococcus* concentrations between 10^1 - 10^3 and 10^4 - 10^6 cells ml^{-1} , respectively. Every effort was made to have uniform *K. brevis* cell concentrations during all experiments, but this was not always possible because cultures were difficult to maintain at high densities in volumes necessary for use in grazing experiments and were prone to unexpected catastrophic die-off. For the same reasons, it was not always possible to use the same *K. brevis* culture isolate in each set of

incubation experiments. *K. brevis* isolates used in each grazing experiment were examined to ascertain that cultures were healthy at the start of each experiment (i.e. cells exhibit positive phototaxis).



Fig. 1. A confocal laser scanning microscope image of *Synechococcus* (CCFWC 502) cells grown in *f/2* medium. The scale bar = 5 μm .

To calculate cellular N and C concentrations for *Synechococcus*, samples from culture bottles were enumerated using flow cytometry (FCM), as described below, and samples were filtered onto pre-combusted (450°C for 2 hours) Whatman GF/F filters and frozen for later analysis of particulate C (PC) and N (PN). Prior to analysis, samples

were dried at 40 °C for at least 48 hours and pelletized into tin discs. Samples were analyzed on a Europa automated N and C analyzer (ANCA) and then normalized per cell (pmol cell⁻¹).

Confocal laser scanning microscopy (CLSM) and epifluorescent microscopy (EM) were used to determine *Synechococcus* cell length, width, and depth (Fig. 1). *Synechococcus* cell biovolume ($\times 10^6 \mu\text{m}^3 \text{ml}^{-1}$) was calculated based on microscopic measurements according to Sun & Liu (2003) to determine that cellular N and C content were realistic for the *Synechococcus* strain used in these experiments. *Synechococcus* cells as large as $20 \times 10^6 \mu\text{m}^3 \text{ml}^{-1}$ have been observed in Florida Bay during blooms of *Synechococcus* (Phlips et al. 1999).

Flow Cytometry and Microscopy. *Synechococcus* cells were enumerated by gating populations of cells based on forward light scatter and red auto-fluorescence. Total FCM photomultiplier tube intensities used for all *Synechococcus* counts were: forward light scatter (FLS) E01 (10 \times signal), side light scatter (SSC) 319 V, green fluorescence (FL1) 520 V, orange fluorescence (FL2) 659 V, and red fluorescence (FL3) 505 V. Each sample was run with 0.5 μm fluorescent beads as an internal marker (Worden & Binder 2003). FCM sample runs were terminated after 30 seconds or when 10 million total events were recorded at the lowest intake speed. At least 1,000 *Synechococcus* cells were gated from each sample. Final *Synechococcus* concentrations ($P_{\text{RC}}, \text{Synechococcus ml}^{-1}$) were calculated by dividing the number of gated events (forward light scatter against red autofluorescence) by the volume sampled (Equation 1) using FCM.

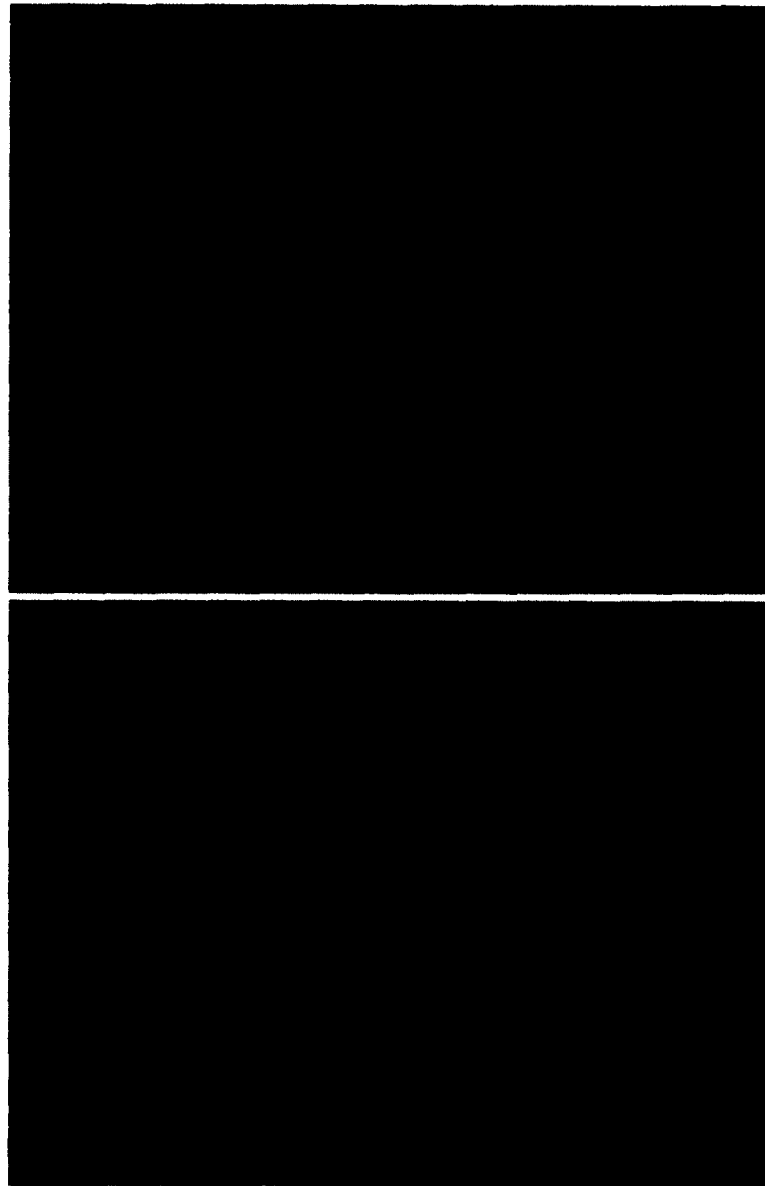


Fig. 2. Two epifluorescent microscope images of *Karenia brevis* cells, grown in f/2 medium, each with a single *Synechococcus* prey inclusion. *Synechococcus* was stained with SYTO 13 (green) for contrast with the red autofluorescence of chloroplasts. Scale bars = 20 μm .

$$P_{RC} = \text{Gated events} / (\text{Volume}_{\text{final}} - \text{Volume}_{\text{initial}}) \quad (1)$$

Karenia brevis (FLS E00 (1× signal), SSC 319 V, FL1 520 V, FL2 659 V, FL3 211 V), *Isochrysis* sp. (FLS E01 (10× signal), SSC 319 V, FL1 520 V, FL2 659 V, FL3 319 V), *Synechocystis* sp. and *C. watsonii* (FLS E01 (10× signal), SSC 319 V, FL1 520 V, FL2 659 V, FL3 505 V), and *P. marinus* (FLS E01 (10× signal), SSC 350 V, FL1 600 V, FL2 550 V, FL3 650 V) were enumerated using the same method as for *Synechococcus* but with different photomultiplier tube intensities. Microscopic and FCM counts of *Synechococcus*, heterotrophic bacteria, and *K. brevis* were plotted on a property-property plot and compared using a linear regression. FCM counts were on average 7 to 14% lower than EM counts ($EM = 1.1 * FCM + 0.45$; $R^2 = 0.997$) (Appendix A).

To verify that prey cells were ingested by *Karenia brevis*, prey inclusions were photographed from subsamples taken from grazing experiments. A 1 ml preserved aliquot from prey-amended *K. brevis* incubation bottles were filtered onto a 5 µm black polycarbonate filter and mounted with Citifluor Antifadent. Slides were examined using EM (Fig. 2) and CLSM scanning the z-axis to ensure that prey cells were actually ingested (Fig. 3).

Grazing Experiments. Initial grazing experiments were time course experiments to determine the optimum incubation length under laboratory conditions. Based on initial grazing experiments that extended for up to 4 days, during which samples were collected daily (data not shown), it was determined that the maximum grazing coefficients (hr^{-1}), or the linear slope of natural log transformed prey abundance with time, were observed within the first day of incubation. In shorter time course experiments (1 day), the slope

of the natural log transformed data was linear for the first 2 to 6 hours, after which time the rate of prey removal decreased dramatically, likely due to *Karenia brevis* reaching digestion rates that are equal to ingestion rates (Li et al. 1999).

Each grazing experiment was done in triplicate in 125 ml PETG bottles.

Triplicate prey control (prey only - no *K. brevis*), *Karenia brevis* control (*K. brevis* - no prey), and prey amended (*K. brevis* plus prey) bottles were incubated for each experimental treatment. Experimental treatments included N-replete and N-deplete, light and dark bottle grazing experiments, as well as day (incubator lights on) and nighttime (incubator lights off) experiments. For each grazing experiment, bottles were gently agitated prior to sample collection to ensure that predator and prey were uniformly distributed in all bottles at the time of sampling. A 2 ml sample was collected and then split into 1 ml aliquots; one aliquot was preserved in 1% (final concentration) glutaraldehyde and stored at 4 °C for microscopic analysis (see above) and the other was immediately analyzed on a BD FACSCalibur flow cytometer to enumerate *K. brevis*, *Synechococcus*, or the abundance of other prey tested (see below).

Calculations. To calculate grazing coefficients ($g; hr^{-1}$) in prey disappearance (PD) experiments, *Synechococcus* concentrations from *Karenia brevis* plus prey incubation bottles were natural log transformed and plotted against time (Sherr & Sherr 1993). The slope of the resulting regression is the grazing coefficient. If there was significant ($p < 0.05$) growth in prey control bottles (prey only), then this was accounted for when calculating grazing coefficients in *K. brevis* plus prey incubations (Frost 1972).



Fig. 3. A confocal laser scanning microscope image showing *K. brevis* containing a *Synechococcus* cell inclusion. *Synechococcus* were stained with SYTO 13 (green) for contrast with the red autofluorescence of *K. brevis* chloroplasts. Scale bar = 10 μm .

Grazing coefficients were calculated based on the linear portion of the natural log transformed prey concentration data for time course experiments.

Clearance rates (CR; $\text{ml } Karenia brevis^{-1} \text{ hr}^{-1}$) were calculated by dividing the grazing coefficient (g) by the grazer concentration (GC ; cells ml^{-1}) (Equation 2).

Ingestion rates (IR; $\text{Synechococcus } K. brevis^{-1} \text{ hr}^{-1}$), which are directly proportional to clearance rates and prey concentration (P_{RC} ; cells ml^{-1}), were also calculated for each experiment (Equation 3).

Synechococcus N- and C-specific assimilation rates (0.5 AE) by *Karenia brevis* were calculated using *Synechococcus* ($PN_{\text{Synechococcus}}$) and *K. brevis* ($PN_{K. brevis}$) N and C cell content (Equation 4), which represent 50% mixotrophic assimilation efficiency (Flynn & Mitra 2009). N- and C-specific assimilation efficiencies do not include loss terms, such as respiration. Therefore N- and C-specific assimilation rates in this study may be overestimates.

$$CR = g / GC \quad (2)$$

$$IR = CR \times P_{RC} \quad (3)$$

$$0.5 \text{ AE} = IR \times PN_{K. brevis} / PN_{\text{Synechococcus}} \quad (4)$$

Statistical Analyses. Grazing coefficients significantly different from zero, indicate active grazing by *Karenia brevis*. ANOVAs were used to test for significant differences in grazing coefficients between treatments (nutrient-deplete versus nutrient-replete or light versus dark). When more than 2 groups were tested, a Tukey's multiple comparisons test was used to determine which treatments were significantly different from each other. A 2-way ANOVA was run in cases where data was pooled to determine if there was a significant difference in grazing coefficients between each experiment as well as the pooled data for each treatment. Finally, an ANCOVA with time as a covariate was run for time course incubations to determine if grazing coefficients were significantly different between treatment incubations (homogeneity of slopes). For ANCOVAs, the statistic reported represents the interaction term (time * treatment).

Nutrient-Replete Grazing. *Karenia brevis* and *Synechococcus* cultures were acclimated on f/2 media with N and P supplied in the form of nitrate and phosphate at concentrations of 8.83×10^{-4} M and 3.63×10^{-5} M, respectively. To determine whether

WFS *K. brevis* isolates, CH2 and SB3, were able to ingest *Synechococcus* (CCFWC 502) in nutrient-replete media under laboratory conditions, 25-hour time course prey disappearance (PD) experiments were conducted and samples were collected at time intervals of 0, 1, 5, 9, 13, 17, 21, and 25 hours. Samples were collected at each time point and a 1 ml aliquot was immediately run on the FCM, as described above, and another 1 ml aliquot was preserved in glutaraldehyde (1% final concentration) for verification of prey inclusions and microscope counts. The natural log transformed *Synechococcus* cell abundance was plotted over time and a linear regression run to determine whether there was grazing in *K. brevis* incubation bottles amended with *Synechococcus* (slope, or grazing coefficient, significantly ($p < 0.05$) different than zero). Since the two nutrient-replete grazing experiments were run in parallel and had similar ambient starting conditions, an ANCOVA with time as a covariate was run to determine whether there were differences in grazing coefficients between *K. brevis* isolates CH2 and SB3.

Nutrient-Deplete Grazing. These experiments were conducted to test whether grazing by *Karenia brevis* on *Synechococcus* was enhanced under nutrient-deplete conditions. Grazing by *K. brevis* isolates CH2 and SB3 on *Synechococcus* (CCFWC 502) was measured in cultures acclimated for 48 hours in f/2 media that was N- and P-deplete. For each experiment, *K. brevis* and *Synechococcus* cells were removed from f/2 media and resuspended in f/2 media with no added N and P. *Synechococcus* cultures (approximately 10^3 cells ml^{-1}) were placed in sterile centrifuge tubes and centrifuged for 20 minutes, after which there was a noticeable pellet of cells at the bottom. The supernatant was decanted and replaced with 0.2 μm filtered modified f/2 media, without

added N and P and the pellet was resuspended and then centrifuged again. This process was repeated 3 times. After the third wash the supernatant was decanted, pellets of *Synechococcus* cells combined and suspended in 30 ml of N- and P-deplete f/2 where they remained for 48 hours before conducting the grazing experiment.

Karenia brevis cells are delicate (Tester et al. 2000); therefore a gentler approach was taken to suspend these cells in nutrient-deplete media. Because cells are $> 10 \mu\text{m}$ in diameter, nutrient-replete cultures of *K. brevis* were gravity filtered using a 47 mm (diameter) $10 \mu\text{m}$ (pore size) polycarbonate filters to concentrate cells (filters were not allowed to go dry). The concentrated *K. brevis* cells were then resuspended in N- and P-deplete f/2 media. This process was repeated three times. Once *K. brevis* and *Synechococcus* cells had been acclimated to the nutrient-deplete media for at least 48 hours, they were placed into triplicate bottles and grazing experiments were conducted over a 24-hour period as described above. For each experiment, samples were taken at 0, 1, 2, 4, 6, and 24 hours. Linear regressions were run on natural log transformed *Synechococcus* cell abundance data to determine if the grazing coefficients were significantly different from zero. To verify that media was N- and P-deplete, concentrations of nitrate (plus nitrite) and phosphate were measured using an Astoria Pacific nutrient analyzer according to manufacturer's specifications (Astoria® Analyzer Nitrate+Nitrite A177 and Ortho-Phosphate 305-A204) (Appendix B).

Because prey amendments were not held constant between nutrient-replete and nutrient-deplete experiments, grazing rates could not be compared for the two sets of experiments.

Light and Dark Grazing. Grazing by nutrient-replete *Karenia brevis* isolates JC4 and CH2 were examined in light and dark bottle incubations. For experiments with *K. brevis* JC4, experiments were done without a dark acclimation period while for *K. brevis* CH2, cultures were first acclimated in dark bottles for at least 48 hours prior to starting grazing experiments. Experiments were sampled over a 24-hour period taking samples for cell counts at 0, 1, 2, 4, 6, and 24 hours. Grazing coefficients for light and dark experiments were estimated using linear regressions (\ln prey concentration versus time).

Functional Response to Varying Concentrations of Synechococcus. To determine the relationship between prey concentration and grazing coefficients, triplicate sets of borosilicate glass culture tubes were filled with 30 ml of uniformly dense (3.3×10^2 cells ml^{-1}) *Karenia brevis* (JC4) culture and amended with live *Synechococcus* cells at five different initial concentrations (3.89×10^4 , 4.88×10^4 , 7.29×10^4 , 1.85×10^5 , and 4.19×10^5 *Synechococcus* ml^{-1}). Samples (2 ml) were collected initially, to verify predator and prey abundance at the start of the incubation, and after a 4-hour incubation period to determine grazing coefficients at each prey density. Samples were collected and preserved for FCM and cell counts as described above and grazing coefficients were calculated using Equation 6. An ANOVA was run to compare grazing coefficients calculated from each replicate as well as a Tukey's multiple comparisons test to determine if there were significant differences in grazing coefficients between treatments (five prey concentrations). Equation 6, a modification of the Ivlev curve (Ivlev 1955), was fit to the data to describe ingestion rate as a function of prey concentration, where IR_{max} is the maximum ingestion rate and α is a constant that describes the initial slope of the curve.

$$g = -1/t * \ln(P_{RCf} / P_{RCi}) \quad (5)$$

$$IR = IR_{max} (0.83 - e^{(-\alpha * P_{RC})}) \quad (6)$$

Grazing on Heat-Killed Synechococcus. This experiment was designed to determine whether *Karenia brevis* (CH2 and JC4) could ingest heat-killed cells at comparable rates as live prey. This experiment was done because use of heat-killed cells would: 1) provide uniformity and flexibility in conducting grazing experiments because stocks of uniform cell density could be preserved for use in both the field and laboratory, and 2) allow me to measure both grazing and photosynthetic C uptake (or uptake of other C and N compounds) in the same incubation bottles (see Chapter 3). Grazing experiments were similar to those described above; however, prey cells were now heat-killed (60 °C for 1 hr) prior to their addition to treatment bottles. *Synechococcus* was heat-killed as described by Sherr et al. (1987), but not stained. Heat-killed *Synechococcus* cells were stored at -80 °C, thawed, and enumerated using FCM to ensure that cells density and autofluorescence remained the same before and after preparation and storage. Samples were taken at 0, 1, 2, 4, 6, and 24 hours for cell counts or preservation as described above. Linear regressions were run on the natural log transformed *Synechococcus* cell data from prey amended incubation bottles to determine if there was a grazing response.

To determine whether there were statistically significant differences in grazing coefficients by *Karenia brevis* SB3 amended with heat-killed versus live *Synechococcus*, I conducted parallel grazing experiments using both as prey. Heat-killed and live *Synechococcus* were supplied at similar concentrations in these experiments. Triplicate bottles were incubated for 24 hours and samples were collected after 0, 1, 2, 4, 6, and 24

hours and either analyzed or stored as described above to measure *Synechococcus* and *K. brevis* cell concentrations. Grazing coefficients were calculated as described above and an ANCOVA was run to test the homogeneity of slopes over time to determine whether there were differences in grazing coefficients between heat-killed versus live prey treatments.

The Effect of Nutrients on Ingestion Rates. Grazing by *Karenia brevis* (SB3) on heat-killed *Synechococcus* was examined in nutrient-replete and -deplete incubations to determine whether nutrient availability affects grazing by *K. brevis* on *Synechococcus*. Treatment incubations were done in 125 ml PETG bottles with 6 replicate prey control (*Synechococcus* only), predator control (*K. brevis* only), and prey-amended (*K. brevis* with *Synechococcus*) bottles. For these experiments, prey amendments were held constant for nutrient-replete and -deplete treatments. Samples were collected after a 4-hour incubation period (within the range where grazing was linear over time) to measure cell abundance and to verify prey inclusions using FCM and EM/CLSM, respectively. Grazing coefficients (g; hr^{-1}) were calculated using Equation 5 (Frost 1972) where P_{RC_i} and P_{RC_f} are the average concentrations of *Synechococcus* cells at the start and end of the incubation period ($t = 4$ hours), respectively. Grazing coefficients from nutrient-replete and -deplete prey amended bottles were compared using an ANOVA to determine if there was a statistical difference between the two nutrient treatments.

The Effect of Light on Ingestion Rates. To determine whether *Karenia brevis* (SB3) ingestion rates on heat-killed *Synechococcus* varied between day and night periods within a diel cycle, I began grazing experiments using triplicate nutrient-replete and nutrient-deplete cultures (prepared as described above) 2 hours after the lights came on

(during the “day”) and 2 hours after the incubation lights turned off (at “night”). Samples were collected at 0 and 4 hours to measure cell abundance and to verify prey inclusions using FCM and EM/CLSM, respectively. Because there was no statistical difference between nutrient-replete and -deplete treatments for this experiment (see Results below), I also pooled triplicate incubations from nutrient-replete and -deplete incubations for the day and night treatments, thus increasing my number of replicates from $n = 3$ to $n = 6$. Grazing coefficients were calculated using Equation 5 ($t = 4$) and a 2-way ANOVA was used to compare grazing coefficients between treatments (day versus night).

Grazing on Other Phytoplankton. Four other phytoplankton species were tested to determine whether *Karenia brevis* could ingest them. These were: *Isochrysis* sp., *Synechocystis* sp., *Prochlorococcus marinus*, and *Crocospaera watsonii*. These experiments were conducted in the light using nutrient-replete f/2 media. Grazing experiments were conducted over a 24-hour sampling period similar to those conducted for *Synechococcus* as described above. Samples were collected and preserved for FCM and microscopic cell counts at 0, 1, 2, 4, 6, and 24 hours. Linear regressions were also run on log transformed cell abundance data to determine if there was a grazing response by *K. brevis* on each prey species.

Grazing on Bacteria. To examine *Karenia brevis* grazing on co-occurring heterotrophic bacteria in the laboratory, dilution experiments were conducted using whole culture water and 0.2 μm filtered f/2 media (Landry & Hassett 1982). Four individual dilution experiments were done using 2 different *K. brevis* isolates, SB3 and CH2, to measure the grazing of *K. brevis* on co-occurring, heterotrophic bacteria found in cultures. *K. brevis* cultures were diluted with 0.2 μm filtered f/2 media by 0, 30, 50, and

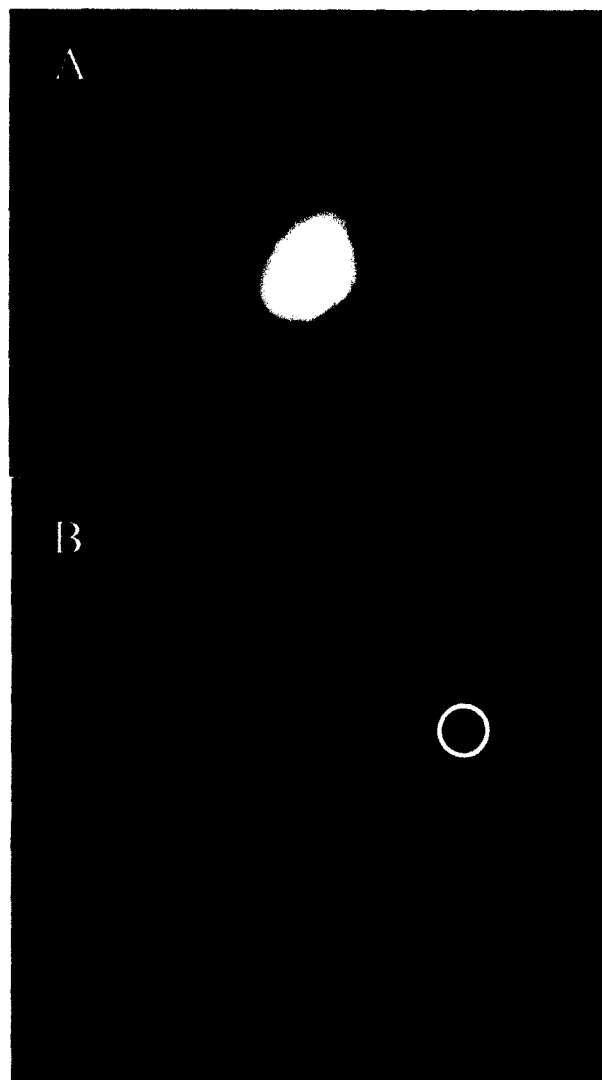


Fig. 4. An epifluorescent microscope image (A) and confocal laser scanning microscope image (B) of heterotrophic bacteria cell inclusions within a *K. brevis* cell (white circle) in nutrient-replete f/2 medium. Bacteria cells were stained with SYTO 13.

70%. Incubations lasted 5 days due to low heterotrophic bacterial ingestion rates.

Incubations were terminated after 5 days when final samples (2 ml) were taken, stored, and cell abundances enumerated. Grazing coefficients were calculated using Equation 5 and compared for each dilution in the dilution series using a linear regression.

Karenia brevis cells were counted using FCM as described above. Bacterial cells were enumerated using FCM and the nucleic acid stain, SYTO 13 (del Giorgio et al. 1996, Troussellier et al. 1999). SYTO 13 was added to each sample at a final concentration of 5 μM and samples were stored in complete darkness for at least 10 minutes similar to Troussellier et al. (1999). *K. brevis* cells were gated based on forward light scatter (FLS) and red fluorescence, as described above, and stained bacteria by FLS and green fluorescence. Ingested heterotrophic bacteria stained with SYTO 13 were verified through EM and CLMS using the method described above (Fig. 4).

Results

Cultures. The average length and width of *Synechococcus* (CCFWC 502) cells were $3.79 \pm 0.12 \mu\text{m}$ and $1.97 \pm 0.07 \mu\text{m}$, respectively. I calculated that the average cell biovolume ($n = 20$) was $13.33 \pm 0.54 \times 10^6 \mu\text{m}^3 \text{ml}^{-1}$ based on Equation 12-H in Sun and Liu (2003). This *Synechococcus* isolate was similar in biovolume to natural populations of *Synechococcus* in Florida Bay (up to $20 \times 10^6 \mu\text{m}^3 \text{ml}^{-1}$; Philips et al. 1999) and to *Synechococcus* collected on the WFS in this study ($10.76 \pm 2.08 \times 10^6 \mu\text{m}^3 \text{ml}^{-1}$ ($n = 20$); Chapter 4). Cellular N and C concentrations in cultured *Synechococcus* were 0.17 ± 0.05 and $1.04 \pm 0.14 \text{ pmol cell}^{-1}$, respectively (Table 1) and PC:PN ratios were about 6.1. *Synechococcus* cells used in this study were larger and had higher N and C quotas than

those used in other grazing studies (Caron et al. 1991, Verity et al. 1992, Bertilsson et al. 2003).

Average *Karenia brevis* cellular N concentrations for nutrient replete *K. brevis* isolates SB3, CH2, and JC4 were 39.2 ± 10.7 , 51.8 (56.9, 46.8), 59.6 ± 9.1 pmol cell⁻¹, respectively (Table 1). Cellular C concentrations were 411.9 ± 63.9 , 446.2 (497.9, 394.4), and 406.6 ± 62.5 pmol cell⁻¹ for SB3, CH2, and JC4 (Table 1), respectively.

Nutrient-Replete Grazing. Initial grazing experiments were conducted using two *Karenia brevis* isolates, CH2 and SB3, to determine whether *K. brevis* isolates from the GOM growing in nutrient-replete culture media in the laboratory could ingest *Synechococcus*, as had been found in studies with other *K. brevis* isolates (Jeong et al. 2005b, Glibert et al. 2009). Initial prey and predator concentrations in *K. brevis* CH2 incubations were $6.03 \pm 2.12 \times 10^4$ and $2.6 \pm 0.2 \times 10^3$ cells ml⁻¹, respectively. The

Table 1. Average particulate N (PN) and C (PC) concentration (pmol cell⁻¹) in *K. brevis* and *Synechococcus* isolates used for this study. Number of replicates or replicate values (for CH2) are in parentheses.

Isolate	PN	PC	PC:PN (mol:mol)
SB3	39.2 ± 10.7 (5)	411.9 ± 63.9 (5)	10.5
CH2	51.8 (56.9, 46.8)	446.2 (497.9, 394.4)	8.6
JC4	59.6 ± 9.1 (8)	406.6 ± 62.5 (7)	6.8
CCFWC 502	0.17 ± 0.05 (9)	1.04 ± 0.14 (9)	6.1

calculated grazing coefficient for *K. brevis* CH2 over the first 5 hours, where the natural log transformed prey abundance was linear over time, was $0.30 \pm 0.09 \text{ hr}^{-1}$ and was significantly different than zero ($R^2 = 0.77$, $n = 9$, $F = 23.76$, $p = 0.0018$) (Fig. 5A, Table 2). There was significant *Synechococcus* growth in prey control bottles over the incubation period ($R^2 = 0.42$, $n = 24$, $F = 15.91$, $p = 0.001$), therefore prey growth rates were accounted for when calculating grazing coefficients from *K. brevis* plus prey incubation bottles for this experiment. Calculated clearance and ingestion rates were $12.0 \pm 3.5 \times 10^{-5} \text{ ml } K. \text{ brevis}^{-1} \text{ hr}^{-1}$ and $7.2 \pm 3.3 \text{ } Synechococcus \text{ } K. \text{ brevis}^{-1} \text{ hr}^{-1}$ (Table 2), respectively. An N- and C-specific prey assimilation rate was also calculated for *K. brevis* cells growing mixotrophically in treatment bottles using calculated ingestion rates and the cellular N and C content of the *K. brevis* and *Synechococcus* isolate used. I calculated N- and C-specific prey assimilation rates (Equation 4) assuming an assimilation efficiency of *Synechococcus* N and C of 50%, the lower end of the range estimated for a model mixotrophic protist (Flynn & Mitra 2009). This resulted in N- and C-specific assimilation rates for *K. brevis* CH2 of 0.28 d^{-1} and 0.20 d^{-1} , respectively (Table 3).

Nutrient-replete *Karenia brevis* isolate SB3 exhibited a grazing response similar to that of *K. brevis* CH2. Initial prey and predator concentrations in this experiment were $6.03 \pm 2.78 \times 10^4$ and $2.4 \pm 0.3 \times 10^3 \text{ cells ml}^{-1}$, respectively. *Synechococcus* cell concentrations in control bottles exhibited a significant increase over time ($R^2 = 0.42$, $n = 24$, $F = 16.12$, $p = 0.001$) indicating prey growth during the 5-hour incubation period. Accounting for *Synechococcus* growth in prey amended bottles, a grazing coefficient of $0.35 \pm 0.09 \text{ hr}^{-1}$ was calculated over the first 5 hours of the experiment, when the natural

log transformed prey abundance was linear over time, ($R^2 = 0.81$, $n = 9$, $F = 29.88$, $p < 0.0001$) (Fig. 5B). Calculated clearance and ingestion rates were $14.6 \pm 4.3 \times 10^{-5}$ ml *K. brevis*⁻¹ hr⁻¹ and 8.8 ± 4.8 *Synechococcus K. brevis*⁻¹ hr⁻¹ (Table 2). N- and C-specific prey assimilation rates for *K. brevis* SB3 were 0.46 d⁻¹ and 0.27 d⁻¹, assuming 50% assimilation efficiency (Table 3).

Prey cell concentrations were similar at the beginning of each nutrient-replete experiment; therefore, an ANCOVA with time as a covariate was run to compare grazing coefficients for the two *K. brevis* isolates, CH2 and SB3. There was no significant difference ($n = 18$, $F = 0.591$, $p = 0.758$) in grazing coefficients between the two isolates compared in this experiment.

Nutrient-Deplete Grazing. A second set of experiments were conducted using *Karenia brevis* CH2 and SB3 acclimated to nutrient-deplete conditions prior to grazing experiments. These experiments were conducted to determine whether *K. brevis* could graze *Synechococcus* under nutrient limited conditions. These experiments were not directly comparable with nutrient replete experiments described above because *Synechococcus* additions were higher and this could have stimulated grazing. In these experiments, *Synechococcus* and *K. brevis* CH2 concentrations were $2.82 \pm 0.24 \times 10^5$ *Synechococcus* ml⁻¹ and $0.3 \pm 0.06 \times 10^3$ *K. brevis* ml⁻¹, respectively; and *Synechococcus* and *K. brevis* SB3 concentrations were $4.39 \pm 0.15 \times 10^5$ *Synechococcus* ml⁻¹ and $2.3 \pm 0.4 \times 10^3$ *K. brevis* ml⁻¹, respectively. *Synechococcus* abundance in prey control bottles showed no significant change over the entire 24-hour incubation period ($R^2 = 0.22$, $n = 18$, $F = 4.52$, $p = 0.05$ and $R^2 = 0.02$, $n = 18$, $F = 0.36$, $p = 0.56$ for the two experiments, respectively). For *K. brevis* CH2, the grazing coefficient was not significantly different

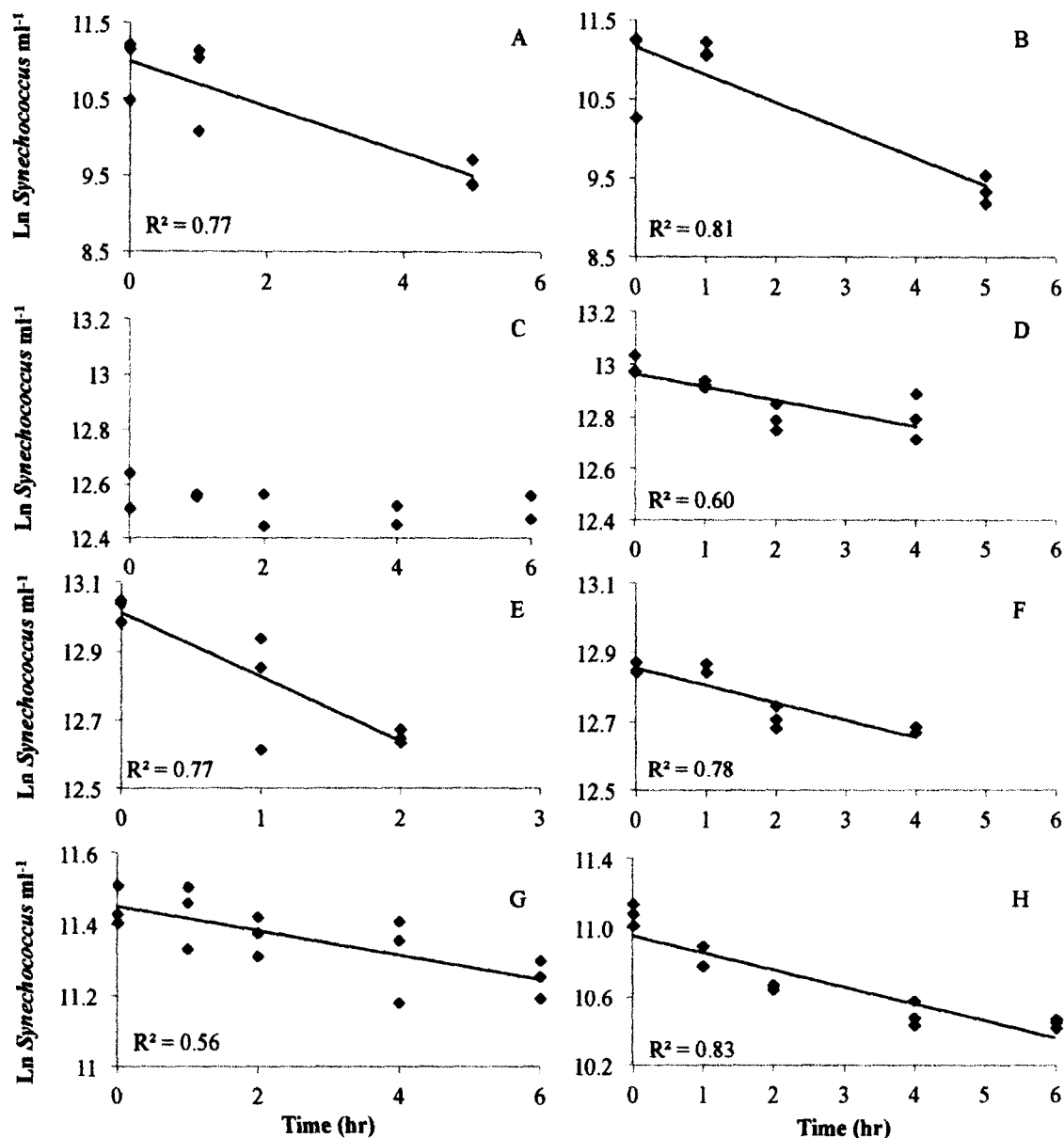


Fig. 5. Live *Synechococcus* (CCFWC 502) abundance in nutrient-replete (A-B), nutrient-deplete (C-D), light (E) and dark (F) bottles, and heat-killed *Synechococcus* (G-H) abundance in prey-amended incubation bottles of *Karenia brevis* isolates CH2 (A, C), SB3 (B, D), and JC4 (E, F). *Synechococcus* abundance (*Synechococcus* ml⁻¹) was natural log transformed and fit with a linear regression using a least squares fit. The slope of the linear regression is equal to the grazing coefficient (hr⁻¹).

Table 2. *K. brevis* grazing coefficients (*g*), clearance rates, and ingestion rates on *Synechococcus* CCFWC 502 in laboratory experiments. Grazing coefficients were calculated from linear regressions using natural log transformed prey cell concentration over time. Clearance and ingestion rates were calculated using Equations 2 and 3. Standard deviations are in parentheses.

Experiment and <i>K. brevis</i> Isolate	Initial <i>Synechococcus</i> concentration (cells ml ⁻¹)	<i>g</i> (hr ⁻¹)	Clearance rate (×10 ⁻⁵ ml <i>K. brevis</i> ⁻¹ hr ⁻¹)	Ingestion rate (<i>Synechococcus K. brevis</i> ⁻¹ hr ⁻¹)	n
Nutrient-replete CH2 (NR1)	6.03 (2.12) ×10 ⁴	0.30* (0.09)	12.0 (3.5)	7.2 (3.3)	9
Nutrient-replete SB3 (NR2)	6.03 (2.78) ×10 ⁴	0.35* (0.09)	14.6 (4.3)	8.8 (4.8)	9
Nutrient-deplete CH2 (ND1)	2.82 (0.24) ×10 ⁵	n.s.	n.s.	n.s.	9
Nutrient-deplete SB3 (ND1)	4.39 (0.15) ×10 ⁵	0.05* (0.03)	2.2 (1.3)	9.7 (5.6)	11
Light JC4	4.53 (0.16) ×10 ⁵	0.19* (0.01)	8.2 (1.0)	37.1 (4.9)	8
Dark CH2	3.82 (0.06) ×10 ⁵	0.06* (0.01)	12.6 (2.3)	48.0 (9.0)	9
Heat-killed prey CH2 (HK1)	9.36 (0.51) ×10 ⁴	0.03* (0.01)	43.0 (19.0)	40.5 (18.1)	15
Heat-killed prey JC4 (HK1)	6.44 (0.42) ×10 ⁴	0.10* (0.005)	63.1 (12.3)	40.7 (8.3)	13
Live v heat-killed prey SB3 (Live)	1.73 (0.11) ×10 ⁵	0.06* (0.04)	14.5 (11.1)	23.4 (18.1)	8
Live v heat-killed prey SB3 (Heat-killed)	2.28 (0.12) ×10 ⁵	0.05* (0.004)	9.5 (5.5)	21.8 (12.6)	9

n.s. - not significantly different than zero

*Significant grazing coefficient (Linear regression Ln prey cell concentration with time $p < 0.05$).

Table 3. N- and C-specific prey assimilation rates for *K. brevis* isolates during laboratory grazing experiments in nutrient-replete and -deplete media, light and dark bottles, and using heat-killed prey. N- and C-specific prey assimilation rates were estimated using *Synechococcus* and *K. brevis* N and C cell content, calculated ingestion rates and assuming a 50% assimilation efficiency (0.5 AE Equation 4) (Flynn and Mitra 2009). Standard deviations are in parentheses.

Experiment and <i>K. brevis</i> Isolate	Calculated <i>K. brevis</i> specific assimilation rate (d ⁻¹)	
	N (0.5 AE)	C (0.5 AE)
Nutrient-replete CH2 (NR1)	0.28 (0.17)	0.20 (0.10)
Nutrient-replete SB3 (NR2)	0.46 (0.30)	0.27 (0.15)
Nutrient- deplete CH2 (ND1)	0.00 (0.00)	0.00 (0.00)
Nutrient-deplete SB3 (ND2)	0.50 (0.35)	0.29 (0.18)
Light JC4	1.27 (0.42)	1.13 (0.27)
Dark CH2	1.89 (0.79)	1.34 (0.34)
Heat-killed prey CH2 (HK1)	1.70 (0.93)	1.13 (0.54)
Heat-killed prey JC4 (HK2)	1.39 (0.51)	1.25 (0.36)
Live v heat-killed prey SB3 (Live)	1.22 (1.30)	0.71 (0.72)
Live v heat-killed prey SB3 (Heat-killed)	1.13 (0.78)	0.66 (0.40)

from zero ($R^2 = 0.26$, $n = 9$, $F = 2.44$, $p = 0.16$) (Fig. 5C), thus clearance and ingestion rates (Table 2), as well as N- and C-specific assimilation rates (Table 3) were not calculated. For *Karenia brevis* SB3, grazing coefficients were calculated over the first 4 hours (Fig. 5d) of the incubations and were $0.05 \pm 0.02 \text{ hr}^{-1}$ ($R^2 = 0.60$, $n = 11$, $F = 10.81$, $p = 0.009$). Calculated clearance and ingestion rates were $2.2 \pm 1.3 \times 10^{-5} \text{ ml } K. brevis^{-1} \text{ hr}^{-1}$ and $9.7 \pm 5.6 \text{ Synechococcus } K. brevis^{-1} \text{ hr}^{-1}$ (Table 2). N- and C-specific

assimilation rates for SB3 in treatments bottles were 0.50 d^{-1} and 0.29 d^{-1} , assuming 50% and assimilation efficiency, respectively (Table 3).

Light and Dark Grazing. Light and dark bottle incubations were conducted to determine whether ingestion by *Karenia brevis* on *Synechococcus* occurred under light and dark conditions. For these experiments, *Synechococcus* and *K. brevis* JC4 concentrations in light bottles amended with prey were $4.53 \pm 0.16 \times 10^5 \text{ Synechococcus ml}^{-1}$ and $2.2 \pm 0.3 \times 10^3 \text{ K. brevis ml}^{-1}$, respectively, in light bottles. No change in *Synechococcus* cell concentration was observed in prey control bottles over the 24-hour incubation period ($R^2 = 0.08$, $n = 18$, $F = 1.38$, $p = 0.26$). In this experiment grazing was significant ($R^2 = 0.77$, $n = 8$, $F = 15.37$, $p = 0.008$) and average grazing coefficients over the first 4 hours of incubation were $0.19 \pm 0.01 \text{ hr}^{-1}$ (Fig. 5E). Calculated clearance and ingestion rates were $8.2 \pm 1.0 \times 10^{-5} \text{ ml K. brevis}^{-1} \text{ hr}^{-1}$ and $37.1 \pm 4.9 \text{ Synechococcus K. brevis}^{-1} \text{ hr}^{-1}$ (Table 2), respectively. N- and C-specific assimilation rates for JC4 in treatments bottles were 1.27 d^{-1} and 1.13 d^{-1} , assuming 50% assimilation efficiency, respectively (Table 3). In dark bottles, *Synechococcus* and *K. brevis* CH2 concentrations were $3.82 \pm 0.06 \times 10^5 \text{ cells ml}^{-1}$ and $0.4 \pm 0.04 \times 10^3 \text{ cells ml}^{-1}$, respectively. As for light bottles, there was no *Synechococcus* growth in dark prey control bottles ($R^2 < 0.01$, $n = 18$, $F = 0.005$, $p = 0.94$) over the course of the 24-hour grazing experiment. Grazing was also significant in dark bottles ($R^2 = 0.78$, $n = 9$, $F = 20.0$, $p = 0.003$) and the calculated grazing coefficient over the first 4 hours of the experiment was $0.06 \pm 0.01 \text{ hr}^{-1}$ (Fig. 5F). Calculated clearance and ingestion rates were $12.6 \pm 2.3 \times 10^{-5} \text{ ml K. brevis}^{-1} \text{ hr}^{-1}$ and $48.0 \pm 8.9 \text{ Synechococcus K. brevis}^{-1} \text{ hr}^{-1}$ (Table 2), respectively. N- and C-specific

Table 4. Comparison of grazing coefficients (g , hr^{-1}), clearance rates (CR , $\times 10^{-5} \text{ ml } K. \text{ brevis}^{-1} \text{ hr}^{-1}$), and ingestion rates (IR , $\text{Syn } K. \text{ brevis}^{-1} \text{ hr}^{-1}$) by *Karenia brevis* (JC4) on heat-killed *Synechococcus* in 4-hour grazing experiments that were initiated 2 hours after incubation lights turned on (day) and 2 hours after incubation light turned off (night) and by nutrient-replete and -deplete *K. brevis* (SB3). Initial *Synechococcus* ($\times 10^5 \text{ cells ml}^{-1}$) and *K. brevis* ($\times 10^3 \text{ cells ml}^{-1}$) cell concentrations were enumerated using FCM. *K. brevis* N- and C-specific assimilation rates were estimated using *Synechococcus* and *K. brevis* N and C cell content, calculated ingestion rates, and assuming a 50% assimilation efficiency (Flynn and Mitra 2009). Standard deviations from replicate bottles are in parentheses ($n = 6$).

	Initial <i>Synechococcus</i>	Initial <i>K.</i> <i>brevis</i>	<i>K. brevis</i> N- & C-specific assimilation rate (d^{-1})		g	CR	IR
			N (0.5 AE)	C (0.5 AE)			
Day	90.0 (5.9)	2.1 (0.08)	0.14 (0.07)	0.09 (0.04)	0.06 (0.02)	3.0 (1.1)	2.7 (1.0)
Night	90.4 (5.9)	2.2 (0.08)	0.24 (0.13)	0.15 (0.07)	0.11 (0.04)	5.0 (2.1)	4.6 (1.9)
Nut-R	161.6 (3.5)	1.2 (0.2)	0.32 (0.19)	0.07 (0.09)	0.05 (0.02)	3.9 (1.7)	6.4 (2.8)
Nut-D	173.4 (10.5)	1.2 (0.1)	0.32 (0.39)	0.12 (0.22)	0.05 (0.03)	4.3 (2.3)	7.2 (3.9)

assimilation rates for CH2 in treatments bottles were 1.89 d^{-1} and 1.34 d^{-1} , assuming 50% assimilation efficiency, respectively (Table 3). The light and dark grazing experiments were not directly comparable because different *K. brevis* isolates and *Synechococcus* additions were used, both of which may influence grazing.

Karenia brevis Functional Response to Varying Prey Density. When *K. brevis* (JC4) cultures ($0.33 \pm 0.03 \times 10^3 \text{ K. brevis ml}^{-1}$) were amended with varying concentrations of *Synechococcus* as prey (range of 3.89×10^4 to $4.19 \times 10^5 \text{ Synechococcus ml}^{-1}$), grazing coefficients were significantly different between the 5 prey concentration treatments ($n = 15$, $F = 28.64$, $p < 0.0001$). *K. brevis* ingestion rates increased with increasing prey density until reaching a maximum ingestion rate of $27 \text{ Synechococcus K. brevis}^{-1} \text{ hr}^{-1}$ (Equation 6). I calculated a lower feeding threshold at which *K. brevis* is able to ingest *Synechococcus* of $1.86 \times 10^4 \text{ Synechococcus ml}^{-1}$ by extrapolating the Ivlev curve to zero (Fig. 6). In this study, *Synechococcus* prey additions were consistently above the lower feeding threshold for grazing calculated for *K. brevis*.

Grazing on Heat-killed Synechococcus. During the first experiment using heat-killed *Synechococcus*, *Synechococcus* concentrations were $9.36 \pm 0.51 \times 10^4 \text{ cells ml}^{-1}$ and *Karenia brevis* (CH2) concentrations were $0.08 \pm 0.03 \times 10^3 \text{ cells ml}^{-1}$. Grazing on heat-killed *Synechococcus* was significant ($R^2 = 0.56$, $n = 15$, $F = 12.34$, $p = 0.004$) and the grazing coefficient was $0.03 \pm 0.01 \text{ hr}^{-1}$ (Fig. 5G). *Synechococcus* cell abundance in control bottles showed no significant change over time ($R^2 < 0.01$, $n = 18$, $F = 0.001$, $p = 0.97$), which was expected because *Synechococcus* cells were heat-killed. Calculated clearance and ingestion rates were $43.0 \pm 19.0 \times 10^{-5} \text{ ml K. brevis}^{-1} \text{ hr}^{-1}$ and 40.5 ± 18.1

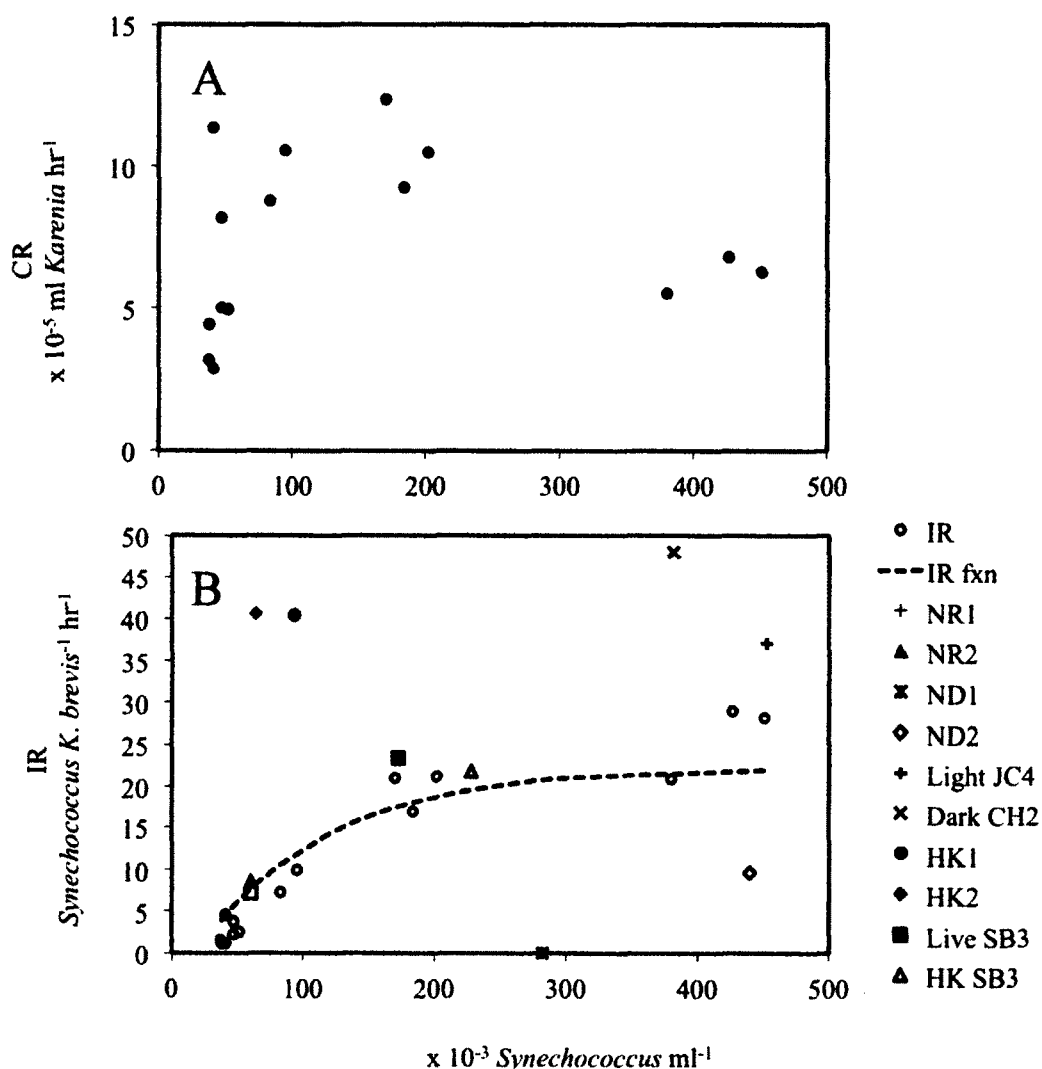


Fig. 6. *K. brevis* (JC4) clearance rates (A) and ingestion rates (B) as a function of *Synechococcus* prey concentration ($\times 10^3 \text{ cells ml}^{-1}$) during 4-hour nutrient-replete incubation experiments. Ingestion rate data (open circles) were fit using a modified Ivlev curve (Ivlev 1955) and is $\text{IR} = 27 * (0.83 - e^{(-0.00001 * \text{Prey})})$, $R^2 = 0.88$ (dashed line) to determine a lower feeding threshold for *K. brevis* feeding on *Synechococcus*, which was $18.6 \times 10^3 \text{ Synechococcus cells ml}^{-1}$. *K. brevis* ingestion rates from Table 2 are plotted to compare nutrient-replete (NR1, NR2), -deplete (ND1, ND2), light (JC4), dark (CH2), heat-killed (HK1, HK2), and live (Live SB3) versus heat-killed (HK SB3) responses to *Synechococcus* concentrations.

Synechococcus K. brevis⁻¹ hr⁻¹ (Table 2), respectively. N- and C-specific assimilation rates for CH2 in bottles amended with prey were 1.70 d⁻¹ and 1.13 d⁻¹, assuming a 50% assimilation efficiency, respectively (Table 3).

During the second experiment using heat-killed *Synechococcus* and *Karenia brevis* JC4, concentrations of heat-killed *Synechococcus* cells were $6.44 \pm 0.42 \times 10^4$ *Synechococcus* ml⁻¹ and *K. brevis* JC4 concentrations were $0.2 \pm 0.03 \times 10^3$ *K. brevis* ml⁻¹. *Synechococcus* in prey control bottles did not grow ($R^2 = 0.02$, $n = 18$, $F = 0.40$, $p = 0.54$) because they were dead. Grazing was significant ($R^2 = 0.83$, $n = 13$, $F = 61.53$, $p < 0.001$) and grazing coefficients were 0.1 ± 0.005 hr⁻¹ (Fig. 5H). Calculated clearance and ingestion rates were $63.1 \pm 12.3 \times 10^{-5}$ ml *K. brevis*⁻¹ hr⁻¹ and 40.7 ± 8.3 *Synechococcus K. brevis*⁻¹ hr⁻¹ (Table 2), respectively. N- and C-specific assimilation rates for JC4 in bottles amended with prey were 1.39 d⁻¹ and 1.25 d⁻¹, assuming 50% assimilation efficiency, respectively (Table 3).

Heat-killed Versus Live Grazing. In experiments to determine whether ingestion rates were comparable on live versus heat-killed prey, *Synechococcus* cell concentrations in live and heat-killed treatments were 1.73 ± 0.11 and $2.28 \pm 0.12 \times 10^5$ cells ml⁻¹, respectively. Average *Karenia brevis* SB3 cell concentrations were $0.5 \pm 0.1 \times 10^3$ cells ml⁻¹. *K. brevis* grazing coefficients on live *Synechococcus* cells were 0.06 ± 0.04 h⁻¹ versus 0.05 ± 0.004 h⁻¹ on heat-killed *Synechococcus* (Fig. 7); and were not significantly different (ANCOVA; $n = 17$, $F = 0.002$, $p = 0.97$). Calculated clearance and ingestion rates, for live *Synechococcus* treatments were $14.5 \pm 11.1 \times 10^{-5}$ ml *K. brevis*⁻¹ hr⁻¹ and 23.4 ± 18.5 *Synechococcus K. brevis*⁻¹ hr⁻¹, respectively (Table 2). For heat-killed *Synechococcus* treatments, clearance rates were $9.5 \pm 5.5 \times 10^{-5}$ ml *K. brevis*⁻¹ hr⁻¹ and

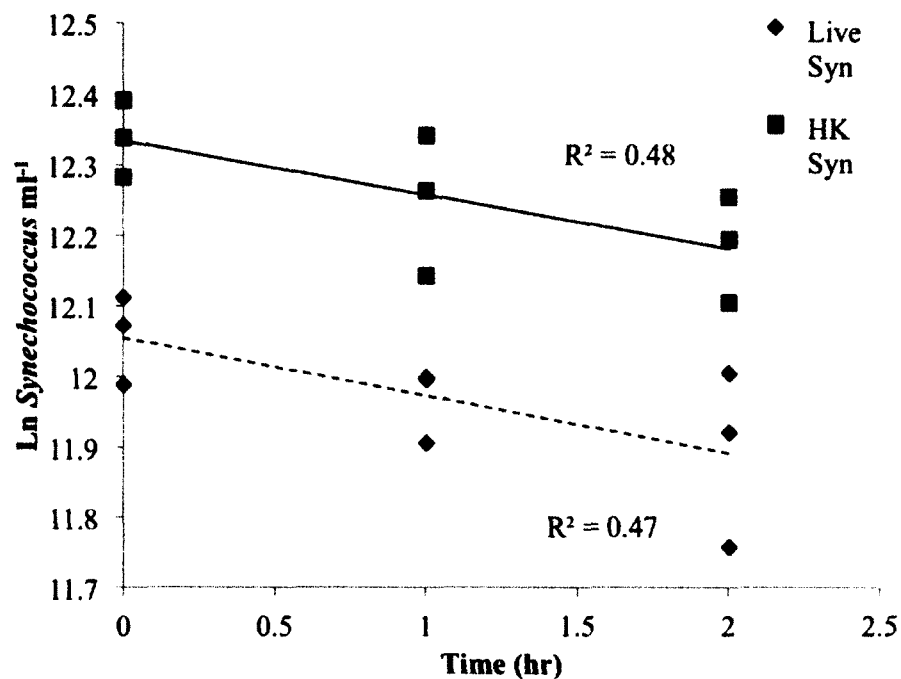


Fig. 7. Disappearance of live (diamonds) and heat-killed (HK, squares)

Synechococcus (Syn) over a 2-hour incubation period. *Karenia brevis* isolate SB3 was used in both sets of triplicate incubation bottles. Grazing was significant in both treatments and the slopes, when compared using an ANCOVA with time as a covariate at a significance level of $p < 0.05$, were not statistically different ($p = 0.97$).

ingestion rates were 21.8 ± 12.6 *Synechococcus K. brevis*⁻¹ hr⁻¹ (Table 2). N-specific assimilation rates for live and heat-killed *Synechococcus* treatments bottles were 1.22 d⁻¹ and 1.13 d⁻¹, respectively, and C-specific assimilation rates for live and heat-killed *Synechococcus* were 0.66 d⁻¹ and 0.71 d⁻¹, respectively, using a 50% assimilation efficiency (Table 3).

The Effect of Nutrients on Ingestion Rates. The effect of nutrients on ingestion rates was measured directly in a third set of experiments using *Karenia brevis* SB3 and constant prey amendments. For these experiments, *Synechococcus* and *K. brevis* cell concentrations were $1.62 \pm 0.04 \times 10^5$ and $1.2 \pm 0.2 \times 10^3$ cells ml⁻¹, respectively, in nutrient-replete prey amended bottles; and $1.73 \pm 0.11 \times 10^5$ and $1.2 \pm 0.1 \times 10^3$ cells ml⁻¹, respectively in nutrient-deplete prey amended bottles. *K. brevis* grazing coefficients on heat-killed *Synechococcus* were 0.05 ± 0.02 and 0.05 ± 0.03 hr⁻¹ during nutrient-replete and -deplete incubations, respectively (Table 4). Based on the results of the ANOVA test, grazing coefficients in nutrient-replete and -deplete treatments were not significantly different from each other ($n = 12$, $F = 0.165$, $p = 0.69$).

The Effect of Light on Ingestion Rates. To compare daytime and nighttime ingestion rates directly, heat-killed *Synechococcus* was supplied at a constant amount in light and dark incubations. *Synechococcus* and *Karenia brevis* JC4 cell concentrations were 90.0 ± 5.9 and $2.1 \pm 0.1 \times 10^3$ cells ml⁻¹, respectively, in day (2 hours after incubation lights turned on) bottles amended with prey; and $9.04 \pm 0.59 \times 10^4$ and $2.2 \pm 0.1 \times 10^3$ cells ml⁻¹, respectively in night incubations (2 hours after lights were turned off). *K. brevis* grazing coefficients on heat-killed *Synechococcus* were 0.06 ± 0.02 and 0.11 ± 0.04 hr⁻¹ during day and night incubations, respectively (Table 4). Based on the

results of the 2-way ANOVA, grazing coefficients in day and night treatments were not significantly different from each other ($n = 12$, $F = 4.406$, $p = 0.069$). Grazing coefficients from nutrient treatments were pooled within day and night treatments. There was no significant difference between grazing coefficients between nutrient-replete and -deplete pooled data using a 2-way ANOVA ($n = 12$, $F = 0.585$, $p = 0.466$).

Grazing on Other Phytoplankton. Grazing was not detected in nutrient-replete *Karenia brevis* (SB3) cultures amended with the diazotrophic cyanobacterium *Crocospaera watsonii*, the cyanobacterium *Synechocystis* sp., or the haptophyte *Isochrysis* sp. at densities of 3.3 ± 0.6 , 4.2 ± 0.1 , and $4.1 \pm 1.1 \times 10^3$ cells ml^{-1} , respectively (Fig. 8A-C). Using an ANCOVA with time as a covariate (time*treatment), natural log transformed prey concentrations in prey control bottles were compared to determine if there was growth in prey control bottles. *C. watsonii* growth in prey amended and prey control bottles were statistically insignificant ($n = 18$, $F = 1.92$, $p = 0.19$). Growth in *Isochrysis* sp. prey amended and prey control bottles were also statistically insignificant ($n = 24$, $F = 0.10$, $p = 0.958$). Finally, growth in *Synechocystis* sp. prey amended and prey control bottles were insignificant ($n = 24$, $F = 1.2$, $p = 0.34$). Prey inclusions for these three organisms were not observed in *K. brevis* using EM/CLSM.

Karenia brevis CH2 did graze on *Prochlorococcus marinus* (CCMP 1986). The average observed grazing coefficients for *P. marinus* were $0.27 \pm 0.02 \text{ hr}^{-1}$ (Fig. 8D) and were significantly different than zero ($R^2 = 0.81$, $n = 9$, $F = 23.48$, $p = 0.003$). For this experiment *P. marinus* were provided as prey at concentrations of $6.87 \pm 0.65 \times 10^4$ cells ml^{-1} and the initial *K. brevis* CH2 concentrations were $0.7 \pm 0.2 \times 10^3$ cells ml^{-1} . *P.*

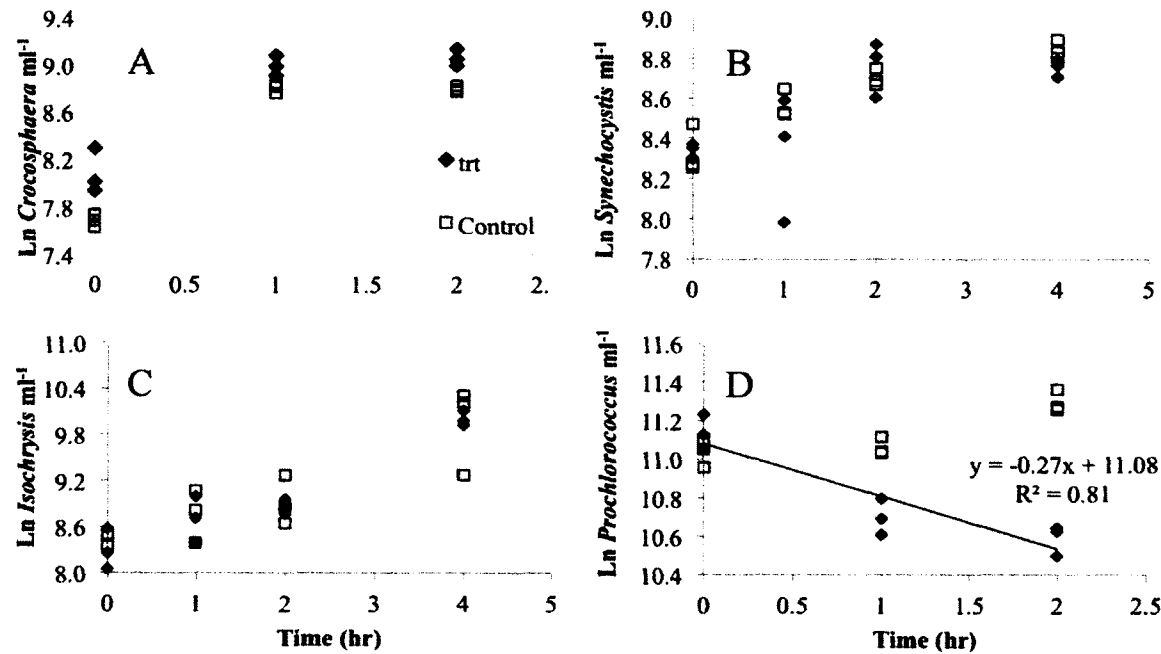


Fig. 8. Cell abundance (ln transformed) of four species offered to *K. brevis* as prey over 2-4 hour incubation periods. *K. brevis* grazing coefficients were below the level of detection for *Crocosphaera* (A), *Synechocystis* (B), and *Isochrysis* (C) at the concentrations used in each experiment. In each of these experiments an ANCOVA was run to determine if growth was significantly different in prey-amended (filled diamonds) bottles and control (open squares) bottles with no *K. brevis*. No prey removal was detected in bottles amended with *Crocosphaera* (A), *Synechocystis* (B), and *Isochrysis* (C). *Prochlorococcus* (D) removal (ln transformed cell abundance) by *K. brevis* was significant over time.

Table 5. Dilution experiments (5 day incubations) using whole culture water from two *K. brevis* isolates, CH2 and SB3.

Initial cell concentrations reported here are from triplicate bottles from the 100% whole water treatment. Bacteria growth rates reported here were estimated by extrapolating the regression to the y-intercept. Linear regressions for each experiment can be found in Fig. 9. Standard deviations from triplicate bottles are in parentheses.

Experiment	<i>K. brevis</i> Isolate	Initial <i>K. brevis</i> (cells ml ⁻¹)	Initial bacteria concentration (cells ml ⁻¹)	Bacteria growth rate (d ⁻¹)	<i>g</i> (hr ⁻¹)	Clearance rate (×10 ⁻⁵ ml <i>K. brevis</i> ⁻¹ hr ⁻¹)	Ingestion rate (bacteria <i>K. brevis</i> ⁻¹ hr ⁻¹)
I	CH2	1.5 (0.4) ×10 ³	6.17 (1.14) ×10 ⁴	0.11	0.003* (0.002)	0.2 (0.1)	0.1 (0.1)
II		0.4 (0.1) ×10 ³	6.28 (0.38) ×10 ⁴	0.14	0.004* (0.002)	1.6 (1.1)	1.0 (0.7)
III		1.3 (0.3) ×10 ³	1.30 (0.11) ×10 ⁶	0.09	0.002* (0.001)	0.2 (0.1)	2.5 (1.4)
IV	SB3	0.7 (0.1) ×10 ³	3.10 (0.28) ×10 ⁵	0.13	0.004* (0.003)	1.0 (0.7)	3.1 (2.1)

*Significant grazing coefficient

(Linear regression, $p < 0.05$).

marinus abundance in control bottles increased significantly over time ($R^2 = 0.45$, $n = 18$, $F = 12.841$, $p = 0.002$) and this growth was accounted for when calculating grazing coefficients (Frost 1972). Calculated clearance and ingestion rates for *K. brevis* grazing on *Prochlorococcus* were $39.6 \pm 11.5 \times 10^{-5} \text{ ml } K. \text{ brevis}^{-1} \text{ hr}^{-1}$ and $27.3 \pm 8.3 \text{ Synechococcus } K. \text{ brevis}^{-1} \text{ hr}^{-1}$, respectively.

Grazing on Bacteria. Heterotrophic bacteria, in *Karenia brevis* culture bottles were first observed using EM and when examining cultures for bacteria using flow cytometry and SYTO 13. Four dilution experiments were conducted using two culture isolates of *K. brevis* and the heterotrophic bacteria that co-occurred in the culture bottles. Dilution experiments I, II, and III measured grazing on bacteria by *K. brevis* CH2 and dilution experiment IV measured bacterial grazing by *K. brevis* SB3 (Table 5). Grazing coefficients were 0.003 ± 0.002 , 0.004 ± 0.002 , 0.002 ± 0.001 , and $0.004 \pm 0.003 \text{ hr}^{-1}$ for experiments I, II, III, and IV (Fig. 9, Table 5), respectively. The intrinsic growth rates of the heterotrophic bacteria were also estimated for each experiment using the method of Landry & Hassett (1982) and were 0.11, 0.14, 0.09, and 0.13 d^{-1} for dilution experiments I, II, III, and IV (Table 5), respectively. Grazing coefficients for dilution experiments I ($R^2 = 0.60$, $n = 9$, $F = 10.34$, $p = 0.015$), II ($R^2 = 0.49$, $n = 10$, $F = 13.85$, $p = 0.006$), III ($R^2 = 0.63$, $n = 12$, $F = 9.54$, $p = 0.011$), and IV ($R^2 = 0.37$, $n = 11$, $F = 5.98$, $p = 0.035$) were significantly different than zero over the dilution series (Fig. 9). Clearance rates and ingestion rates from all four dilution experiments were plotted against bacterial cell concentration to determine the functional response of *K. brevis* grazing on heterotrophic bacteria (Fig. 10). *K. brevis* ingestion rates of heterotrophic bacteria were fitted to a modified Ivlev curve (Equation 6).

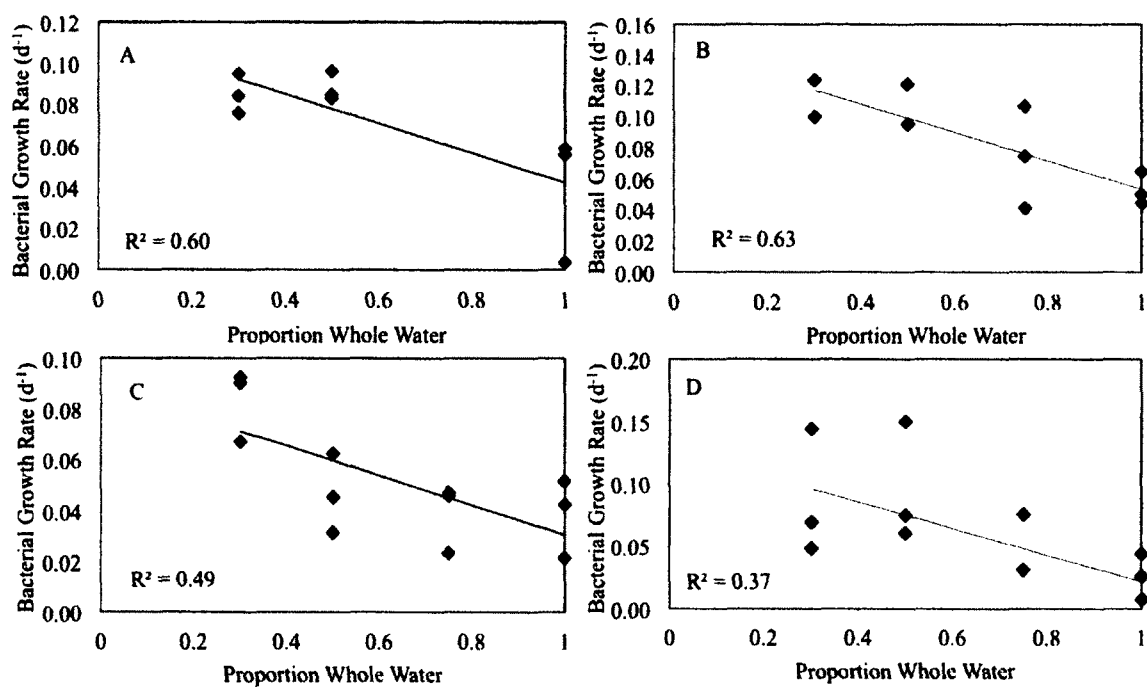


Fig. 9. Four dilution experiments ($t = 5$ days) using whole *K. brevis* culture water amended with f/2 media. *K. brevis* isolate CH2 was used in experiments I-III (A-C) and SB3 in experiment IV (D). Data fitted with a linear regression are significant over the dilution series.

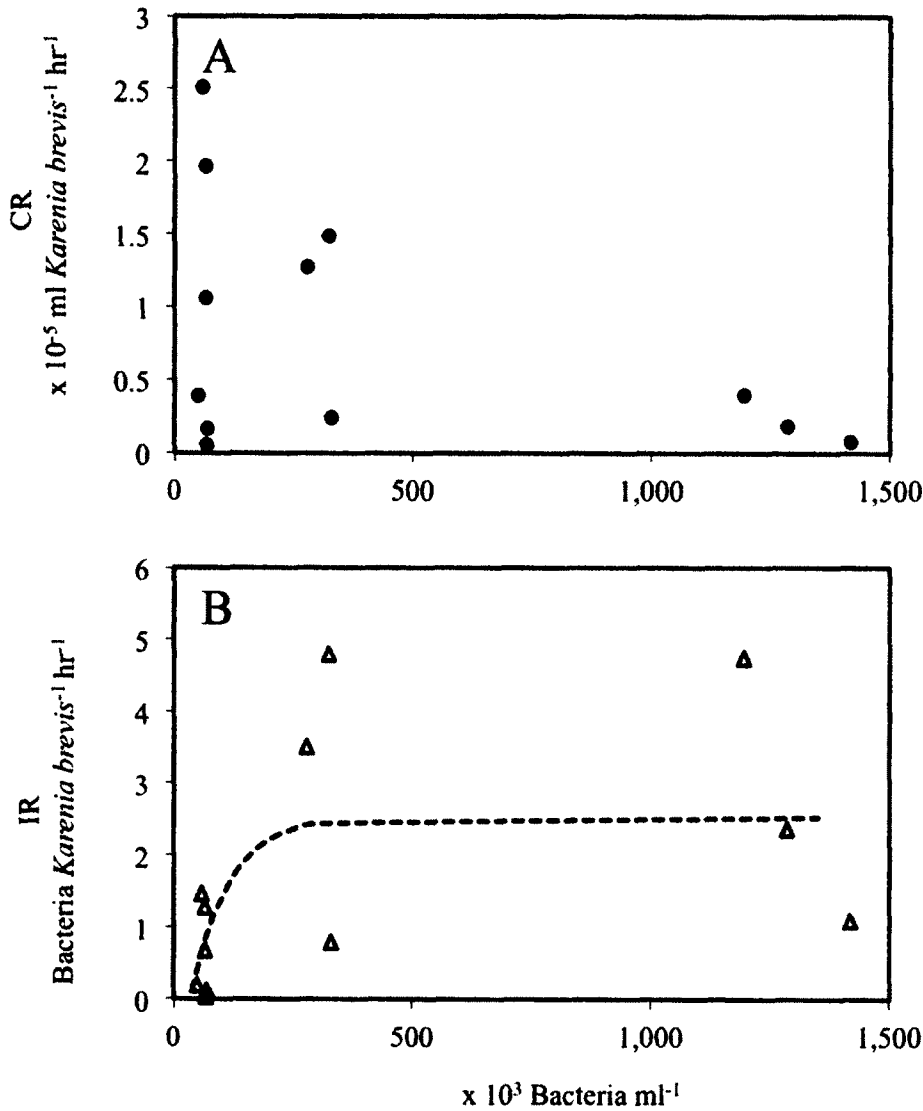


Fig. 10. *K. brevis* clearance rates (A) and ingestion rates (B) rates as a function of heterotrophic bacteria prey concentration ($\times 10^3 \text{ cells ml}^{-1}$) from four dilution experiments. The dashed line is a modified Ivlev curve (Ivlev 1955) fit to the data and is $IR = 3.9 * (0.65 - e^{(-0.0000126 * \text{Bacteria})})$, $R^2 = 0.43$ (dashed line). Based on this Ivlev curve, the lower feeding threshold of *K. brevis* on co-occurring heterotrophic bacteria cells was $34.2 \times 10^3 \text{ cells ml}^{-1}$.

Discussion

Many dinoflagellates are mixotrophic (Jeong et al. 2005a, b) and the nutritional benefits they gain from this capability may be fundamental to their success in the environment (Burkholder et al. 2008). Augmenting nutrient uptake through mixotrophic grazing on picoplankton and heterotrophic bacteria may enable mixotrophic phytoplankton to out-compete co-occurring phytoplankton that are strictly autotrophic and “bloom” in the environment. In a broad culture survey, Jeong et al. (2005b) found that 18 species of red tide forming dinoflagellates, including some not previously thought to be mixotrophic (Legrand et al. 1998) such as *Karenia brevis*, were able to ingest *Synechococcus* and concluded that dinoflagellates may rival nanoflagellates as important grazers on this ubiquitous group of cyanobacteria.

In the current study, I demonstrate that *Karenia brevis* isolates (CH2, SB3, and JC4) from the WFS in the GOM also graze on *Synechococcus*. *K. brevis* isolates SB3 and CH2 had statistically similar *Synechococcus* ingestion rates when growing in nutrient-replete media under photic conditions (Table 2, Fig. 5A, B). In experiments using live prey cells supplied at different concentrations, ingestion rates by the three *K. brevis* isolates ranged from 7.2 to 48.0 *Synechococcus K. brevis*⁻¹ hr⁻¹. This agrees well with previously reported ingestion rates for other *K. brevis* isolates; 5.0 *Synechococcus K. brevis*⁻¹ hr⁻¹ (unidentified CCMP isolates, Jeong et al. 2005b) and 0.96 to 83.8 *Synechococcus K. brevis*⁻¹ hr⁻¹ (CCMP 2228 and 2229, Glibert et al. 2009). Ingestion rates by WFS *K. brevis* isolates can be estimated using heat-killed *Synechococcus*, which is important as it allowed me to provide constant prey density in incubation experiments

so I could directly compare ingestion rates in nutrient-replete and -deplete *K. brevis* cultures and in day and night incubations.

N and P limitation have been shown to stimulate mixotrophic grazing by other dinoflagellate species (Li et al. 1999, 2000; Smalley et al. 2003). In one study, N limitation was shown to stimulate mixotrophy in *Poterioochromonas malhamensis*, but the effect varied with light level (Caron et al. 1990). In the current study, grazing was not enhanced under nutrient-limited growth; rather ingestion rates by cells acclimated to a nutrient-deplete media were similar to those grown in a nutrient replete media (Table 4). These results suggest that nutrient limitation may not play a role in controlling grazing by *Karenia brevis*. Thus, grazing by *K. brevis* in the GOM could potentially occur at similar rates in eutrophic waters in Tampa Bay as well as in the oligotrophic water column on the edge of the WFS where *K. brevis* blooms occur. The ability to graze at similar rates in these contrasting environments would allow *K. brevis* to continue to grow in the presence of other phytoplankton competing for scarce nutrient resources.

Light availability has also been shown to stimulate or inhibit grazing (Burkholder et al. 2008, Smalley et al. 2003) and digestion rates have been shown to be light dependent for herbivorous protists (Strom 2001). Legrand et al. (1998) found that there was no difference in ingestion rates by *Heterocapsa triquetra* on fluorescently labeled algae (FLA) during light and dark incubations. However, others have observed that certain species of dinoflagellates preferentially graze on picoplankton under low light (Sanders et al. 1990, Glibert et al. 2009). Results presented here demonstrate *Karenia brevis* has similar ingestion rates when growing mixotrophically under photic conditions during the day and at night in the dark (Table 4). Although grazing by *K. brevis* on

Synechococcus was not examined under a range of light levels, grazing in the absence of light may allow *K. brevis* 'seed' populations to remain viable below the euphotic zone and allow populations to continue to thrive when cell densities are high and self-shading can limit photosynthetic C acquisition.

Prey removal was linear, using a natural log scale, only during the first 2 to 6 hours of incubation experiments (Fig. 5), after which prey removal rates declined. This was likely due to of *Karenia brevis* grazing reaching digestion rates that were similar to ingestion rates (Li et al. 1999) or a decrease in encounter rates between predator and prey, the latter is unlikely because prey removal never resulted in depletion of prey in prey-amended incubation bottles below the lower feeding threshold (1.86×10^4 *Synechococcus* ml⁻¹). Ingestion rates can also decrease over time if digestion rates are slow (Bockstahler & Coats 1993b, Smalley & Coats 2002). Previous studies examining grazing by *K. brevis* have employed incubations of varying length; from minutes (Jeong et al. 2005b) to days (Glibert et al. 2009) (Table 6). I found that ingestion rates were saturated after 4-6 hours and so short incubation times (on the order of hours) were more appropriate for grazing experiments examining grazing by *K. brevis* on *Synechococcus*.

Similarly, *Karenia brevis* ingestion rates are also sensitive to prey density. In a functional response experiment with *K. brevis* populations of 0.33×10^3 *K. brevis* ml⁻¹, ingestion rates increased as *Synechococcus* cell concentrations increased until saturation around $\sim 1.95 \times 10^6$ *Synechococcus* ml⁻¹ (Fig. 6). I calculated a lower feeding threshold for *K. brevis* feeding on *Synechococcus* of 1.86×10^4 *Synechococcus* ml⁻¹, based on a modified Ivlev fit to functional response data (Fig. 6). In every grazing

Table 6. Ingestion rates of *Synechococcus* by multiple *K. brevis* isolates in laboratory experiments employing different incubation times.

<i>K. brevis</i> Isolate	Prey Isolate	Grazing Occurred	Incubation Time	Ingestion rate (Prey <i>K. brevis</i> ⁻¹ hr ⁻¹)	Reference ₀
Unidentified CCMP strain	<i>Synechococcus</i> - Genbank Accession Number DQ023295	Yes	Minutes	5.0	Jeong et al. 2005b
CCMP 2229	<i>Synechococcus</i> - CCMP 1768	Yes	Days	0.96 - 83.8	Glibert et al. 2009
CCMP 2228	<i>Synechococcus</i> - CCMP 1768	Yes	Days	1.04 - 79.3	
CH2	<i>Synechococcus</i> - CCFWC 502	Yes	Hours	7.2 - 48.0	This study
	<i>Synechococcus</i> - Heat-killed CCFWC 502	Yes	Hours	40.5	
	<i>Prochlorococcus</i> CCMP 1986	Yes	Hours	27.3	
	Heterotrophic bacteria	Yes	Days	0.1 - 2.5	
	<i>Synechococcus</i> - CCFWC 502	Yes	Hours	37.1	
JC4	<i>Synechococcus</i> - Heat-killed CCFWC 502	Yes	Hours	40.7	
SB3	<i>Synechococcus</i> - CCFWC 502	Yes	Hours	8.8 - 9.7	
	<i>Synechocystis</i> - CCFWC 493	No	Hours	B.L.D.	
	<i>Isochrysis</i> - CCFWC 363	No	Hours	B.L.D.	
	<i>Crocospaera</i> <i>watsonii</i> -WH8501	No	Hours	B.L.D.	
	Heterotrophic bacteria	Yes	Days	3.1	

experiment, where *Synechococcus* was offered as prey, prey abundance was above the lower feeding threshold and never fell below the lower feeding threshold during the incubation period. In the Gulf of Mexico, *Synechococcus* abundance is present at approximately 10^4 *Synechococcus* ml^{-1} (Paul et al. 2000), similar to the feeding threshold found in laboratory experiments here. Using the ingestion and assimilation rates reported here, this would support N- and C-specific turnover times of 0.05 to 1.89 and 0.04 to 1.34 d^{-1} , respectively, for a *K. brevis* population with a cell density of 10^6 cells l^{-1} (Table 3). These N- and C-specific turnover times would support cellular doubling times of about 0.37 to 15.07 and 0.52 to 16.85 days, respectively, consistent with growth rates of *K. brevis* observed previously (Redalje et al. 2008, Sipler et al. In Revision).

Growth rates for *Karenia brevis* on the WFS have been shown to range from <0.1 to $> 1.0 \text{ d}^{-1}$ (Redalje et al. 2008, Sipler et al. In Revision). At a modest growth rate of 0.2 d^{-1} (0.29 divisions d^{-1}), I calculate that *K. brevis* would need to ingest approximately 1.2 *Synechococcus* hr^{-1} to support their growth strictly through ingestion of prey. This ingestion rate would increase to about 4.3 *Synechococcus* hr^{-1} at a growth rate of 0.7 d^{-1} (1.0 divisions d^{-1}). These estimates of ingestion rates calculated here fall within the range of the ingestion rates observed in this study. However, this calculation does not take in to account loss terms related to assimilation efficiencies for *K. brevis* grazing on prey (i.e. respiration). Thus, grazing by *K. brevis* on *Synechococcus* on the WFS can theoretically support high growth rates and/or maintenance of high cell densities, similar to those observed annually during blooms.

Mixotrophic ingestion of prey cells has been shown to increase growth rates of many phagotrophic (Stoecker et al. 2006) and mixotrophic (Li et al. 1999, Jeong et al.

2004, Adolf et al. 2006) dinoflagellates, including *K. brevis* (Glibert et al. 2009).

However, some heterotrophic nanoflagellates have been shown to ingest *Synechococcus* but not digest it (Boenigk et al. 2001). While Jeong et al. (2005b) first observed that *K. brevis* were capable of ingesting prey, Glibert et al. (2009) were the first to suggest that *K. brevis* growth was enhanced by the addition of *Synechococcus* prey under laboratory conditions. In the latter study, the direct transfer of *Synechococcus* cell N to *K. brevis* was observed. The N- and C-specific assimilation rates calculated here (Tables 3 and 4) suggests that grazing can potentially contribute substantially to *K. brevis* growth. Further, growth rate estimates for *K. brevis* that are based on photosynthesis alone may underestimate population growth if phagotrophic ingestion of picocyanobacteria contributes significantly to population growth in the environment.

In my direct comparison between nutrient-deplete and nutrient-replete *Karenia brevis*, grazing was not enhanced in nutrient-deplete cultures; rather ingestion rates by cells acclimated to a nutrient-deplete medium were similar to those grown in a nutrient replete medium (Table 4). These results suggest that nutrient concentrations may not affect grazing by *K. brevis* allowing populations to graze in eutrophic embayments where blooms accumulate as well as in the oligotrophic water column on the edge of the WFS where blooms are thought to initiate. The ability to graze at similar rates in these contrasting environments provide *K. brevis* a competitive advantage during both bloom initiation, when grazing might allow them nutritional alternatives while competing with autotrophic phytoplankton for scarce dissolved nutrient resources, and during bloom maintenance, when high cell densities may cause light limitation.

Light availability has also been shown to stimulate or inhibit grazing (Burkholder et al. 2008, Smalley et al. 2003) and digestion rates have been shown to be light dependent for some herbivorous protists (Strom 2001). Legrand et al. (1998) found that there was no difference in ingestion rates by *Heterocapsa triquetra* on fluorescently labeled algae (FLA) during light and dark incubations. However, others have observed that certain species of dinoflagellates preferentially graze on picoplankton under low light conditions (Sanders et al. 1990). While I did not examine grazing under a range of light levels, results presented here demonstrate *Karenia brevis* has similar ingestion rates of *Synechococcus* during the day, when it is light, and at night, in the dark (Table 4). The ability to graze in the dark may allow *K. brevis* 'seed' populations to remain viable below the euphotic zone or allow *K. brevis* populations to continue to thrive when cell densities are high and self-shading can limit photosynthetic C acquisition.

Karenia brevis displays a unique behavioral pattern of surface aggregation into 0 - 5 m surface layer during daylight hours, and random dispersal at night rather than downward migration (Heil 1986, Kamykowski et al. 1998, Walsh and Steidinger 2001). While this may concentrate *K. brevis* in well-lit surface water to access light to power photosynthetic C fixation during the day, it is likely to reduce ingestion rates because the prey to predator ratio would be low and thus unfavorable for encountering prey (Glibert et al. 2009). In contrast, random dispersal into the water column during nighttime may increase the prey to predator ratio and increase *K. brevis* encounter rates with prey within surface waters at night. Thus, *K. brevis* behavior may facilitate photoautotrophic growth during the day and mixotrophic grazing at night.

In addition to grazing on *Synechococcus*, this study demonstrates for the first time that *Karenia brevis* can also graze on two other important unicellular picoplankters, *Prochlorococcus marinus* and heterotrophic bacteria. *Prochlorococcus* spp. are found at cell concentrations as high as 10^5 cells ml^{-1} in the GOM (Paul et al. 2000, Jochem 2001) and bacterial cell densities are generally on the order of 10^4 - 10^6 cells ml^{-1} (Weinbauer & Suttle 1996, Long et al. 2008), the same order of magnitude as those used in this study. This suggests that *K. brevis* may be capable of ingesting an array of pico- and nanoplankton and these could contribute to their growth and nutrition in the GOM where *Synechococcus*, *Prochlorococcus* (Paul et al. 2000), and heterotrophic bacteria (Weinbauer et al. 1996, Long et al. 2008) are all abundant. Cell-specific ingestion rates on heterotrophic bacteria by *K. brevis* were lower than cell-specific ingestion rates on *Synechococcus* (Table 2, 5, Jeong et al. 2005b, Glibert et al. 2009). This may have been due to the long incubation times during bacterial grazing experiments or some synergistic relationship between bacteria and *K. brevis* in cultures. Identifying the full range of prey available to *K. brevis* on the WFS and their prey preferences is required to provide a more complete understanding of nutritional factors contributing to bloom initiation and maintenance for this organism.

The three species of picoautotrophs that were not ingested by *Karenia brevis* are much larger than the picoplankton that were ingested suggesting that *K. brevis* may prefer smaller prey. The estimated spherical diameter (ESD) of *Synechococcus* (CCFWC 502) cells was about 1.97 ± 0.07 μm (Fig. 1), which is similar to the cell volume reported for *Synechococcus* from blooms in Florida Bay (Phlips et al. 1999). ESDs for *Isochrysis*, *Synechocystis*, and *Crocospaera* have been estimated to be approximately 5 (Jeong et al.

2009), 2.35 (Hammer et al. 2001), and 5 μm (Goebel et al. 2008), respectively. Although the three species that were not ingested by *K. brevis* in this study were also supplied at relatively low cell densities (10^3 cells ml^{-1}) and this may have precluded their ingestion. In this study, I calculated a lower feeding threshold of 1.86×10^4 *Synechococcus* ml^{-1} (Fig. 7), however in another study, *K. brevis* incorporated N from *Synechococcus* when it was supplied at just $\sim 10^2$ *Synechococcus* ml^{-1} (Table 4 in Glibert et al. 2009); however, in that study the number of cells grazed (*Synechococcus* *K. brevis* $^{-1}$ h^{-1}) was calculated based on the transfer of ^{15}N from ^{15}N -labeled *Synechococcus* to unlabeled *K. brevis* rather than using the prey disappearance or prey inclusion methods with heat-killed prey. Transfer of isotope from *Synechococcus* to *K. brevis* could have also occurred via regeneration or release of ^{15}N -labeled dissolved N by *Synechococcus* and uptake by *K. brevis* rather than through ingestion during the 24 hour incubation period, as has been observed in incubation experiments with other cyanobacteria (Mulholland et al. 2004, Submitted)

While *Karenia brevis* did not graze on *Crocospaera watsonii*, *Synechocystis* sp., and *Isochrysis* sp. in this study when they were supplied at concentrations below the lower feeding threshold for grazing on *Synechococcus*, they may do so in nature where their abundance can be much higher. Alternatively, grazing on different prey groups may be isolate-specific. *K. brevis* SB3 was the only isolate used in testing grazing on *Isochrysis*, *Synechocystis*, and *C. watsonii*. All of the WFS *K. brevis* isolates examined to date have ingested *Synechococcus* at rates within the range measured in previous studies (Jeong et al. 2005b, Glibert et al. 2009).

While mixotrophic dinoflagellates may benefit and gain a competitive advantage by eating co-occurring picoplanktonic prey cells (Thingstad et al. 1996), in other cases both prey and predator may benefit. Sanders et al. (2001) concluded that mixotrophs, such as *Ochromonas* sp., may not be able to store N and P. Cellular leakage of these nutrients from the dinoflagellate may feed heterotrophic bacteria that in turn are grazed by the mixotroph. It is also likely that *Karenia brevis* shares some sort of relationship, other than predator-prey, with the heterotrophic bacteria in culture. Many dinoflagellates, including *K. brevis*, are difficult to grow axenically. Despite early efforts to grow *K. brevis* axenically (Ray & Wilson 1957), efforts to date have been unsuccessful (discussed in Kusek et al. 1999). Phagotrophic ingestion of heterotrophic bacterial contaminants by *K. brevis* and the stimulation of bacterial growth by *K. brevis* may offer some nutritional advantage to both.

Conclusions. While many potential sources of nutrients are available to *Karenia brevis* on the WFS, the total available N pool is insufficient to fuel large blooms of long duration (Walsh & Steidinger 2001, Mulholland et al. 2006, Vargo et al. 2008). In this study, I determined that *Karenia brevis* isolates from the WFS can graze on the cyanobacteria *Synechococcus* and *Prochlorococcus marinus*, as well as heterotrophic bacteria. Grazing may offer yet another source of nutrients that can contribute to bloom initiation or help sustain large and prolonged blooms of this organism. Under laboratory conditions, N-specific ingestion rates of *Synechococcus* by WFS *K. brevis* isolates ranged from 0.23 to 8.16 pmol N *K. brevis*⁻¹ hr⁻¹, respectively, more than previously thought (Glibert et al. 2009). Results presented here demonstrate that nutrient acquisition and growth of *K. brevis* could be substantially augmented by mixotrophic grazing and that N-

and C-specific ingestion rates of prey should be measured and included in nutrient budgets for *K. brevis* blooms on the WFS.

CHAPTER III

***KARENIA BREVIS* AS A MIXOTROPH: AUTOTROPHIC, PHAGOTROPHIC, AND OSMOTROPHIC METABOLISMS COMPARED**

Introduction

Karenia brevis, formerly known *Gymnodinium breve* (Aldrich 1962), has been shown to occasionally contribute as much as 100% of the annual primary production on the West Florida Shelf (WFS). Primary productivity during monospecific *K. brevis* blooms has been shown to be as high as $3.8 \text{ g C m}^{-2} \text{ d}^{-1}$ (Vargo et al. 1987) and growth rates of *K. brevis* bloom populations have been reported as high as 0.3 d^{-1} on the WFS (Van Dolah et al. 1999, Walsh & Steidinger 2001). However, chlorophyll normalized primary production has been shown to be lower within blooms of *K. brevis* than in natural water samples collected outside of bloom-affected areas (Bendis et al. 2004), suggesting that bloom populations are less productive. This could be due to light limitation of photosynthesis in dense blooms or photoinhibition of shade-adapted bloom populations (Bendis et al. 2004). Other phytoplankton are known to increase cellular chlorophyll concentrations in response to light limitation to maintain high growth rates (Falkowski 1991, MacIntyre et al. 2002). Here I hypothesize that autotrophic carbon (C) uptake may be augmented by heterotrophic C uptake during blooms resulting in lower rates of chlorophyll normalized primary productivity.

High primary productivity generates a concomitantly high nutrient demand (N and P) to support balanced production (Odum et al. 1955, Vargo et al. 1987, Heil et al. 2004, Hitchcock et al. 2010). In addition to new nutrient inputs from terrestrial runoff,

rivers and estuaries, atmospheric deposition, and upwelling (Pribble & Janicki 1999, Vargo et al. 2008), inorganic and organic nitrogen (N) and phosphorus (P) compounds recycled *in situ* may also contribute to bloom maintenance (Sinclair et al. 2006a, b, Killberg-Thoreson 2011). There are a variety of sources of regenerated N that are available to support high rates of primary productivity within blooms. For example, *Trichodesmium* exudates include NH_4^+ and dissolved organic N compounds (Capone et al. 1994, Glibert & Bronk 1994, Mulholland et al. 2004a) that are bioavailable to *Karenia brevis* on the WFS (Mulholland et al. 2004b, 2006, Submitted). In addition, nutrients are regenerated through microbial interactions within blooms (McCarthy et al. 1980) and through the death and decay of fish and other aquatic organisms (Walsh et al. 2006).

Regeneration of organic matter produces a variety of organic compounds that may be bioavailable to phytoplankton. It has been shown that organic N uptake is important for maintaining *Karenia brevis* biomass on the WFS (Mulholland et al. 2006, Bronk et al. Submitted), especially when light is limiting (Sinclair & Kamykowski 2008, Sinclair et al. 2009). For example, *K. brevis*-normalized urea uptake rates were comparable to or higher than NH_4^+ and NO_3^- uptake in cultures and natural populations (Mulholland et al. 2006, Killberg-Thoreson 2011). I hypothesize that the C from these organic compounds as well as other dissolved organic C (DOC) compounds may contribute to *K. brevis* growth. Concentrations of dissolved organic C (DOC) in the GOM range from 83 μM in offshore waters to 131 μM inshore (Guo et al. 1994).

Our understanding of the metabolic capabilities of *Karenia brevis* has changed over the last 50 years. *K. brevis* was originally thought to be a strict autotroph (Aldrich 1962). It was later shown that *K. brevis* could incorporate amino acids directly into

protein (Baden & Mende 1979), thereby taking advantage of both N and C from these compounds. *K. brevis* is now known to be capable of phagotrophy (Jeong et al. 2005b, Glibert et al. 2009, Chapter 2) which offers a broad spectrum of nutritional subsidies to its photoautotrophic metabolism. *K. brevis* is capable of ingesting *Synechococcus*, *Prochlorococcus*, heterotrophic bacteria and possibly other species that remain to be identified (Jeong et al. 2005b, Glibert et al. 2009, Chapter 2). However, it is unknown how *K. brevis* balances photoautotrophic and heterotrophic metabolisms to achieve high growth rates and bloom in the environment.

Mixotrophy among harmful algal species, such as *Karenia brevis*, appears to be more common than previously thought (Jeong et al. 2005a, b, Raven et al. 2009, Yoo et al. 2010). Although mixotrophs were formerly thought to thrive primarily in oligotrophic environments where nutrient concentrations are limiting (Sander 1991, Arenovski et al. 1995), eutrophic ecosystems are also favorable environments for many mixotrophic species because while nutrients can be plentiful, light can limit photosynthesis (Stoecker et al. 1997, Adolf et al. 2008, Anderson et al. 2008, Burkholder et al. 2008) as well as nutrient ratio imbalances (Li et al. 2000, Smalley et al. 2003). I previously demonstrated that *K. brevis* was capable of grazing under both nutrient-replete and -deplete conditions and in both the light and dark periods (Chapter 2) suggesting that phagotrophy may be an integral component of this organisms metabolism provided sufficient prey are available.

The metabolic cost versus nutritional benefit of autotrophic versus heterotrophic nutrient acquisition by mixotrophs is not completely understood for most species, but there are many theoretical advantages to mixotrophy. Mixotrophs may have a competitive advantage over obligate auto- and heterotrophic organisms because they can potentially

access a wider range of nutrient sources over a longer period of the day (Bockstahler & Coats 1993a, b). Mixotrophy offers organisms access to both dissolved and particulate nutrient pools and because heterotrophic growth does not require light energy, mixotrophs can potentially acquire C for growth at night when photoautotrophy is limited (Putt 1990, Adolf et al. 2006). In addition, many mixotrophs may ingest their autotrophic competitors.

Some dinoflagellate species have higher growth rates when they are growing mixotrophically versus when they are growing under strictly autotrophic conditions (Li et al. 1999, Adolf et al. 2006). Hypothetically, heterotrophic C acquisition may be less metabolically costly than autotrophic growth and so more cellular C can be apportioned to growth rather than maintaining photosynthetic machinery (Raven 1997, Adolf et al. 2006). It has also been shown that photosynthetic rates are reduced in some mixotrophs that are grown in the presence of prey compared with those growing autotrophically without prey cells (Skovgaard et al. 2000, Adolf et al. 2006). By switching between the two modes of metabolism, a mixotroph may increase nutritional benefits while also lowering their energetic expenditures (Caron et al. 1990, Rothhaupt 1996, Skovgaard 1996, Stoecker 1998). The effect of phagotrophy on photosynthetic C incorporation by *Karenia brevis* is unknown.

Here I compare C acquisition by *Karenia brevis* from photoautotrophic uptake of dissolved inorganic C (DIC), C uptake via phagotrophic ingestion of picoplanktonic prey, and osmotrophic uptake of amino acid C. I simultaneously compared phagotrophic grazing by *K. brevis* on heat-killed *Synechococcus* with photosynthetic uptake of DIC to determine if primary productivity is reduced when *K. brevis* is amended with prey.

Amino acid C uptake was also compared in *K. brevis* cultures amended with heat-killed *Synechococcus* and in cultures with no added prey to determine whether osmotrophic C uptake modified by the presence of prey.

Methods

Culture Acclimation. Cultures of *Karenia brevis* (CCFWC 251, JC4 and CCFWC 254, SB3) were grown in a temperature controlled incubation room (22 to 26 °C) on a 12:12 light:dark cycle and supplied with 70 -100 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ using 20W “cool white” fluorescent light bulbs. Cultures were not axenic, but bacterial contamination was kept at a minimum by transferring cultures in exponential phase growth in a laminar airflow hood (NuAire; downflow 70 ft min⁻¹, inflow 105 ft min⁻¹) using aseptic techniques. Cultures were acclimated to nutrient-replete or -deplete media for at least 48 hours prior to the initiation of experiments, as described in Chapter 2.

Nutrient-Replete DIC Uptake Versus Grazing. DIC uptake (as bicarbonate) and phagotrophic grazing of heat-killed *Synechococcus* by nutrient-replete *Karenia brevis* (JC4) were compared in parallel light and dark bottle incubations. *K. brevis* cells were maintained on nutrient-replete medium and enumerated as described in Chapter 2. Heat-killed *Synechococcus* (CCFWC 502) were prepared and enumerated as described in Chapter 2. Previously, I demonstrated that comparable grazing coefficients (g, hr⁻¹) were observed in *K. brevis* cultures amended with live or heat-killed *Synechococcus* cells (Fig. 7, Table 2). Triplicate 125 ml incubations included: heat-killed *Synechococcus* controls, a *K. brevis* control, and *K. brevis* amended with heat-killed *Synechococcus*. A 10% addition (200 μM) of highly enriched (99%) $\text{H}^{13}\text{CO}_3^-$ was added to incubation bottles to

measure photosynthetic C uptake. Grazing and DIC uptake were measured in parallel light and dark bottle incubations for nutrient-replete cultures. Samples were collected to measure cell abundance after 0, 6, and 12 hours. Before a sample was removed, each bottle was gently mixed to ensure that concentrations of predator and prey were uniformly distributed. A 2 ml sample was collected and split into 1 ml aliquots for visualization of inclusions using EM/CLSM or cell counts using FCM. Samples were preserved in 1% (final concentration) glutaraldehyde, and stored at 4 °C. Samples for FCM analyses were analyzed immediately as described in Chapter 2 to enumerate *K. brevis* and *Synechococcus*. Clearance rates, ingestion rates, and grazing coefficients were calculated using Equations 2, 3, and 4, respectively, over the first 6 hours of the incubation when prey disappearance was most likely to have decreased linearly over time on a natural log scale (Chapter 2).

To measure DIC uptake, tracer additions (~10%) of highly enriched (99%) $\text{H}^{13}\text{CO}_3^-$ was added to each incubation bottle at the start of the light period. A 50 ml aliquot was immediately removed from each incubation bottle (T_0) and filtered through a pre-combusted (400 °C for 2 hr) Whatman GF/F filter and frozen until analysis. A second 50 ml sample was removed after a 12-hour incubation period and filtered and stored as described above to estimate $\text{H}^{13}\text{CO}_3^-$ uptake into particulate organic matter over the 12-hour light period. Each filter was dried at 40 °C for at least 48 hours before being pelletized in tin discs for analysis on a Europa Scientific 20-20 isotope ratio mass spectrometer (IRMS) equipped with an automated N and C analyzer (ANCA).

DIC uptake rates were calculated based on the mixing model used by Mulholland et al. (2006) with modifications to account for isotopically light C contributed from heat-

killed *Synechococcus* retained on GF/F filters in prey amended incubations containing live *Karenia brevis*. C from non-photosynthesizing, dead *Synechococcus* C (PC_{Syn}) in prey amended samples was estimated by calculating the particulate carbon concentration in *Synechococcus* control bottles without added ^{13}C bicarbonate. To calculate C uptake by *K. brevis* in prey amended bottles, I subtracted the particulate C due to *Synechococcus* from the total particulate C (PC_{total}) in prey amended incubation bottles (Equation 7) to calculate a particulate C value for *K. brevis* only ($PC_{K. brevis}$). I multiplied the sample atom % by the total particulate C (atom % PC_{filter}) to calculate total ^{13}C atoms retained in particles ($^{13}C_{K. brevis}$) on the filter (Equation 8). Then, I calculated the atom % for *K. brevis* only (atom % $PC_{K. brevis}$) by dividing ^{13}C atoms on the filter by the particulate C due to *K. brevis* only ($PC_{K. brevis}$) (Equation 9). The calculated atom % sample and the particulate C for *K. brevis* only were used to calculate specific uptake rates based on a mixing model (Equation 10) (Mulholland et al. 2006). To calculate absolute uptake rates, specific uptake rates were multiplied by the particulate C concentration of *K. brevis* cells in the incubation bottle ($PC_{K. brevis}$).

$$PC_{Kbrevis} = PC_{total} - PC_{Syn}, \quad (7)$$

$$^{13}C_{K brevis} = PC_{filter} \times \text{atom \% } ^{13}C_{filter} \times 100 \quad (8)$$

$$\text{atom \% } C_{K brevis} = ^{13}C_{K brevis} / PC_{K brevis} \quad (9)$$

$$C \text{ uptake} = ((\text{atom \% } PC_{K brevis})_{final} - (\text{atom \% } ^P PC_{K brevis})_{initial}) / ((\text{atom \% enrichment DIC} - \text{atom \% } PC_{K brevis})_{initial} \times \text{time}) \times [PC_{K brevis}] \quad (10)$$

Nutrient-Deplete DIC Uptake Versus Grazing. Autotrophic bicarbonate uptake and phagotrophic grazing of *Synechococcus* by *Karenia brevis* (SB3) was also examined in parallel light and dark bottles in nutrient-deplete media. Cultures were acclimated for

48 hours on f/2 media with no added N or P as described in Chapter 2. Incubation set up (triplicate bottles), prey and isotope amendments, and sampling schedule were the same as described above for the nutrient-replete experiment. Clearance rates, ingestion rates, grazing coefficients, and uptake rates were also measured and calculated as described above.

Nutrient-Replete C and N Uptake from Amino Acids Versus Grazing. As for DIC uptake experiments, amino acid uptake and nutrient-replete grazing were measured in experiments using *Karenia brevis* SB3 and heat-killed *Synechococcus* during the day. Amino acid concentrations were measured using the DFAA procedure and Turner Fluorometer at the beginning of the grazing experiment (T_0) using methods modified from Parsons et al. (1984). I assumed a C:N ratio of 4 for the amino acid mixture used in this experiment. Grazing was measured in triplicate bottles over a 4-hour incubation period taking 2 ml subsamples at 0 and 4 hours to measure cell abundance by FCM as described in Chapter 2. Grazing coefficients were calculated using Equation 5. Amino acid uptake was measured in each of the triplicate bottles similar to the DIC uptake experiment described above. A highly enriched (^{13}C 98%; ^{15}N 96-99% Cambridge Isotope Laboratories, Inc.) ^{15}N and ^{13}C labeled amino acid mixture was added ($0.145 \mu\text{mol N l}^{-1}$ and $0.579 \mu\text{mol C l}^{-1}$ final concentration) to 3 sets of triplicate 25 ml incubations: *K. brevis* control (without *Synechococcus*), *K. brevis* amended with heat-killed *Synechococcus*, and a heat-killed *Synechococcus* control (without *K. brevis*). This addition represented an enrichment of approximately 18.4% for amino acids. Two amino acid uptake experiments, each lasting 30 minutes, were done; one initiated at the beginning (T_0) of the grazing experiment and the other at the end (T_F) to determine

whether amino acid uptake by *K. brevis* was affected by the availability of prey. After 30 minutes, uptake experiments were terminated by filtering samples through pre-combusted (450 °C for 2 hours) Whatman GF/F filters and filters were frozen until analysis. Each filter was dried and pelletized in a tin disk prior to analysis on a Europa Scientific 20-20 IRMS equipped with an ANCA. Similar modifications to Equations 7-10 for particulate N (PN) and C (PC) were made to estimate amino acid C and N uptake by *K. brevis* only and remove the isotopically light contribution of heat-killed *Synechococcus* also collected on the filter, as was done in the combined DIC uptake and grazing experiments described above. Grazing samples were taken and handled or stored as described above. Samples to quantify cell concentrations and the natural abundance of C and N were also collected at each time point. *K. brevis* and *Synechococcus* cell numbers were measured as described in Chapter 2 using FCM.

Amino acid N and C uptake were calculated using Equations 11 and 12, respectively (Mulholland et al. 2006).

$$\text{N uptake} = (((\text{atom \% PN}_{K \text{ brevis}})_{\text{final}} - (\text{atom \% PN}_{K \text{ brevis}})_{\text{initial}}) / (\text{atom \% enrichment N source pool} - \text{atom \% PN}_{K \text{ brevis}})_{\text{initial}} \times \text{time}) \times [\text{PN}_{K \text{ brevis}}] \quad (11)$$

$$\text{C uptake} = (((\text{atom \% PC}_{K \text{ brevis}})_{\text{final}} - (\text{atom \% PC}_{K \text{ brevis}})_{\text{initial}}) / (\text{atom \% enrichment C source pool} - \text{atom \% PC}_{K \text{ brevis}})_{\text{initial}} \times \text{time}) \times [\text{PC}_{K \text{ brevis}}] \quad (12)$$

Statistical Analysis. Average grazing coefficients and uptake rates were compared using ANOVA tests similar to Chapter 2. Each grazing experiment employed a 4 or 6 hour incubation period, which was previously determined to be the within the range where *Synechococcus* removal by *Karenia brevis* was linear on a natural log scale. ANOVA tests were used to determine whether or not there were significant differences in

grazing coefficients or uptake rates between treatments (nutrient-deplete versus nutrient-replete or light versus dark). When more than 2 groups were tested, a Tukey's multiple comparisons test was used to determine which treatments were significantly different from each other.

Results

Nutrient-Replete DIC Uptake Versus Grazing. Initial concentrations of heat-killed *Synechococcus* were $5.46 \pm 0.49 \times 10^4$ *Synechococcus* ml⁻¹ (Table 7). Cell concentrations did not change over the incubation period and there was no uptake of H¹³CO₃ by heat-killed *Synechococcus* in prey control bottles (Table 7), which was expected because cells were dead. Initial *Karenia brevis* JC4 cell concentrations in incubation bottles were $0.07 \pm 0.01 \times 10^3$ *K. brevis* ml⁻¹.

Grazing by *Karenia brevis* JC4 was observed in all prey-amended treatment incubations and grazing coefficients were 0.02 ± 0.01 and 0.02 ± 0.01 in light and dark prey amended bottles (Table 7), respectively, and not significantly different from each other (ANOVA, n = 6, T = 0.701, p = 0.427). Ingestion rates were 13.9 ± 4.7 *Synechococcus K. brevis*⁻¹ hr⁻¹ in light treatment bottles and 10.8 ± 8.0 *Synechococcus K. brevis*⁻¹ hr⁻¹ in dark treatment bottles (Table 7). Ingestion and clearance rates were similar to those measured in previous experiments using WFS isolates (Chapter 2) and were in the range of those in studies using other *K. brevis* isolates (Jeong et al. 2005b, Glibert et al. 2009, Table 2). Using cellular C concentrations measured previously for the

Table 7. Grazing coefficients, clearance, and ingestion rates of nutrient replete *K. brevis* JC4 on heat-killed *Synechococcus* in triplicate light (L) and dark (D) bottles over a 6-hour incubation period. C-specific uptake rates from prey ingestion are compared to inorganic C uptake rates in prey amended and unamended *K. brevis* cultures over a 12-hour period. Standard deviations from triplicate bottles are in parentheses. Values (*) are significantly different than each other or (#) significantly different than the other 3 groups at the $p < 0.05$ level (Tukey's multiple comparison).

		Initial <i>Syn</i> ($\times 10^4$ cells ml ⁻¹)	Initial <i>K.</i> <i>brevis</i> ($\times 10^3$ cells ml ⁻¹)	<i>g</i> (hr ⁻¹)	Clearance rate ($\times 10^{-5}$ ml <i>K.</i> <i>brevis</i> ⁻¹ hr ⁻¹)	Ingestion rate (<i>Syn K. brevis</i> ⁻¹ hr ⁻¹)	C-specific uptake (pmol C cell ⁻¹ hr ⁻¹)		
								Prey	DIC
Kb+Syn	L	5.17 (0.70)	0.07 (0.01)	0.02 (0.01)	26.0 (9.0)	13.9 (4.7)	14.4 (5.3)	1.8 (0.3)*	
	D	5.35 (0.24)	0.07 (0.01)	0.02 (0.01)	20.3 (14.9)	10.8 (8.0)	11.2 (8.4)	1.5 (0.2)	
Kb	L		0.07 (0.01)					1.0 (0.1)*	
	D		0.07 (0.01)					0.1 (0.02) [#]	
Syn	L	5.92 (0.49)						0.0 (0.0)	
	D	5.38 (0.21)						0.0 (0.0)	

Table 8. Grazing coefficients, clearance, and ingestion rates of nutrient-deplete *K. brevis* SB3 on heat-killed *Synechococcus* in triplicate light (L) and dark (D) bottles over a 6-hour incubation period. C-specific uptake rates from prey ingestion are compared to inorganic C uptake rates in prey amended and unamended *K. brevis* cultures over a 12-hour incubation period. Standard deviations from triplicate bottles are in parentheses. Values (*) are significantly different than each other or (#) significantly different than the other 3 groups at the $p < 0.05$ level (Tukey's multiple comparison).

		Initial <i>Syn</i> ($\times 10^5$ cells ml ⁻¹)	Initial <i>K.</i> <i>brevis</i> ($\times 10^3$ cells ml ⁻¹)	<i>g</i> (hr ⁻¹)	Clearance rate ($\times 10^{-5}$ ml <i>K. brevis</i> ⁻¹ hr ⁻¹)	Ingestion rate (<i>Syn K.</i> <i>brevis</i> ⁻¹ hr ⁻¹)	C-specific uptake (pmol C cell ⁻¹ hr ⁻¹)	
							Prey	DIC
Kb+Syn	L	2.34 (0.06)	0.12 (0.002)	0.019 (0.009)	15.1 (7.3)	37.4 (18.3)	38.8 (19.7)	2.3 (0.5)*
	D	2.40 (0.28)	0.16 (0.01)	0.012 (0.004)	7.0 (3.0)	17.3 (6.0)	17.9 (6.7)	0.8 (0.4)*
Kb	L		0.10 (0.002)					1.9 (0.2)
	D		0.08 (0.003)					0.1 (0.004) [#]
Syn	L	2.47 (0.23)						0.0 (0.0)
	D	2.38 (0.02)						0.0 (0.0)

Synechococcus isolate used in these experiments (Table 1), I calculated C-specific ingestion rates of 14.4 ± 5.2 and 11.2 ± 8.4 pmol C *K. brevis*⁻¹ hr⁻¹ for *K. brevis* in light and dark prey amended bottles, respectively.

In the absence of prey, bicarbonate uptake rates were 1.0 ± 0.1 and 0.05 ± 0.02 pmol C *Karenia brevis*⁻¹ hr⁻¹ in light and dark bottles, respectively (Table 7), and were significantly different from each other ($p = 0.0002$). However, bicarbonate uptake rates were 1.8 ± 0.3 and 1.5 ± 0.2 pmol C *K. brevis*⁻¹ hr⁻¹ in light and dark bottles amended with heat-killed *Synechococcus*, respectively and not significantly different from each other ($p = 0.918$). Uptake of DIC was significantly lower in the light in *K. brevis* cultures that had not been amended with prey relative to those that had ($p = 0.032$).

Nutrient-Deplete DIC Uptake Versus Grazing. Initial concentrations of heat-killed *Synechococcus* in these experiments were $2.40 \pm 0.17 \times 10^5$ *Synechococcus* ml⁻¹, (Table 8). Similar to the nutrient-replete DIC vs. grazing experiment, *Synechococcus* cell concentrations did not significantly change during the incubation and there was no uptake of H¹³CO₃ in prey control bottles due to the fact that *Synechococcus* cells were heat-killed (Table 8). Average *Karenia brevis* SB3 cell concentrations were $0.12 \pm 0.03 \times 10^3$ *K. brevis* ml⁻¹.

Grazing by *Karenia brevis* SB3 was observed in all prey amended incubation bottles and grazing coefficients were 0.019 ± 0.008 and 0.012 ± 0.004 hr⁻¹ in light and dark treatment bottles, respectively, which were not significantly different from light bottles (ANOVA, $n = 6$, $T = 0.550$, $p = 0.638$). Ingestion rates of *Synechococcus* by nutrient-deplete *K. brevis* SB3 were higher in light bottles (37.4 ± 18.3 *Synechococcus* *K. brevis*⁻¹ hr⁻¹) than dark bottles (17.3 ± 6.0 *Synechococcus* *K. brevis*⁻¹ hr⁻¹) (Table 8).

Based on cellular C concentrations of the *Synechococcus* isolate used in these experiments (Chapter 2, Table 1), I calculated C-specific ingestion rates of 38.8 ± 19.7 and 17.9 ± 6.7 pmol C *K. brevis* in light and dark bottles, respectively.

DIC uptake rates were 2.3 ± 0.5 and 0.8 ± 0.4 pmol C *Karenia brevis*⁻¹ hr⁻¹ in nutrient-deplete, prey amended, light and dark treatment bottles, respectively. In light and dark bottles unamended with prey, DIC uptake rates by *K. brevis* SB3 were 1.9 ± 0.2 and 0.07 ± 0.004 pmol C *K. brevis*⁻¹ hr⁻¹, respectively (Table 8). Unlike nutrient replete *K. brevis* JC4, inorganic C uptake by nutrient deplete *K. brevis* SB3 was significantly higher in the light prey amended incubation bottles than in the dark prey amended bottles ($p = 0.006$) and light DIC uptake rates by *K. brevis* SB3 were not statistically different in prey amended and unamended incubations ($p = 0.059$). Like nutrient replete *K. brevis* JC4, dark DIC uptake rates by nutrient deplete *K. brevis* SB3 were significantly higher in the prey amended incubations than in those that had no added prey ($p = 0.022$) using a Tukey's Multiple comparison post hoc test.

Nutrient-Replete C and N Uptake from Amino Acids Versus Grazing. Initial *Synechococcus* cell concentrations were $8.96 \pm 0.37 \times 10^4$ *Synechococcus* ml⁻¹ and *Karenia brevis* SB3 concentrations were $2.7 \pm 0.3 \times 10^3$ *K. brevis* ml⁻¹ for these experiments. Grazing was detected and grazing coefficients over the 4-hour incubation were 0.08 ± 0.01 hr⁻¹. Ingestion rates and calculated C-specific ingestion rates were 2.5 ± 0.4 *Synechococcus* *K. brevis*⁻¹ hr⁻¹ and 2.6 ± 0.03 pmol C *K. brevis*⁻¹ hr⁻¹ (Table 9), respectively.

Amino acid uptake by *Karenia brevis* was measured at two time points during the 4-hour grazing experiment to determine of the effect of grazing on amino acid uptake.

Amino acid concentrations at T_0 were $0.128 \pm 0.001 \mu\text{mol l}^{-1}$ ($n = 6$). Average amino acid C uptake rates were 0.62 ± 0.1 and $0.03 \pm 0.01 \text{ pmol C } K. brevis^{-1} \text{ hr}^{-1}$ in prey-amended and unamended *K. brevis* cultures at the beginning (T_0) of the grazing experiment (Table 10). However, *K. brevis* amino acid C uptake rates at the end (T_F) of the 4-hour grazing experiment were below the limit of detection in prey amended bottles. Amino acid C uptake was $0.07 \pm 0.07 \text{ pmol C } K. brevis^{-1} \text{ hr}^{-1}$ in bottles to which no *Synechococcus* had been added (Table 10). There was a significant difference in C-specific uptake rates of amino acids by *K. brevis* between treatments (with or without *Synechococcus*) at each time point (ANOVA, $n = 12$, $F = 19.359$, $p = 0.001$). *K. brevis* amended with *Synechococcus* at T_0 took up amino acid C at rates significantly higher than *K. brevis* amended with *Synechococcus* at T_F ($p = 0.001$). *K. brevis* not amended with *Synechococcus* at T_0 ($p = 0.003$) and T_F ($p = 0.009$) were lower than *K. brevis* cultures amended with *Synechococcus* at T_0 when compared using a Tukey's multiple comparison test (Table 10). Heat-killed *Synechococcus* did not take up amino acid N or C and were not included in the post hoc comparisons test (Table 10).

Average amino acid N uptake rates were 0.90 ± 0.1 and $0.01 \pm 0.002 \text{ pmol N } K. brevis^{-1} \text{ hr}^{-1}$ at T_0 for prey-amended and unamended *K. brevis*, respectively (Table 11), which were significantly different when compared with a Tukey's post hoc test ($P < 0.001$). *K. brevis* amino acid uptake rates at T_F were $0.62 \pm 0.1 \text{ pmol N } K. brevis^{-1} \text{ hr}^{-1}$ in bottles amended with *Synechococcus* and $0.05 \pm 0.06 \text{ pmol N } K. brevis^{-1} \text{ hr}^{-1}$ in bottles without *Synechococcus*.

Table 9. C-specific uptake rates of *Synechococcus* and amino acids by *K. brevis* were compared in nutrient-replete media while incubation lights were on. Grazing was measured in triplicate bottles over a 4-hour incubation period. Subsamples from the grazing experiment were taken at the beginning (T_0) of the grazing experiment and again at the end (T_F) of the grazing experiment and amended with dually labeled amino acids. Amino acid uptake was measured in triplicate bottles in 30 minute incubations.

	Initial <i>Syn</i> ($\times 10^4$ cells ml^{-1})	Initial <i>K. brevis</i> ($\times 10^3$ cells ml^{-1})	g (hr^{-1})	Clearance rate ($\times 10^{-5}$ $\text{ml } K. brevis^{-1} \text{ hr}^{-1}$)	Ingestion rate (<i>Syn</i> <i>K. brevis</i> $^{-1} \text{ hr}^{-1}$)	C-specific uptake ($\text{pmol C } K. brevis^{-1} \text{ hr}^{-1}$)		
						Prey	AA - T_0	AA - T_F
Kb+Syn	8.96 (0.37)	2.7 (0.3)	0.08 (0.01)	2.7 (0.4)	2.5 (0.4)	2.6 (0.3)	0.62 (0.1)	0.0 (0.0)
Kb							0.03 (0.01)	0.07 (0.07)
Syn							0.0 (0.0)	0.0 (0.0)

Table 10. Amino acid uptake rates at the beginning (T_0) and end (T_F) of a 4-hour grazing experiment measured during two 30-minute incubations. Standard deviations from triplicate bottles are in parentheses. Values (*) are significantly different than the other *K. brevis* experimental groups at the $p < 0.05$ level (Tukey's multiple comparison).

Treatment		Amino Acid (pmol <i>K. brevis</i> ⁻¹ hr ⁻¹)	
		N-specific	C-specific
T_0	Kb+Syn	0.90 (0.1)*	0.62 (0.1)*
	Kb	0.01 (0.002)	0.03 (0.01)
	Syn	0.0 (0.0)	0.0 (0.0)
T_F	Kb+Syn	0.62 (0.1)*	0.0 (0.0)
	Kb	0.05 (0.06)	0.07 (0.07)
	Syn	0.0 (0.0)	0.0 (0.0)

Discussion

Previous studies of mixotrophy by *Karenia brevis* focused on phagotrophic grazing of *Synechococcus* and its contribution to *K. brevis*' N demand (Jeong et al. 2005b, Glibert et al. 2009, Chapter 2). Here I also examined the contribution of grazing to *K. brevis*' C demand. *K. brevis* can account for nearly 100% of the productivity on the WFS during blooms (Vargo et al. 1987). However, chlorophyll a-normalized C fixation rates were lower within blooms than in surrounding waters in one study (Bendis et al. 2004) suggesting, paradoxically, that bloom populations are less productive. Low productivity during blooms may be due to changes in the physiological state of blooms as they initiate and then progress. Nutrient conditions and the physiological status of bloom organisms can change dramatically as blooms develop and progress as does light availability (Mulholland et al. 2009a, b). It is also likely that nutrient conditions that promote bloom initiation are markedly different from those that are observed once blooms are already established. Most sampling of algal blooms commences once blooms are visible to the eye and therefore, well established, and this may bias our view of primary productivity during bloom initiation. During bloom senescence, primary productivity may be low relative to periods of bloom initiation and relative to natural waters unimpacted by blooms. Alternatively, this study suggests that grazing can contribute substantially and even provide the bulk of *K. brevis*' C demand for growth if sufficient prey are available (Table 7, 8).

In this study, I also measured the interactive effects of prey additions, and associated ingestion of prey, on autotrophic DIC uptake and the uptake of dissolved organic N and C from amino acids. C fixation by nutrient-replete (*Karenia brevis* JC4)

and -deplete (*K. brevis* SB3) was significantly higher in light and dark bottles amended with prey (heat-killed *Synechococcus*) than in those that received no prey additions (Table 7, 8) suggesting that mixotrophy enhances primary productivity by *K. brevis*. This could be because grazing alleviates nutrient limitation of growth and photosynthesis. However, these results differ from some previous studies that showed decreases in primary productivity by mixotrophs that were actively grazing (Skovgaard et al. 2000, Adolf et al. 2006). Prey stimulation of C fixation may also result from the presence of growth factor(s) other than N and P, which were supplied to nutrient-replete cultures. The growth medium normally used to grow *K. brevis* (GP medium) contains soil extract, which is thought to provide necessary but unidentified organic growth elements to support their growth in cultures. Prey may provide similar nutritional benefits that remain to be identified.

In this study, C fixation rates by nutrient-replete *Karenia brevis* JC4 amended with heat-killed *Synechococcus* were statistically similar in light and dark bottles (Table 7). However, under nutrient-deplete conditions, C fixation by *K. brevis* SB3 in prey amended bottles was statistically lower in dark bottles than in light bottles, but dark C fixation was still higher in prey-amended bottles than in incubation bottles that had not been amended with prey (Table 8). Dark C fixation is often thought to be negligible in photoautotrophs (Paerl & Mackenzie 1977). But dark C fixation has been shown to be significant in some studies. For example, dark C fixation accounted for more than 50% of the C fixed in light bottles using cultured isolates of *Phaeodactylum* and *Dunaliella*, and increased with increasing phytoplankton cell density in a study by Morris et al. (1971). The species used by Morris et al. were grown in F₁ medium (Guillard & Ryther

1962) at 20 or 25 °C and 14:10 and 16:8 light dark cycle for *Phaeodactylum* and *Dunaliella*, respectively. Like *K. brevis* in this study, *Dinophysis* was also shown to fix C at higher rates in the dark when growing mixotrophically than under photic conditions without prey amendments (Granéli et al. 1997). Granéli et al. suggest that dark C-fixation may be from the uptake of C-labeled dissolved or particulate material in response to low light or dark conditions. In the current study, organic particulate matter (in the form of heat-killed *Synechococcus*) was not enriched with ^{13}C during incubations (Fig. 7, 8). Because two different *K. brevis* isolates were used and two different concentrations of prey were supplied in nutrient-replete and -deplete experiments, strain-related and prey-dependent differences in dark C fixation cannot be ruled out either. Above the lower feeding threshold of 1.86×10^4 *Synechococcus* ml^{-1} , *K. brevis* ingestion rates on *Synechococcus* increase exponentially until grazing is saturated (Fig. 6B).

Light and nutrient availability have been shown to stimulate or inhibit grazing by other mixotrophs (Burkholder et al. 2008, Smalley et al. 2003). It has been observed that certain species of dinoflagellates preferentially graze on picoplankton under low light and nutrient levels (Smalley et al. 2003). In contrast, Legrand et al. (1998) found that there was no difference in grazing by *Heterocapsa triquetra* on fluorescently labeled algae (FLA) in light and dark incubations. Grazing by *Karenia brevis* on live and heat-killed *Synechococcus* was not statistically different in light versus dark or nutrient-replete versus -deplete incubations (Chapter 2; Table 4, 7, 8). However, while I compare prey stimulation of light and dark C fixation for both experiments, I could not draw conclusions regarding nutrient stimulation of grazing because as stated above, the higher ingestion rates measured in the nutrient-deplete incubations done as part of this study

could also have been due to the greater initial prey amendments made to nutrient-deplete bottles versus nutrient-replete bottles or strain-specific differences. Up to some saturating prey concentration ($\sim 1.95 \times 10^6$ *Synechococcus* ml⁻¹), ingestion rates by *K. brevis* increase with increasing prey concentration (Fig. 6). While the concentrations of prey amendments were always above the lower feeding threshold, prey concentrations were lower than those needed to saturate the grazing response. In the GOM, *Synechococcus* concentrations are generally above the lower feeding threshold and below the saturating concentration (Phlips et al. 1999, Paul et al. 2000).

In addition to grazing, *Karenia brevis* is known to take up a variety of organic compounds including amino acids (Baden & Mende 1979, Killberg-Thoreson 2011, Bronk et al. Submitted). Amino acids are a small component of the dissolved organic matter pool but this pool is very labile and can be both produced and rapidly taken up by heterotrophic bacteria and phytoplankton (Kirchman et al. 1989, Mulholland & Lomas 2008). Organisms, such as *Trichodesmium*, which are an important component of the phytoplankton community on the WFS, release DOM (Bronk et al. 2004, Mulholland et al. 2006) including amino acids (Capone et al. 1994, Glibert et al. 1994), and so these compounds are likely available to *K. brevis* on the WFS. Previous studies examining the uptake of organic compounds by cultured and natural populations of *K. brevis* were focused on N uptake from these compounds and did not measure associated C uptake (Bronk et al. 2004, Glibert et al. 2009, Sinclair et al. 2009, Killberg-Thoreson 2011). In this nutrient-replete culture study, amino acid C uptake during the light period was higher in prey-amended treatments (0.62 ± 0.1 pmol C *K. brevis*⁻¹ hr⁻¹) immediately following the prey addition than in unamended *K. brevis* cultures (0.03 ± 0.01 pmol C *K. brevis*⁻¹ hr⁻¹).

¹), but amino acid C uptake was not detectable at the end of the 4 hour incubation period in prey-amended incubations (Table 9). Amino acid C uptake in prey-amended incubations was within the range reported from the WFS, where amino acid C uptake ranged from 0.10 to 1.96 pmol C *K. brevis*⁻¹ hr⁻¹ (Bronk et al. submitted), and in nutrient-replete culture studies (Killberg-Thoreson 2011).

Amino acid N uptake rates by *Karenia brevis* SB3 were also substantially higher (0.90 ± 0.1 pmol N *K. brevis*⁻¹ hr⁻¹) in prey-amended treatments just after prey additions relative to unamended cultures, in which N uptake was negligible (0.01 ± 0.002 pmol N *K. brevis*⁻¹ hr⁻¹) (Table 10), and within the ranges found in another culture study using a different cultured isolate (Sinclair et al. 2009) and in natural populations dominated by *K. brevis* (Killberg-Thoreson 2011), even though *K. brevis* populations and their nutrient prehistory varied between studies. As for C, rates of amino acid N uptake by *K. brevis* were enhanced in prey-amended incubations. Enhanced amino acid uptake associated with grazing could be due to enhanced amino acid availability as a result of amino acid release by grazers (Bronk & Steinberg 2008). As grazing by *K. brevis* on heat-killed *Synechococcus* continued during the 30-minute amino acid uptake experiment, following the 4-hour grazing experiment incubation, the ambient amino acid concentration may be increasing as nutrients are released as a result of grazing. Increasing the amino acid concentration would decrease the atm% as the labeled substrate would become dilute, inevitably increasing the absolute uptake. In this experiment uptake of labeled amino acid C was not detected during the 30-minute incubation after *K. brevis* was incubated for 4 hours with heat-killed *Synechococcus*. Although prey-amended *K. brevis* ceased to take up amino acid C after the 4-hour incubation, amino acid N was still taken up (Table

10) at a rate that may be an underestimate, as described above. This is the first observation of its kind and future studies should be aimed at understanding the relative uptake and release of C and N from nitrogenous organic compounds and associated with grazing by *K. brevis*.

When I compare the uptake of organic N and C from amino acids with N and C acquisition associated with the ingestion prey I found that, similar to DIC uptake, amino acid C uptake by *Karenia brevis* amended with prey was higher than incubations that had no added *Synechococcus* (Tables 7, 8 and 11). Flynn & Fielder (1989) reported an increase in free amino acid uptake when *Oxyrrhis marina* was ingesting dead prey however they did not distinguish between C and N uptake from amino acids. While I found that N uptake rates from amino acids continued to be enhanced even after 4 hours of grazing, C uptake from amino acids was reduced after 4 hours of grazing. In this study amino acid concentrations were not measured in the T_F incubations, therefore it is also likely that I underestimated amino acid N uptake rates in the T_F incubations because grazing activity and cellular degradation most likely increased amino acid concentrations within the incubation bottles amended with heat-killed prey.

Growth rates for some mixotrophic dinoflagellates are higher when they are phagotrophically ingesting prey cells (Raven 1995, Adolf et al. 2006). The results presented here suggest that for *Karenia brevis*, prey ingestion actually stimulates primary production as well (Table 7, 8). This may be because phagotrophy can alleviate nutrient or other growth factor limitations. In another laboratory grazing experiment, *K. brevis* grew at higher rates when supplemented with prey (Glibert et al. 2009). For this study, C-specific assimilation rates of heat-killed *Synechococcus* by *K. brevis* growing in

nutrient-replete media were 0.43 ± 0.17 and $0.33 \pm 0.25 \text{ d}^{-1}$ in light and dark bottles, respectively, while C-specific assimilation rates of prey by *K. brevis* acclimated to nutrient-deplete media but supplied with higher concentrations of prey were 1.15 ± 0.61 and $0.53 \pm 0.21 \text{ d}^{-1}$, in light versus dark bottles, respectively. Although these values represent C-specific assimilation rates that cannot be directly compared due to varying prey concentrations, they provide a range of C-specific assimilation values for two *K. brevis* isolates at prey concentrations and can be compared to the functional response of *K. brevis* ingesting *Synechococcus* (Fig. 6). In both experiments, nutrient-replete and -deplete DIC vs grazing, ingestion rates were approximately 2 times greater than ingestion rates predicted based on the modified Ivlev curved (Fig. 6B). These assimilation rates yield C doubling times for *K. brevis* due to ingestion of *Synechococcus* of about 0.96 and 1.86 days. While these do not take in to account loss terms (i.e. respiration), these doubling times would support a suggested turnover rate of at least 1 d in the GOM on the WFS (Vargo et al. 2008).

Carbon acquisition via autotrophy, osmotrophy, and phagotrophy, were all observed by *Karenia brevis* in this laboratory study using cultured isolates. Results presented here suggest that phagotrophy can be the dominant mode of C acquisition by *K. brevis*, provided suitable prey are available in sufficient quantities. Autotrophic uptake of DIC and osmotrophic uptake of amino acid C also contribute to cellular C acquisition and these modes of nutrient acquisition may dominate in the absence of sufficient prey. In parallel incubations, I estimate that *K. brevis* took up $14.4 \pm 5.3 \text{ pmol C } K. brevis^{-1} \text{ hr}^{-1}$ from *Synechococcus* compared to $1.8 \pm 0.3 \text{ pmol C } K. brevis^{-1} \text{ hr}^{-1}$ from DIC (Table 7) and $0.62 \pm 0.1 \text{ pmol C } K. brevis^{-1} \text{ hr}^{-1}$ from amino acids (Table 9). However, the

estimates of C uptake due to ingestion of prey presented assume assimilation efficiencies of about 50% and do not include respiratory losses and so may overestimate actual C-specific growth from grazing by *K. brevis*. Although the ingestion of prey may potentially dominate the C uptake by *K. brevis* in culture experiments, the availability of prey or other environmental factors may modulate the relative contribution of C from phagotrophy, osmotrophy, and autotrophy supporting *K. brevis* growth and blooms in nature and this remains to be examined.

Conclusions. A wide variety of nutrient sources are available to *K. brevis* on the WFS (Sinclair et al. 2009, Vargo et al. 2008), including inorganic and organic N and C (Bronk et al. submitted), and picoplankton prey (Jeong et al. 2005b, Glibert et al. 2009, Chapter 2). *Karenia brevis* appears to employ multiple nutrient acquisition strategies. These include: phagotrophic grazing on picoplankton, osmotrophic uptake of organic molecules, and photosynthesis. The capacity for utilizing all of these metabolic strategies simultaneously or alternatively in response to environmental conditions may be key to understanding the initiation and persistence of *K. brevis* blooms for weeks to months on the WFS. This nutritional flexibility may also explain how large blooms ($> 100,000$ cells l^{-1}) along the WFS maintain their biomass in nutrient depleted waters. Future research is required to better quantify the uptake of N and C from organic compounds versus the ingestion of prey under the diverse environmental conditions that occur on the WFS as blooms initiate and develop. Quantifying the relative balance in metabolic processes as *K. brevis* blooms progress could provide insights as to how *K. brevis* blooms initiate and become dominant on the WFS annually.

CHAPTER IV

GRAZING ON THE WESTERN FLORIDA SHELF BY *KARENIA BREVIS* ON *SYNECHOCOCCUS*

Introduction

Karenia brevis is a mixotrophic dinoflagellate (Jeong et al. 2005b, Glibert et al. 2009) that forms monospecific blooms annually in the oligotrophic offshore waters in the Gulf of Mexico (GOM) (Steidinger et al. 1998, Yang & Weisberg 1999, Vargo et al. 2008). These blooms are transported into nearshore waters where *K. brevis* can exert harmful effects on Gulf Coast ecosystems and can be directly toxic to humans (Kirkpatrick et al. 2004). Over the last 40 years, *K. brevis* blooms on the West Florida Shelf (WFS) in the GOM have appeared in late spring and fall and have persisted for weeks to months (Walsh & Steidinger 2001). Surface water temperature and salinity ranges observed during blooms are 20 to 28 °C and 31 to 37 salinity, respectively (see Steidinger et al. 1998). Dissolved inorganic nitrogen (DIN) concentrations on the WFS are generally at or near the limit of analytical detection during bloom initiation. The WFS is thought to be nitrogen (N) limited because ample phosphorus (P) is present in the system due to mining of phosphatic rock deposits and agriculture (see Heil et al. 2007).

Initiation of *Karenia brevis* blooms is thought to occur offshore and blooms have been linked to onshore transport (Walsh & Steidinger 2001, Walsh et al. 2006), vertical migration of *K. brevis* cells (Kamykowski et al. 1998), and water current circulation patterns (Tester & Steidinger 1997, Walsh et al. 2006). New N inputs from marine dinitrogen (N₂) fixation are thought to stimulate initiation of these blooms (Lenes et al.

2001, Walsh & Steidinger 2001, Mulholland et al. 2004b, 2006). Eolian transport and input of iron-rich Saharan Desert dust, is thought to stimulate blooms of the diazotrophic cyanobacteria *Trichodesmium* spp. (Walsh & Steidinger 2001), that fix N_2 and release fixed N into the surrounding water (Capone et al. 1994, Glibert & Bronk 1994, Mulholland et al. 2004a, b, Mulholland 2007) in forms that are bioavailable to *K. brevis* and co-occurring algae (Mulholland et al. 2006) including ammonium (NH_4^+) (Mulholland et al. 2004, Sinclair et al. 2009), amino acids (Capone et al. 1994), and other dissolved organic nitrogen (DON) compounds (Glibert & Bronk 1994, Mulholland et al. 2004, Sinclair et al. 2009). Nutrients released by *Trichodesmium* can potentially account for half the nutrients necessary for bloom initiation and biomass production (Mulholland et al. 2006, Vargo et al. 2008).

In addition to N inputs from N_2 fixation, a variety of other N sources may contribute to N inputs on the WFS. These include N inputs from estuaries (Vargo et al. 2004, 2008), where total N concentrations are high relative to offshore waters (Vargo et al. 2008), inputs of N through hurricanes and groundwater (Hu et al. 2006), and atmospheric deposition of nutrients (Pribble & Janicki 1999). Dissolved nutrients are also regenerated from sediments (Sinclair & Kamykowski 2008), through food web interactions (O'Neil et al. 1996), and from decaying *Trichodesmium*, fish, and other biota (Walsh et al. 2006, 2009). Regenerated N is necessary to maintain high cell biomass as blooms persist over time. Allelopathic inhibition of competing taxa (Kubanek et al. 2005) and the lack of predation on *Karenia brevis* (Turner & Tester 1997, 1998, Lester et al. 2001, Schofield et al. 2006) are non-nutrient related mechanisms that may also contribute to persistence *K. brevis* blooms on the WFS.

Inputs of nutrients from upwelling on the WFS are largely driven by wind patterns in the region that affect surface currents (Weisberg et al. 2009). Along the WFS, strong upwelling-favorable winds from the north or northeast reduce stratification and inject nutrient-rich water which generally supports diatom growth (Walsh et al. 2003). In contrast, wind relaxation is thought to reduce upwelling-associated nutrient inputs and result in a higher degree of stratification, conditions thought to foster the growth of cyanobacteria, including diazotrophs (Karl et al. 1995, Paerl 1996), and dinoflagellates (Tyler & Seliger 1978, Smayda & Trainer 2010), such as *Karenia brevis* (Steidinger et al. 1998, Walsh et al. 2006). Stratification also allows cells to accumulate in surface waters via vertical migration and positive phototaxis (Geesey and Tester 1993, Kamykowski et al. 1998). Once initiated, *K. brevis* populations are concentrated and transported to the WFS by currents (Steidinger & Haddad 1981, Yang & Weisberg 1999) and thermal and salinity fronts (Vargo et al. 2008).

Recently, it was discovered that *Karenia brevis* has the ability to ingest the unicellular marine cyanobacteria, *Synechococcus* (Jeong et al. 2005b, Glibert et al. 2009). Prior to this study (Chapter 2, 3), grazing by *K. brevis* (CCMP2228, CCMP2229, and an unidentified CCMP strain) on *Synechococcus* (Genbank Accession Number DQ023295 from the East China Sea and CCMP1768 from the GOM) had only been measured in nutrient-replete cultures under photic conditions (Jeong et al. 2005b, Glibert et al. 2009). Glibert et al. (2009) suggested that mixotrophic grazing by *K. brevis* might support the maintenance of *K. brevis* biomass during blooms. Alternatively, grazing might provide *K. brevis* an abundant nutrient source unavailable to co-occurring autotrophic phytoplankton during bloom initiation phase.

In this study, we conducted grazing experiments using natural populations of *K. brevis* collected during blooms dominated by *Karenia brevis* during October 2007, 2008, and 2009; during 2007, there was a co-occurring *Trichodesmium* bloom. In order to determine whether *K. brevis* would also graze in the absence of light, we conducted light and dark bottle experiments in 2008 and 2009. While it has been shown that grazing by *K. brevis* on *Synechococcus* is significant in cultured populations (Jeong et al. 2005b, Glibert et al. 2009, Chapter 2, 3), this is the first report, to our knowledge, of grazing by natural populations of *K. brevis* on *Synechococcus* in the GOM on the WFS.

Methods

Study Area. Grazing by natural populations of *Karenia brevis* on *Synechococcus* was examined during cruises aboard the *R/V Pelican* on the WFS in the GOM in October 2007, 2008, and 2009. Grazing experiments were conducted at stations where concentrations of *K. brevis* were relatively high ($\geq 10^1$ cells ml^{-1}) during each cruise (Fig. 11, Table 11), a condition necessary to detect grazing coefficients (hr^{-1}) and prey inclusions (Chapter 2). During a 2010 cruise, *K. brevis* cell concentrations too low to conduct grazing experiments (5 cells ml^{-1}). *Synechococcus* (CCFWC 502) isolated from the WFS and maintained at FWRI on GP media with soil extract (Loeblich & Smith 1968) was supplied as prey in all field experiments.

Biomass, Cell Abundance, and Nutrient Concentrations. At each station where grazing was measured, a CTD mounted on a rosette equipped with 12 or 20 l Niskin bottles was deployed. Surface water salinity, temperature, and fluorescence were measured and samples were collected from near the surface to measure *Karenia brevis*

and *Synechococcus* abundance, particulate carbon (PC) and nitrogen (PN), and dissolved nutrient concentrations (see below). In addition, surface water samples were collected at every station along the cruise transect (up to 12 stations daily) from Niskin bottles to map surface (upper 1 m) concentrations of *Synechococcus* and *K. brevis* during 2008, 2009, and 2010 cruises. Water samples were collected into 50 ml Falcon tubes and immediately preserved with unacidified Lugol's solution to enumerate *K. brevis* onboard. Prior to microscopic enumeration of *K. brevis*, tubes were gently mixed to ensure the sample was homogeneous. One ml of sample was then placed into a multi-well culture dish and another drop of unacidified Lugol's solution was immediately added. Once cells settled to the bottom, each *K. brevis* cell within the well was counted using a CK2 inverted light microscope at 100× magnification. Samples collected for later enumeration of *Synechococcus* cells were preserved in glutaraldehyde (1% final concentration), and stored at 4 °C until they were counted using a BD FACSCalibur (15 mW 488nm air cooled argon-ion laser) flow cytometer (FCM). *Synechococcus* cells natural populations and from each grazing experiment were enumerated by gating populations of cells based on forward light scatter against red auto-fluorescence. Total FCM photomultiplier tube intensities used for all *Synechococcus* counts were: forward light scatter (FLS) E01 (10× signal), side light scatter (SSC) 319 V, and red fluorescence (FL3) 505 V. Each sample was run with 0.5 µm fluorescent beads as an internal marker (Worden and Binder 2003). FCM sample runs were terminated after 30 seconds or when 10 million total events were recorded at the lowest intake speed. At least 1,000 *Synechococcus* cells were counted from each sample. Final *Synechococcus* concentrations (P_{RC} , cells ml⁻¹) were calculated

by dividing the number of gated events (forward light scatter against red autofluorescence) by the volume sampled (Equation 1) by the FCM.

Water samples for nutrient analyses were collected from Niskin bottles and filtered through GF/F Whatman filters (nominal pore size of 0.7 μm) to remove particles, and filtrate was placed in polyethylene bottles and stored frozen until analyses of NO_3^- , NH_4^+ , PO_4^{3-} , total dissolved N (TDN), total dissolved phosphorus (TDP), and urea. Samples for dissolved silicate analysis were filtered through Pall Supor (0.45 μm) membrane filters. Dissolved phosphate (PO_4^{3-}) (Grasshoff & Koroleff 1983, Murphy & Riley 1962), nitrate plus nitrite (hereafter referred to as NO_3^-) (Grasshoff & Koroleff 1983, Hanson 2000, Wood et al. 1967), total dissolved P (TDP) (Solorzano & Sharp 1980), and urea (Grasshoff & Koroleff 1983) were analyzed using a Bran+Luebbe/SEAL nutrient autoanalyzer. Total dissolved N (TDN), dissolved SiO_2 , and ammonium (NH_4^+) were analyzed on the same autoanalyzer according to the manufacturer's specifications (Bran+Luebbe 2004).

Water samples were also collected onto combusted (450 °C for 2 hours) GF/F filters for quantification of PC and PN and chlorophyll *a* (Chl *a*). Filters for PC and PN analysis were placed into combusted aluminum foil and stored frozen over desiccant until analysis. PC and PN were analyzed after combustion using an elemental analyzer (Thermo Electron 2004). Chl *a* samples were collected onto GF/F filters, placed into combusted aluminum foil, and analyzed within 2 weeks of collection (Holm-Hansen et al. 1965).

Field Incubation Experiments. At stations where *Karenia brevis* was abundant, additional seawater was collected for grazing experiments using a bucket. This

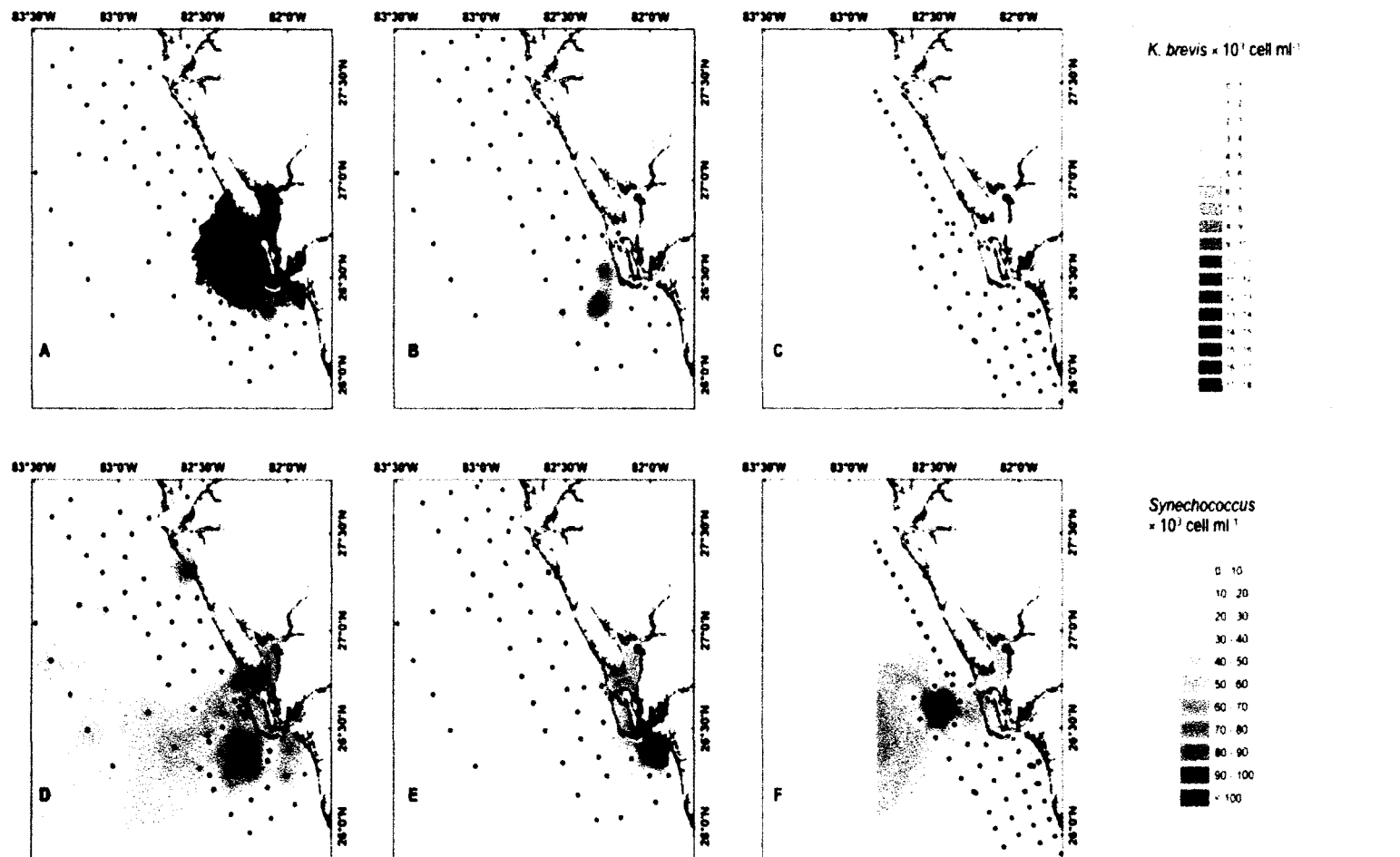
minimized stress on cells, and allowed collection of concentrated, surface-aggregated *K. brevis* cells. Whole water from the bucket was gently filtered through a 64 μm mesh net into another clean bucket to remove large zooplankton and colonies of *Trichodesmium*. Epifluorescent and light microscopic investigation of preserved water samples, verified that the 64 μm mesh effectively removed these groups. Microzooplankton grazers can contribute to grazing on picoplankton, including *Synechococcus* (Strom & Strom 1996, Campbell & Carpenter 1986), but this group of heterotrophs was not counted.

Consequently, ingestion rates reported here may overestimate grazing by *K. brevis* if microzooplankton were also grazing on *Synechococcus* in incubation bottles (Strom & Strom 1996). *K. brevis* were enumerated using light microscopy. In samples examined using epifluorescence (Fig. 12) and confocal laser scanning microscopy (Fig. 13), *K. brevis* was the only organism found containing autofluorescent prey inclusions. Imaging prey inclusions using confocal laser scanning microscopy (CLSM) within *K. brevis* from the WFS was improved using a Kalman filter to reduce background noise (Fig. 14).

In 2007, incubation bottles were prepared using whole water from a station at 26.54°N and -82.91°W and grazing by natural populations of *K. brevis* was measured using two methods: the prey disappearance (PD) method (Sherr & Sherr 1993b, Landry et al. 1995, Vasquez-Dominguez et al. 1999), which measures the disappearance of prey cells in prey-amended whole water incubation bottles relative to unamended whole water incubation bottles and cultures of prey in the absence of grazers; and the prey inclusion (PI) method, which measures prey inclusions normalized to *K. brevis* concentrations over time (Sherr et al. 1987). For the former, disappearance of *Synechococcus* was monitored in preserved subsamples using FCM. For the latter, epifluorescent and CLSM were used

Table 11. Date, station location (latitude, longitude), salinity (PSU), temperature (°C), Chl *a* (µg Chl l⁻¹), *K. brevis* and *Synechococcus* (*Syn*) cell concentrations (cell ml⁻¹), and nutrient concentrations (µM) during four annual cruises in October on the WFS. CTD data was collected by FWRI and nutrient data was collected and measured by MOTE Marine Laboratory.

	10/17/07	10/10/08	10/03/09	10/10/10
Latitude	26.27	26.64	26.35	26.31
Longitude	-82.02	-82.26	-82.30	-81.92
Salinity (PSU)	37.10	33.77	36.11	
Temp (°C)	28.25	27.87	30.66	
Chl <i>a</i> (µg Chl l ⁻¹)	4.03	1.16	2.68	
<i>K. brevis</i> (cell ml ⁻¹)	6.9 × 10 ³	0.38 × 10 ³	0.016 × 10 ³	0.005 × 10 ³
<i>Syn</i> (cell ml ⁻¹)	2.24 × 10 ⁶	1.76 × 10 ⁴	1.38 × 10 ⁴	7.41 × 10 ⁴
PC (µM)	32.0	17.3	16.3	
PN (µM)	2.61	2.13	1.78	
PC:PN	12.3	8.10	9.15	
PO ₄ ⁻³ (µM)	0.19	0.29	0.13	
TDP (µM)	0.36	0.68	0.39	
NO ₃ ⁻ (µM)	0.04	0.04	0.04	
NH ₄ ⁺ (µM)	0.14	0.14	0.07	
Urea (µM)	0.40	0.83	0.63	
TDN (µM)	11.9	12.9	9.00	
SiO ₂ (µM)	24.6	5.31	4.66	



to visualize prey inclusions microscopically.

In 2007, whole water from a station at 26.54°N and 82.91°W was placed into 125 ml Polyethylene Terephthalate Glycol-modified (PETG) bottles and triplicate bottles were amended with $8.6 \pm 0.7 \times 10^3$ *Synechococcus* cells ml⁻¹ and another set of triplicate bottles were not amended with *Synechococcus*. The *Synechococcus* addition represented a 0.4% enrichment of the natural *Synechococcus* population ($2.2 \pm 0.02 \times 10^6$ *Synechococcus* cells ml⁻¹) present at the station where samples were collected. Incubation bottles were then placed in deck incubators supplied with continuously flowing surface seawater and neutral density screen to simulate ambient temperature and light conditions. Following methods described by Jeong et al. (2005), I conducted a time course grazing experiment during which 4 ml subsamples were collected from incubation bottle after 0, 5, 10, 30, and 60 minutes to enumerate *Karenia brevis*, *Synechococcus*, and *Synechococcus* inclusions within *K. brevis*. Samples were placed into 5 ml cryovials, preserved with 1% glutaraldehyde (final concentration), and stored at 4 °C until they were analyzed at Old Dominion University. *Synechococcus*, *K. brevis*, and prey inclusions were counted using a combination of epifluorescent microscopy (EM), CLSM, and FCM as described above.

In 2008, grazing was examined during another *Karenia brevis* bloom on the WFS at a station located at 26.64°N and -82.26°W. During 2008, both light and dark bottle incubations were conducted. Surface water samples were obtained using a clean bucket and dispensed into 125 ml PETG incubation bottles. Incubation bottles were prepared as described for the experiment done in 2007; however, in 2008, prey-amended incubation bottles received $1.16 \pm 0.10 \times 10^5$ *Synechococcus* cells ml⁻¹. These additions resulted in



Fig. 12. Epifluorescent image of a *Karenia brevis* cell with one *Synechococcus* cell inclusion (white arrow) from an incubation bottle amended with *Synechococcus* CCFWC 502. Scale bar = 10 μm .

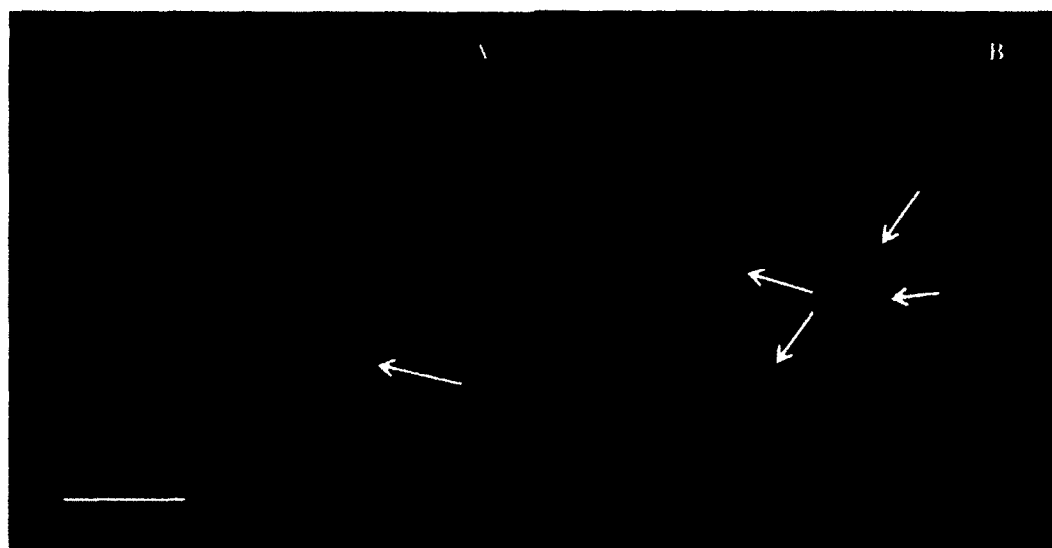


Fig. 13. Confocal laser scanning microscope image of *K. brevis* cells with one (A) and four (B) *Synechococcus* inclusions (white arrows) from WFS water amended with *Synechococcus*. Prey inclusions were not concentrated in a single area within the *K. brevis* cell.

a 13-fold increase in *Synechococcus* abundance relative to the natural surface populations. As in 2007, bottles were incubated in deck incubators, equipped with flow-through seawater and neutral density screen to simulate temperature and light levels in near-surface waters, or in the dark. Incubations were initiated at dawn and lasted until dusk, a period of 11 hours. Samples were collected from incubation bottles at the start and end of the incubation period to measure *Synechococcus* and *K. brevis* abundance. Subsamples were also examined to verify *Synechococcus* inclusions within *K. brevis* cells (Fig. 12, 13, 14). Samples for cell counts were preserved in glutaraldehyde (1 % final

concentration) and unacidified Lugol's solution for laboratory and shipboard cell counts, respectively.

In 2009, I examined ingestion rates in light and dark bottles during another *Karenia brevis* bloom at a station along the WFS at 26.35 °N, -82.30 °W. Incubations were set up as described above, with triplicate whole water and prey-amended whole water bottles, and incubated for 11 hours. For these experiments, prey-amended bottles received $3.7 \pm 0.2 \times 10^3$ *Synechococcus* cells ml⁻¹, an enrichment of approximately 3.7 % relative to the natural abundance of *Synechococcus* in surface water. Subsamples were taken at dawn and dusk, the beginning and end of the incubation period, respectively, and preserved in glutaraldehyde (1 % final concentration) or unacidified Lugol's solution for enumeration of *Synechococcus* and *K. brevis*. For light and dark bottle treatments, grazing coefficients were compared using an ANOVA ($p < 0.05$).

To calculate grazing coefficients, I assumed that *Karenia brevis* was the sole grazer in our bottle experiments. Grazing coefficients (hr⁻¹) were calculated from PD experiments (Sherr & Sherr 1993b, Landry et al. 1995, Vasquez-Dominguez et al. 1999) using Equation 4. In the case that *Synechococcus* growth rates in prey control incubations were found to be insignificant ($p > 0.05$), prey growth in prey-amended incubations was not taken into account when calculating grazing coefficients. However, if I observed growth in the prey control bottles, prey growth was accounted for when calculating grazing coefficients according to Frost (1962). Clearance rates (ml *Karenia brevis*⁻¹ hr⁻¹), a measure of the volume of water that *K. brevis* processes to encounter a prey cell, were calculated using Equation 2. Ingestion rates (*Synechococcus* *K. brevis*⁻¹ hr⁻¹) were also calculated for each experiment using Equation 3.

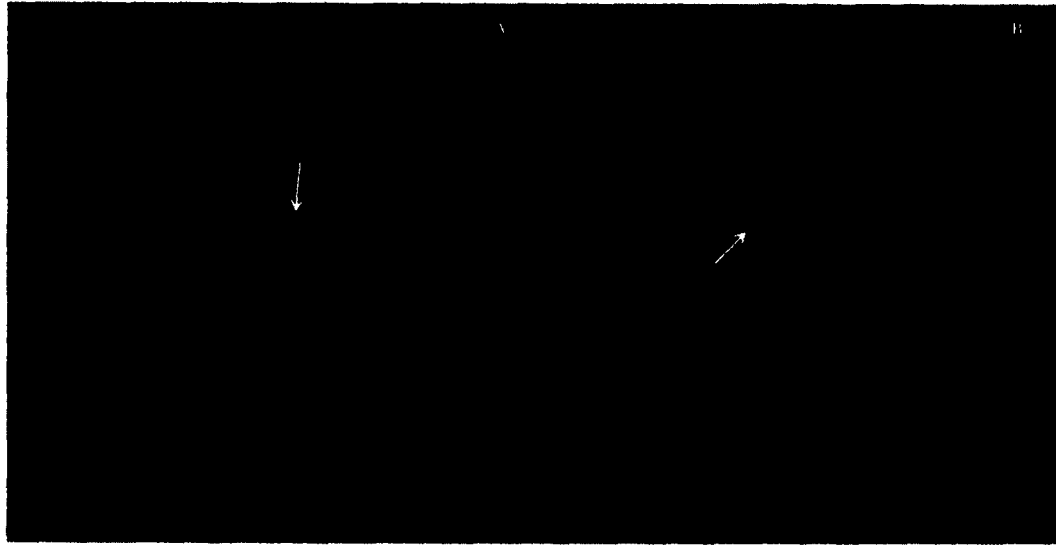


Fig. 14. Confocal laser scanning microscope image of *Karenia brevis* cells with *Synechococcus* inclusions (white arrows) from prey-amended incubations on the WFS in 2008. A Kalman filter was used to reduce background fluorescence (A and B).

To determine the number of prey inclusions, 1 ml of sample was filtered and *Karenia brevis* cells were enumerated microscopically by counting 10 random fields and at least 250 cells at $200\times$ magnification using EM. Ingested *Synechococcus* cells were counted using a modification of the method described by Jeong et al. (2005). Samples were slowly scanned in a random direction at $600\times$ magnification counting at least 30 dinoflagellates while searching for prey inclusions, which appeared as small ($1\text{--}2\text{ }\mu\text{m}$), solid red-orange cells within each dinoflagellate (Fig. 12, 13, 14). Each filter was scanned in this way 10 times, starting at a new and random location each time. *Synechococcus* cell inclusions were visualized using autofluorescence (Jeong et al. 2005b). This method of visualization may have underestimated grazing as prey

inclusions could be obscured behind chloroplasts (Fig. 15), which also appear as large red intracellular components. When an inclusion was found, microscope settings were switched from EM to CLSM so that the image could be scanned along the z-axis, to ensure that the *Synechococcus* was actually ingested rather than attached to the cell surface. Each *K. brevis* cell was scanned in 0.2 μm slices through the z-axis and the magnification was increased from 600 \times to 3,000 - 6,000 \times magnification by digital zooming (5 - 10 \times magnification) to optimize prey inclusion visualization.

In order to calculate grazing coefficients using the PI method, observed prey inclusions were normalized to grazer abundance and then plotted versus time (Sherr et al. 1987). The slope of the linear portion of the data was equal to the ingestion rate (*Synechococcus Karenia brevis*⁻¹ hr⁻¹). Clearance rates were calculated by dividing the ingestion rates by prey cell concentrations and grazing coefficients were calculated by multiplying clearance rates by the grazer concentrations.

To test whether grazing coefficients calculated from PD and PI methods were significantly different, a one sample t-test was run comparing the mean grazing coefficient from the PI method to the mean grazing coefficient from the PD method at a significance level of $p < 0.05$.

Prey Inclusions in Natural Populations. Water samples (50 ml) were collected during *Karenia brevis* blooms during cruises in 2007, 2008, and 2009 and preserved with glutaraldehyde (1% final concentration) to microscopically search for prey inclusions (as described above) in natural populations of *K. brevis* and determine whether there was evidence of grazing *in situ* (Sherr et al. 1987, Jeong et al. 2005b).

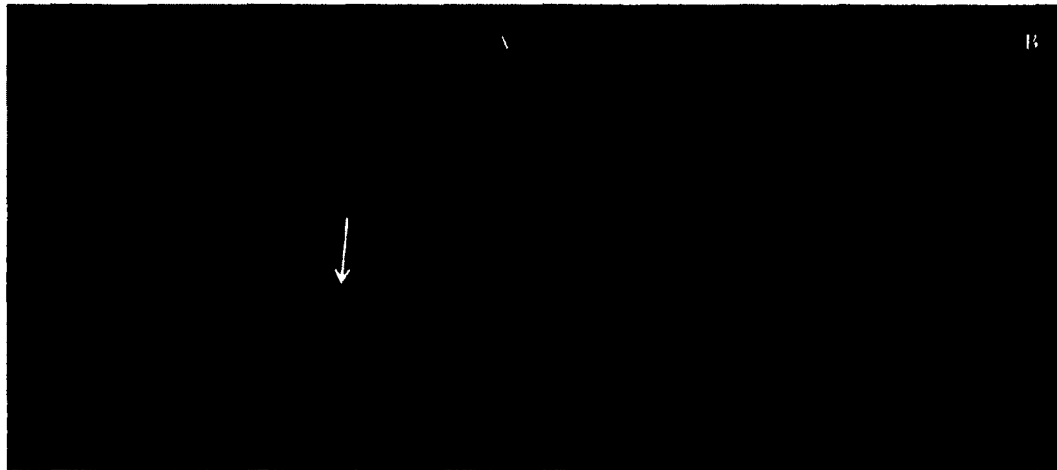


Fig. 15. Confocal laser scanning image of a *Karenia brevis* cell with one *Synechococcus* inclusion (white arrow) in view (A) and hidden (B) by a chloroplast.

Results

Karenia brevis *Abundance and Environmental Conditions.* During cruises along the WFS between 2007 and 2010, blooms of *Karenia brevis* on the order of 10^3 cells ml^{-1} in 2007, and 10^2 cells ml^{-1} in 2008 and 2009 were observed, which were densities great enough to observe a grazing response. During the 2007 *K. brevis* bloom, there was a co-occurring *Trichodesmium* bloom and I examined grazing using two methods, the PI method that had been used in previous studies (Sherr et al. 1987, Jeong et al. 2005b), and a PD method adapted for this study and used also in laboratory experiments described in Chapters 2 and 3 (Sherr and Sherr 1993b, Jeong et al. 2005b). In 2008 and 2009, *K. brevis* cell densities were lower than cell densities measured in 2007 and *Trichodesmium* were present at background concentrations, defined as $0.001 - 0.0035 \times 10^3$ colonies ml^{-1} (Hood et al. 2004), but not abundant. Grazing experiments were not conducted in

October 2010 because *K. brevis* cell densities were 5 cells ml⁻¹, which were too low to do so during this cruise.

In 2007, sustained winds from the north and northeast created upwelling favorable conditions for almost 2 weeks prior to the cruise (Fig. 16A). At the station where the grazing experiment was initiated, surface salinity and temperature were 37.10 and 28.25 °C, respectively (Table 11). Average concentrations of NH₄⁺, NO₃⁻, and urea were 0.14 μM, 0.04 μM, and 0.40 μM, respectively. Concentrations of PO₄⁻³ and dissolved silica were 0.19 μM and 24.6 μM, respectively. TDN and TDP concentrations were 11.9 and 0.36 μM, respectively. Concentrations of Chl *a* were 4.03 μg l⁻¹ and PC and PN concentrations were 32.0 and 2.61 μM, respectively. In addition to there being abundant *Trichodesmium* above background concentrations, defined as $> 0.0035 \times 10^3$ colonies ml⁻¹ (Hood et al. 2004), *Synechococcus* concentrations were also high ($2.2 \pm 0.02 \times 10^6$ cells ml⁻¹) at the station where grazing experiments were done. *Karenia brevis* cell concentrations were $6.9 \pm 0.7 \times 10^3$ cells ml⁻¹ (Table 11), a concentration that can produce enough brevetoxin to kill fish (Walsh et al. 2006). Indeed, dead fish were observed throughout the 2007 cruise.

In 2008, upwelling favorable winds lasted for a shorter duration directly before sampling occurred on the WFS, and *Karenia brevis* abundance was near background concentration (~1 cells ml⁻¹). On October 10, 2008, at the station where grazing experiments were conducted, surface salinity and temperatures were 33.8 and 27.9 °C, respectively. NH₄⁺, NO₃⁻, and urea concentrations were lower than in 2007 and were 0.1, 0.04, and 0.8 μM, respectively (Table 11). Concentrations of PO₄⁻³ and silicate were 0.3 and 5.3 μM, respectively. Concentrations of TDN and TDP were 12.9 and 0.7 μM,

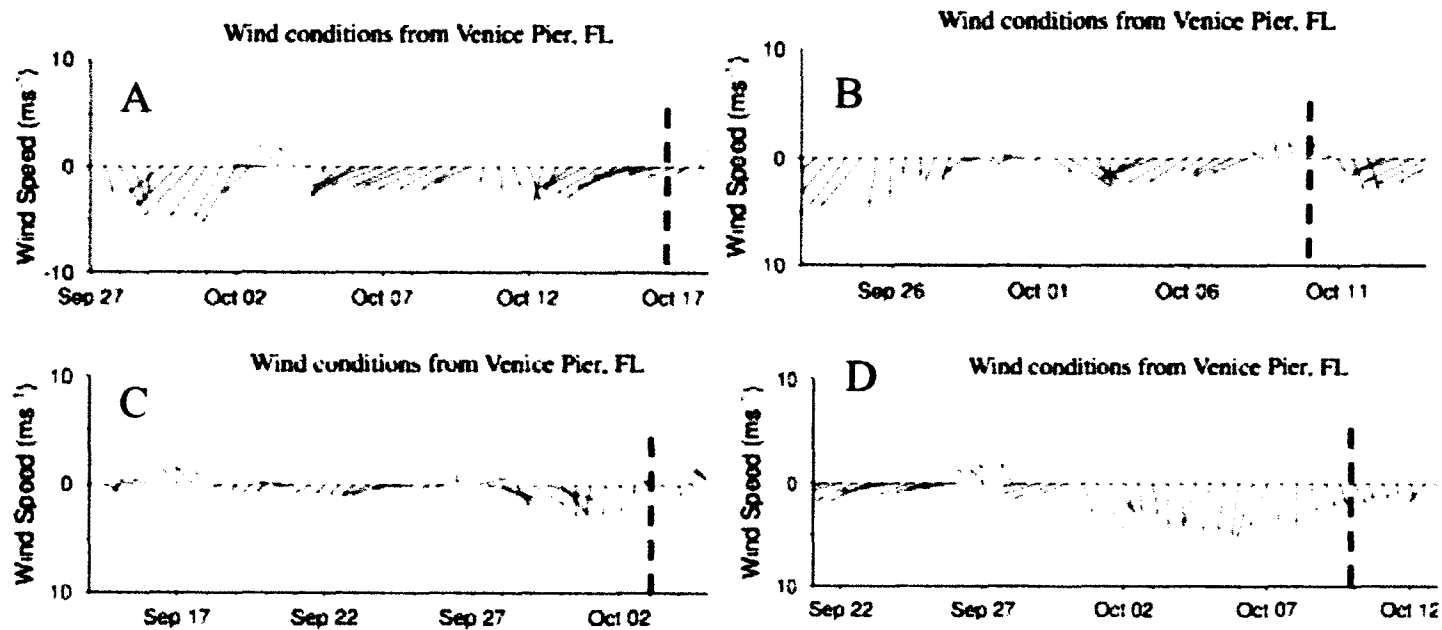
respectively. Chl *a* concentrations were $1.2 \mu\text{g l}^{-1}$ and PC and PN concentrations were 17.3 and $2.13 \mu\text{M}$, respectively. *Synechococcus* cell concentrations were $1.76 \pm 0.15 \times 10^4 \text{ cells ml}^{-1}$ (Table 11), 2 orders of magnitude lower than in 2007. Surface *K. brevis* cell concentrations were $0.38 \times 10^3 \text{ cells ml}^{-1}$.

In October 2009, wind speeds were less intense in the weeks preceding the cruise with upwelling favorable winds from the north/northeast occurring only in the 2 days prior to sampling (Fig. 16C). *Karenia brevis* cell concentrations were 10^1 to $10^2 \text{ cells ml}^{-1}$ during this cruise (Fig. 11B). At the station where grazing experiments were conducted, surface water temperature was 30.7°C , much higher than in 2007 and 2008 (Table 11) and salinity was 36.11. Surface *K. brevis* cell concentrations were just $0.016 \times 10^3 \text{ cells ml}^{-1}$, much lower than the two previous years. *Synechococcus* concentrations at the station where grazing experiments were conducted were similar to those measured in 2008. During 2009, nutrient concentrations were similar to or lower than in the previous 2 years (Table 11). Chl *a* concentrations were $2.7 \mu\text{g l}^{-1}$, which was higher than in the previous years. However, PC and PN concentrations were lower than in both previous years and were 16.28 and $1.78 \mu\text{M}$, respectively.

In October 2010, *Karenia brevis* populations were very low ($0.005 \times 10^3 \text{ cells ml}^{-1}$) (Table 11); concentrations were too low to conduct grazing incubations experiments.

Field Incubation Experiments. In 2007, grazing coefficients for natural populations of *Karenia brevis* amended with *Synechococcus* were $0.008 \pm 0.002 \text{ hr}^{-1}$ and clearance rates were $0.01 \pm 0.004 \times 10^5 \text{ ml K. brevis}^{-1} \text{ hr}^{-1}$ (Table 12) using the PD method. Grazing coefficients and clearance rates using the PI method were significantly

Fig. 16. Wind data were obtained from NOAA's National Weather Service (NWS) (<http://tidesandcurrents.noaa.gov/hab/>). Wind speed and direction were measured from buoys and are 12-hour averages. The length of line indicates wind speed and the angle indicates direction. Pink indicates that the wind direction was upwelling favorable near the coast. Blue lines indicate winds that were not upwelling favorable near the coast. Black dashed vertical lines represent day which samples for grazing experiments were taken in 2007 (A), 2008 (B), 2009 (C), or where highest *K. brevis* cell concentrations were measured in 2010 (D).



lower ($n = 6$, $t = 22.579$, $p = 0.002$) at $0.001 \pm 0.00007 \text{ hr}^{-1}$ and $0.002 \pm 0.001 \times 10^5 \text{ ml } K. brevis^{-1} \text{ hr}^{-1}$, respectively (Table 12).

In 2008, ambient *Karenia brevis* cell concentrations were $0.42 \pm 0.05 \times 10^3 \text{ cells ml}^{-1}$ (Table 13), lower by 2 orders of magnitude than in 2007 (Table 12). However, grazing coefficients for *K. brevis* on *Synechococcus* in 2008, were more than three times higher in dark bottle incubations than in parallel light incubations, 0.01 ± 0.004 and $0.003 \pm 0.0009 \text{ hr}^{-1}$, respectively (Table 11); however, the difference was not significant ($n = 6$, $F = 2.694$, $p = 0.176$).

During the small *Karenia brevis* bloom in 2009, grazing was examined in both light and dark bottle incubations. Initial *Synechococcus* concentrations in prey-amended light and dark bottles were 14.1 ± 0.04 and $14.3 \pm 1.1 \times 10^3 \text{ cells ml}^{-1}$, respectively (Table 12). There was significant growth of *Synechococcus* in unaltered bloom water in light and dark bottles ($n = 6$, $F = 2.355$, $p = 0.032$), therefore *Synechococcus* growth was accounted for when estimating prey removal in prey-amended bottles (Frost 1972). Grazing coefficients in 2009, were higher in both dark and light incubation bottles (0.07 ± 0.05 and $0.09 \pm 0.04 \text{ hr}^{-1}$, respectively) than those observed in 2008 and 2007 (Table 12) and the difference between light and dark bottles was not significant ($n = 6$, $F = 0.345$, $p = 0.589$).

Observations of Prey Inclusions in Natural Populations. Although natural water samples collected within blooms were examined during 2007, 2008, and 2009, no autofluorescent prey inclusions were observed in *Karenia brevis* cells where blooms were present.

Table 12. Initial cell concentrations of *Synechococcus* and *K. brevis* from whole water (WW) or prey-amended (PA) incubation bottles during a *K. brevis* bloom in October 2007, 2008, and 2009. Grazing coefficients, clearance and ingestion rates using PI and PD incubation methods are shown (n = 3). In 2007 rates were calculated over a one-hour incubation period under photic conditions. In 2008 and 2009, incubations lasted 11 hours and were conducted in light (L) and dark (D) bottles. Standard deviations are in parentheses. Values (*) are significantly different at the $p < 0.05$ level.

Year	Lat	Long		n (cell counts)	<i>Synechococcus</i> (cell ml ⁻¹)	<i>K. brevis</i> (×10 ³ cell ml ⁻¹)	Incubation method	<i>g</i> (hr ⁻¹)	Clearance rate (×10 ⁻⁵ ml <i>K. brevis</i> ⁻¹ hr ⁻¹)	Ingestion rate (<i>Synechococcus K. brevis</i> ⁻¹ hr ⁻¹)
2007	26.54	-82.91		6		6.9 (0.7)				
			WW	3	2.24 (0.18) ×10 ⁶					
			PA	3	2.25 (0.26) ×10 ⁶					
							PD (L)	0.008* (0.002)	0.01 (0.004)	2.4 (0.8)
							PI (L)	0.0001* (0.00007)	0.002 (0.001)	0.04 (0.02)
2008	26.64	-82.26		6		0.42 (0.04)				
			WW	3	1.06 (0.08) ×10 ⁴					
			PA	3	1.35 (0.06) ×10 ⁵					
							PD (D)	0.01 (0.004)	2.6 (0.9)	1.9 (0.8)
							PD (L)	0.003 (0.0009)	0.6 (0.2)	0.4 (0.2)
2009	26.35	-82.30		6		0.08 (0.03)				
			WW	3	1.07 (0.10) ×10 ⁴					
			PA	3	1.41 (0.04) ×10 ⁴					
							PD (D)	0.07 (0.05)	91.7 (56.3)	9.7 (6.0)
							PD (L)	0.09 (0.04)	109.8 (38.6)	15.5 (5.4)

Discussion

Until recently, *Karenia brevis* was thought to be obligate photoautotroph that blooms in areas where ambient dissolved inorganic and organic N and P concentrations are low (Aldrich 1962, Steidinger et al. 1998, Havens et al. 2004). However, it has now been demonstrated that *K. brevis* is a mixotroph that can also graze on *Synechococcus*, although results to date are from cultured isolates (Jeong et al. 2005b, Vargo et al. 2008, Glibert et al. 2009, Chapter 2, 3). Results presented here show, for the first time, that natural populations of *K. brevis* on the WFS can phagotrophically ingest *Synechococcus* (CCFWC 502).

Although prey inclusions were not observed in natural populations of *Karenia brevis*, *Synechococcus* is abundant in surface waters on the WFS (Paul et al. 2000, Philips et al. 1989, 1999, Fig. 11) at concentrations greater than the lower feeding threshold for grazing (Chapter 2, Fig. 6). *Synechococcus* concentrations in surface waters during cruises ranged from 10^3 to 10^6 cells ml^{-1} (Fig. 11); however, there was no significant correlation ($n = 44$, $p = 0.478$) between *K. brevis* and *Synechococcus* abundance (Fig. 17). This is not surprising as there is likely a lag correlation between predator and prey abundance and the sampling program was not designed to test hypotheses related to mixotrophic grazing. An inverse relationship between *Synechococcus* and *K. brevis* concentrations was observed during a transect through an area of increasing *K. brevis* abundance in 2007 on the WFS (Sipler et al., in Revision), but this was strictly correlative; however, this may be caused by *K. brevis* grazing pressure. Predator-prey abundances are frequently out of synch when predation is strong.

One reason why prey inclusions may not have been observed in natural populations of *Karenia brevis* is that ingested prey are rapidly digested making it difficult to visualize inclusions. This is consistent with the observation that grazing coefficients estimated using the PI method were much lower than those calculated using the PD method in 2007 (Table 12). While this discrepancy could be due to a variety of reasons, including difficulty in visualizing inclusions because they are easily hidden by

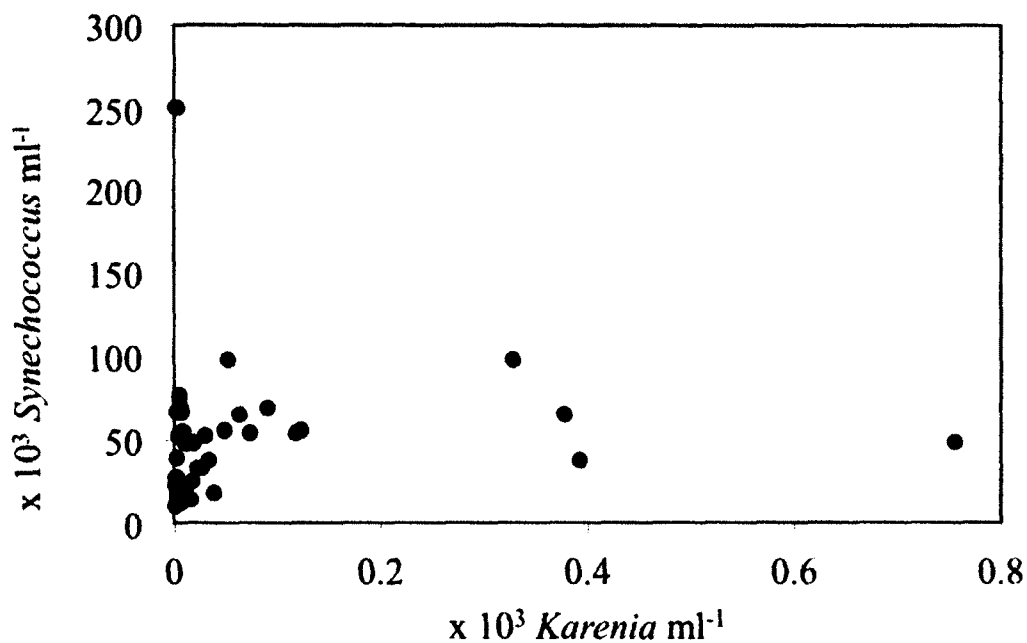


Fig. 17. Relationship between field populations of *Karenia brevis* and *Synechococcus* on the West Florida Shelf from October 2008, 2009, and 2010.

chloroplast autofluorescence (Fig. 15), an obvious methodological artifact is that only undigested *Synechococcus* cells can be observed as inclusions within *K. brevis*. Digested cells and partially digested cells are not observed as inclusions, and this could have resulted in underestimates of *K. brevis* ingestion and grazing coefficients using the PI method. Additionally, it has been found that some cells purge prey inclusions after being fixed with an aldehyde (Sieracki et al. 1987) and this can result in underestimates of grazing using the PI method. This caveat may have made seeing prey inclusions in natural water (with no prey amendments) more difficult to visualize. A better method for preservation may be the Lugol's and sodium thiosulfate method (Sherr & Sherr 1993a), which may reduce the possibility of prey cells becoming egested.

Our calculated ingestion rates of *Synechococcus* by natural populations of *Karenia brevis* from the WFS in 2007, using the PD method, are within the range previously reported for laboratory cultures of *K. brevis* (Jeong et al. 2005b, Glibert et al. 2009). However, while ingestion rates by *K. brevis* on *Synechococcus* can be underestimated using the PI method; ingestion rates calculated using the PD method could potentially overestimate grazing because prey organisms could also disappear as a result of grazing by other heterotrophic organisms present in natural waters. Although they were not counted and were assumed to contribute little to grazing coefficients, ciliates and heterotrophic nanoflagellates are known to graze on *Synechococcus* in coastal environments (Campbell & Carpenter 1986, Strom & Strom 1996), are abundant on the WFS (Strom & Strom 1996, Juhl & Murrell 2005), and may have been in competition with *K. brevis* for prey during incubations as they were most likely not removed by the 64 μm mesh through which whole water was pre-screened. However, microzooplankton

cell densities in the Northern GOM range between 10^1 and 10^2 cells ml^{-1} (Strom & Strom 1996), which is up to 2 orders of magnitude lower than the *K. brevis* cell densities measured during blooms on the WFS. Based on their relative abundance and the relative clearance rates these competing organisms would need to achieve to acquire similar amounts of prey, it is unlikely that they contributed significantly to the observed grazing during our incubation experiments. Therefore, the ingestion rates presented in the current study represent conservative rates.

Ingestion rates of *Karenia brevis* on *Synechococcus* were lower in 2008 than in 2007, based on the PD method (Table 12). This may be due to differences in bloom phase, concentrations of *K. brevis*, or the physiological status of *K. brevis* cells between years. In 2007, the *K. brevis* blooms was relatively large and was already well-established while in 2008, the bloom was initiating and *K. brevis* populations were less dense (Heil et al. Submitted). Natural populations of *Synechococcus* were also denser in 2007 compared to 2008 (Table 11), conditions which may have fostered the higher ingestion rates during that year (Table 12). Mixotrophic grazing is prey concentration-dependent and ingestion rates increase as prey cell abundance increases for many dinoflagellates (Jeong et al. 2005a, b), including *K. brevis* (Fig. 6). The even higher ingestion rates measured in 2009, when natural populations of *Synechococcus* were below the lower feeding threshold (1.86×10^4 *Synechococcus* ml^{-1}) for *K. brevis* grazing on *Synechococcus* under laboratory conditions, further demonstrate the annual variability in grazing and complexity of the relationship between grazing and environmental variables during *K. brevis* blooms.

In laboratory experiments, linear grazing responses were observed for cultured isolates of *Karenia brevis* over 4 to 6 hours when plotted against natural log transformed cell abundance. After 6 hours grazing appears to be saturated and prey removal is reduced as maximum clearance rates attained (Fig. 6A). Rates reported here, for 2008 and 2009 data, therefore may also be underestimates because of the long incubation times.

Mixotrophic grazing by other dinoflagellate species has been shown to be enhanced when dissolved N and P are limiting (Li et al. 1999, 2000; Smalley et al. 2003). In this study, additions of nutrient-replete cultured *Synechococcus* likely added nutrients to prey-amended incubations that could have supported autotrophic *Karenia brevis* growth or inhibited grazing in incubation bottles. However, I demonstrate that *K. brevis* graze equally well on picoplankton under nutrient-replete (f/2 culture media: NO_3^- 8.83×10^{-4} M and PO_4^{3-} 3.63×10^{-5} M) (Jeong et al. 2005b, Glibert et al. 2009, Chapter 2, 3) and nutrient-deplete (modified f/2 with no added N or P) (Chapter 2, 3) conditions. Consequently, I don't believe that nutrients added during cultured prey amendments and the small changes in nutrient concentrations between years in the study area (Table 11) affected grazing by *K. brevis*.

Light and dark cycles may also play an important role in the balance of heterotrophic and autotrophic metabolism in mixotrophic dinoflagellates. Legrand et al. (1998) reported that prey inclusions were more frequent in *Heterocapsa triquetra* cells taken from samples incubated in the dark versus those taken under light conditions. In this study, grazing coefficients by *Karenia brevis* in dark treatment bottles were not significantly different than in light bottles, in 2008 and 2009 (Table 12). This could be

important for sustaining *K. brevis* growth at night or at depth on the WFS. If *K. brevis* is capable of grazing at similar rates in total darkness, then it seems possible that ‘seed’ populations could survive below the euphotic zone or in the disphotic zone, where light is minimal and heterotrophic prey cells are also abundant. The ability to graze at night may allow *K. brevis* to augment light-dependent photosynthesis during the daytime with uptake of nutrients from grazing at night. Other dinoflagellates species have been shown to phagotrophically ingest prey cells in complete darkness or when growing at low light levels and may gain a majority of their energy needs and important nutrients for growth at night via phagotrophic grazing (Skovgaard 1996, Hansen & Nielsen 1997).

Previous research has highlighted the importance and competitive benefits associated with mixotrophy by harmful algal species (Thingstad et al. 1996, Burkholder et al. 2008). It has been suggested that the metabolic cost of phagotrophic ingestion of prey may be less than maintaining autotrophic machinery and that switching between metabolisms may minimize the metabolic cost to nutritional benefit ratio between heterotrophy and autotrophy (Raven 1997, Skovgaard et al. 2000, Adolf et al. 2006). It has been shown that *Karenia brevis* autotrophic (Table 7, 8) and osmotrophic (Table 9) abilities may be enhanced while phagotrophically ingesting prey, which may offer *K. brevis* a metabolic edge over co-occurring phytoplankton that cannot utilize multiple metabolisms by allowing them to obtain C and nutrients from different sources during both day and night on the WFS.

To date, grazing has not been included as a nutrient source in models aimed at describing or predicting bloom initiation, growth, or maintenance. N and C inputs from grazing could significantly alter current models of blooms on the WFS. Vargo et al.

(2004) estimated that *Karenia brevis* populations at a concentration of 3×10^5 cells l^{-1} require 0.056 to $0.267 \mu\text{mol N } l^{-1} d^{-1}$ to grow at a division rate of $0.2 d^{-1}$. Potential hourly phagotrophic N uptake rates, based on *Synechococcus* particulate N concentrations (Table 1) were calculated and range from 0.05 to $13.86 \mu\text{mol N } l^{-1} hr^{-1}$ (Table 13). The calculated range of N uptake from *Synechococcus* prey is within the range of N uptake rates estimated for NH_4^+ , NO_3^- , and urea (Mulholland et al. 2006, Killberg-Thoreson et al. 2012), and *Trichodesmium* exudation (Mulholland et al. 2006) (Table 13). Therefore, N acquisition from nutrient- and light-independent grazing on *Synechococcus* cells, may contribute to the initiation and maintenance of *K. brevis* blooms on the WFS in the GOM.

Conclusions. *Karenia brevis* is a nutritionally flexible mixotrophic dinoflagellate with a wide range of nutrient acquisition strategies and allochthonous and autochthonous nutrient sources available to support its growth. The balance between autotrophic, heterotrophic, and phagotrophic nutrient uptake by *K. brevis* may be key to understanding blooms and how *K. brevis* outcompetes co-occurring phytoplankton on the WFS. However, additional studies are needed to better understand controls on mixotrophic grazing in the laboratory and in nature. In this study, we demonstrate that natural populations of *K. brevis* on the WFS can graze on one of the most numerically abundant cyanobacterial groups, *Synechococcus*. Additional investigations should address grazing in non-bloom GOM waters compared to grazing in *K. brevis* bloom water to determine the grazing impact nanoflagellate grazers have on the *Synechococcus* populations and the diversity of prey available to *K. brevis*, such as other cyanobacteria (*Prochlorococcus*) and heterotrophic bacterial (as suggested by Meyer et al. Submitted). Finally, because many mixotrophs take up dissolved organic compounds that include carbon (Glibert &

Legrand 2006, Bronk et al. Submitted, Table 10), the capacity for osmotrophic carbon uptake in the field should be examined in future studies of this and other bloom-forming mixotrophs.

Table 13. Literature values of N sources available to *K. brevis* from field and laboratory uptake experiments and N-specific uptake rates for each source of N available for uptake on the WFS.

N Source			$\mu\text{mol N l}^{-1} \text{ hr}^{-1}$	$\mu\text{mol N l}^{-1} \text{ d}^{-1}$
Killberg-Thoreson 2011	NH_4^+	Field 2007	0.34 - 4.16	
	NO_3^-	Field 2007	0.07 - 0.60	
	Urea	Field 2007	0.07 - 0.62	
Mulholland et al. 2004a	<i>Trichodesmium</i> exudate		0.15 - 1.15	
Current study	<i>Synechococcus</i>	Field 2007	PI	0.05
			PD	3.01
		Field 2008	Light	0.03
			Dark	0.13
		Field 2009	Light	0.27
			Dark	0.11
		DIC v Grazing	Light	0.21
		Nutrient Replete	Dark	0.17
		DIC v Grazing	Light	0.76
		Nutrient Deplete	Dark	0.47
		Functional Response		1.38
		Nutrient Replete (SB3)		3.10
		Nutrient Replete (CH2)		3.88
		Nutrient Deplete (CH2)		0.54
		Nutrient Deplete (SB3)		3.77
		Light (JC4)		13.86
		Dark (CH2)		3.67
		Heat-killed (CH2)		0.55
		Heat-killed (JC4)		1.38
Vargo et al. 2004	Total N demand			0.0567 - 0.267

CHAPTER V

CONCLUSIONS

The primary goal of this study was to measure grazing by *Karenia brevis* on potentially co-occurring planktonic organisms in the laboratory, using cultured *K. brevis* isolates, and during blooms on the WFS. Here I present data demonstrating that *K. brevis* isolates from the West Florida Shelf (WFS) in the Gulf of Mexico (GOM) graze on *Synechococcus* (Chapters 2 and 3) at rates comparable to those observed in previous studies using other cultured isolates (Jeong et al. 2005b, Glibert et al. 2009).

I also demonstrate that *Karenia brevis* can also ingest *Prochlorococcus marinus* and heterotrophic bacteria (Chapter 2), suggesting that there may be multiple prey organisms that can fuel *K. brevis* blooms on the WFS. Mixotrophic grazing by *K. brevis* on co-occurring plankton may significantly alter our understanding of nutrient acquisition during bloom initiation and progression in the Gulf of Mexico (GOM) as prey ingestion supplies C as well as other macro- and micronutrients and trace elements necessary for growth.

In Chapter 2, I examine grazing by *Karenia brevis* on *Synechococcus* in nutrient-replete and -deplete media, under light and dark conditions, and with varying prey concentrations. Previous studies reported ingestion rates ranging 0.96 - 83.8 *Synechococcus K. brevis*⁻¹ hr⁻¹ for an unidentified strain from the Provasoli-Guillard National Center for Culture of Marine Phytoplankton (CCMP) collection (Jeong et al. 2005b), and CCMP 2228 and 2229 (Glibert et al. 2009). The rates reported for WFS isolates in Chapter 2 are all within the range observed for the CCMP isolates (Table 2).

In this and previous studies prey concentrations and incubation lengths varied and so it is difficult to draw conclusions regarding absolute controls on grazing by *Karenia brevis* (Table 6). Ingestion rates were highly dependent on prey concentrations in experiments designed to determine the functional response of *K. brevis* to prey concentration; below some lower feeding threshold (1.86×10^4 cells ml⁻¹) grazing did not occur, and above some saturating prey concentration ($\sim 1.95 \times 10^6$ cells ml⁻¹), ingestion rates did not increase much with increasing prey concentrations. Further, I determined that the optimal length for grazing experiments was < 4-6 hours, after which time grazing appeared to be saturated. The functional response and time course experiments conducted here are the first such experiments for *K. brevis* and are important for constraining future experiments and interpreting previous results from experiments where prey amendments varied and incubation lengths ranged from 1 to 25 hours.

The major findings presented in Chapter 2 include the following: First, although N and P limitation have been shown to stimulate mixotrophic grazing by other dinoflagellate species (Li et al. 1999, 2000; Smalley et al. 2003), the presence or absence of N and P do not seem to control grazing by *Karenia brevis* on *Synechococcus* (Table 4). However, in nutrient-deplete experiments reported here, cultures had not been acclimated for several generations to a low-nutrient environment, but were acclimated for at least 48 hours prior to incubation, because it was difficult to maintain cultures in continuous and semi-continuous cultures. Therefore, although resuspension of *K. brevis* in medium deplete in N and P did not trigger *K. brevis* grazing on *Synechococcus*, I cannot rule out that longer term nutrient deprivation might induce grazing. At the same time, high nutrient concentrations did not inhibit grazing in this study either suggesting that at least

some grazing by *K. brevis* is constitutive. Results presented here suggest that *K. brevis* can potentially graze at similar rates over a range of nutrient conditions, including those characteristic of inshore and offshore environments, and along a gradient from the nutrient-rich, eutrophic waters near Tampa Bay to the nutrient-impoverished oligotrophic waters in the GOM.

Second, light availability has been shown to stimulate or inhibit grazing among mixotrophs (Burkholder et al. 2008, Smalley et al. 2003). Legrand et al. (1998) found that there was no difference in grazing by *Heterocapsa triquetra* on fluorescently labeled algae (FLA) in the light versus dark. Similarly, for *Karenia brevis*, there was no significant difference in grazing coefficients between day and night incubations (Table 4). The ability to acquire macronutrients, trace elements, and other growth factors through grazing at comparable rates in the light and dark is another competitive advantage that may help *K. brevis* outcompete co-occurring obligate autotrophs. Grazing in low light or dark conditions could also allow ‘seed’ populations present at or in the sediments on the WFS to thrive in the absence of sunlight.

In addition, when blooms are fully developed, biomass can be so great that cells in the water column being self-shaded thereby limiting photoautotrophic growth. Being able to graze in the dark may allow *Karenia brevis* to continue to acquire C and nutrients when other photoautotrophs cannot and this may also offer them nutritional flexibility when light becomes limiting as a result of self-shading. Third, prey abundance has been shown to affect ingestion rates by some mixotrophic harmful algal species (Lin et al. 2004, Jeong et al. 2005b, Adolf et al. 2008). During a functional response experiment, I showed that there was a lower feeding threshold, a prey density below which grazing was

not detected. Further, *K. brevis* ingestion rates increased with increasing prey density up to some upper threshold beyond which grazing was saturated (Fig. 6). In the GOM many nutrient sources are available to *K. brevis* and grazing may be yet another source of nutrients fueling the development and sustenance of large blooms that can reach and remain at 10^6 cells l^{-1} for weeks to months. In 1994, there was a nearly year-long bloom that spanned the west coast of Florida during which cell densities remained above 10^3 to 10^9 cells l^{-1} .

Together my results suggest that grazing by *Karenia brevis* at similar rates during all bloom phases from bloom initiation when cell concentrations are low before nutrient becoming limiting. Grazing on co-occurring plankton may also happen during bloom maintenance when nutrients may become depleted and self-shading may limit light availability. Along the WFS in the GOM, *Synechococcus* concentrations range from 10^4 to 10^6 cells ml^{-1} (Fig. 12 in Chapter 4; Paul et al. 2000), which is at or above the lower feeding threshold of 1.86×10^4 cells ml^{-1} (Fig. 6). Finally, *K. brevis* can also graze on *Prochlorococcus* (Fig. 8D) and heterotrophic bacteria (Table 5) that may also be available on the WFS (Weinbauer et al. 1996, Paul et al. 2000, Jochem 2001, Long et al. 2008). The diversity of prey available to *K. brevis* may include other species not tested in this study or species tested here but provided at insufficient concentrations to induce grazing in this study under nutrient replete conditions in the light (Fig. 8A, B, C).

Previous studies (Jeong et al. 2005b, Glibert et al. 2009) have suggested that mixotrophy among harmful algal species, including *Karenia brevis*, is a mode of metabolism that may allow blooms to sustain biomass based on increased growth rates when grazing on *Synechococcus*. Mixotrophic ingestion of prey cells has been shown to

increase growth rates in many phagotrophic dinoflagellates (Jeong et al. 2004, Adolf et al. 2006, Stoecker et al. 2006, Glibert et al. 2009). *K. brevis* growth rates were not calculated during the short-term incubations. Instead, N- and C-specific assimilation rates, based on cell N or C content and ingestion rates, were calculated for *K. brevis* grazing on *Synechococcus* (Table 3, 4). These rates are comparable to the range of growth rates reported for *K. brevis* under bloom conditions on the WFS (Redalje et al. 2008, Hitchcock et al. 2010); however, the N- and C-specific assimilation rates do not take loss terms, such as respiration, into account. Increased N and C assimilation from phagotrophic grazing and the ability to ingest potential competitors on the WFS may give *K. brevis* an additional competitive advantage that at least partially allows such large monospecific blooms annually as some mixotrophs exhibit a competitive advantage over obligate autotrophs and heterotrophs (Bockstahler & Coats 1993a, b).

I also estimate that rates of N uptake due to prey ingestion were on the same order of magnitude as that of N uptake from NH_4^+ , NO_3^- , and urea (Killberg-Thoreson 2011), and *Trichodesmium* exudate (Mulholland et al. 2006) (Table 13). My estimate of N uptake from phagotrophy alone exceeded the daily N requirement of for a *Karenia brevis* population of 3×10^5 cells l^{-1} (Table 13, Vargo et al. 2004). N acquisition from prey ingestion may contribute to the development or maintenance of the high biomass observed during *K. brevis* blooms.

In addition to evaluating ingestion rates with respect to nutrients, light, and prey availability, I determined that *Karenia brevis* ingested heat-killed *Synechococcus* at statistically similar rates as live *Synechococcus* (Fig. 7). This is important as it allows one to measure the effects of prey abundance and grazing on photosynthesis and

dissolved N uptake by *K. brevis* in incubation experiments amended with heat-killed prey. Heat-killed prey may also be stained and used in field experiments. In addition, the use of heat-killed prey allows the preparation of uniformly dense stocks to use in prey amendment experiments thereby reducing variability in ingestion rates due to prey density. Because of differences in densities of live cultures used in these studies, variability in ingestion rates observed here was likely due in part to differences in prey density between experiments.

In Chapter 3, using heat-killed *Synechococcus*, I compared autotrophic and osmotrophic C uptake with C uptake estimated from ingestion of prey in laboratory experiments. When *Karenia brevis* were supplied *Synechococcus* as prey, phagotrophy was the dominant source of C uptake (Table 7, 8, 9). Surprisingly, when *K. brevis* was provided prey, DIC uptake was higher than in cultures that had not been amended with heat-killed prey. Although my estimate of C acquired via ingestion of prey was higher than that from bicarbonate and amino acids, I was unable to measure the uptake efficiency of C from prey. Higher C uptake from prey cells than from inorganic C have been attributed to lower uptake efficiency of prey cells for other mixotrophic protists (Adolf et al. 2006).

When amended with heat-killed prey, nutrient-replete *Karenia brevis* fixed inorganic C at similar rates in light bottles and in cultures that had been in the dark for 48 hours (Table 7). Additionally, amendments of heat-killed prey stimulated C fixation by *K. brevis* (Table 7). These results suggest that ingestion of prey may alleviate some other limitation on C fixation by *K. brevis*. Amino acid C uptake by *K. brevis* was also stimulated by the addition of heat-killed prey, similar to DIC uptake, likely due to

enhanced availability of organic compounds due to sloppy feeding and release of cellular material from heat-killed cells (Bronk & Steinberg 2008). The effect of grazing on the production of dissolved organic matter, in particular highly bioavailable compounds such as amino acids, and the subsequent uptake of these compounds should be addressed in future work but was beyond the scope of this study.

In Chapter 4, I examined ingestion rates by *Karenia brevis* during blooms on the WFS in 2007, 2008, and 2009. Although grazing by natural populations of *K. brevis* on co-occurring picoplankton was not observed, I found that *K. brevis* populations from the WFS ingested *Synechococcus* when water samples containing high densities of *K. brevis* were amended with cultured *Synechococcus* strain CCFWC 502. This is the first time that grazing by natural populations of *K. brevis* was observed and suggest that blooms in the GOM may not be a result of strictly autotrophic growth, as previously thought (Aldrich 1962). In 2007, a *K. brevis* bloom was well established and co-occurred with a *Trichodesmium* bloom. As a result, dissolved nutrient concentrations were high. In addition, *Synechococcus* were abundant (Table 11). During this bloom, I calculated grazing by *K. brevis* on *Synechococcus* using two methods, the PI and PD methods in short incubations similar to Jeong et al (2005b). Although I microscopically observed inclusions of *Synechococcus* using the PI method, this method can underestimate ingestion for a number of reasons that are discussed in Chapter 4.

In 2008 and 2009, grazing on *Synechococcus* by *Karenia brevis* was measured using the PD method in both light and dark bottles. The ingestion rates observed on the WFS measure using the PD method ranged from 0.4 to 15.5 *Synechococcus K. brevis*⁻¹ hr⁻¹ and were within the range of those found in the laboratory demonstrating for the first

time natural populations of *K. brevis* can ingest picoplankton on the WFS during blooms. Ingestion rates measured in light and dark bottle experiments done using natural populations collected during cruises in 2008 and 2009, support my laboratory findings that light availability does not affect grazing by *K. brevis* on *Synechococcus* (Table 12).

Grazing by *Karenia brevis* on picoplankton in the GOM may also shorten the trophic transfer of dissolved organic material through the microbial food web (Azam 1983) as dinoflagellates can be a direct link between picoplankton and zooplankton (Berggreen et al. 1988, Jeong 1995). Certain species of mixotrophic dinoflagellates are also grazed by zooplankton (Teegarden & Cembella 1996), including *K. brevis* (Breier & Buskey 2007, Cohen et al. 2007, Kubanek et al. 2007). However, grazing on *K. brevis* has been shown to be a poor food source for some zooplankton species causing minor sublethal effects on *Acartia tonsa* and lethal effects on *Temora turbinata* feeding on *K. brevis* (Cohen et al. 2007). *A. tonsa* fed on *K. brevis* have also been shown to exhibit lowered egg production (Collumb & Buskey 2004).

While this study makes an initial comparison of autotrophic versus phagotrophic C uptake by *Karenia brevis*, future studies are needed to better quantify the relative contributions of inorganic and organic nutrient uptake versus grazing to the N and C nutrition of *K. brevis* on the WFS (Fig. 18). Further, the relative balance of different nutrient acquisition strategies may change as blooms initiate and progress. Based on my initial hypotheses, I constructed a “triangle” model to describe theoretically how three modes of C metabolism might contribute to *K. brevis* blooms on the WFS (Fig. 18). I initially speculated that during coastal upwelling events or when there are substantial inorganic nutrient inputs from estuarine sources (Vargo et al. 2004), *K. brevis* may take

up inorganic nutrients and grow primarily as photoautotrophs (Fig. 18). However, nutrient inputs can also stimulate the growth of potential prey organisms such as *Synechococcus*, and this could shift cellular metabolism to favor phagotrophic ingestion of C, especially if inorganic nutrients became depleted. In the event that *Trichodesmium* blooms preceded or co-occurred with *K. brevis* blooms, this could provide a source of organic nutrients that could fuel C acquisition from organic compounds. Similarly, as blooms mature, dead and decaying cells can potentially supply organic compounds that could be taken up by *K. brevis*.

In reality, what laboratory studies indicated was that *Karenia brevis* could acquire C from multiple sources simultaneously and that grazing and high concentrations of prey actually enhanced primary production by *K. brevis*. Grazing was independent of nutrient and light availability and inorganic C fixation occurred even at night when prey were provided (Table 7). Based on results presented here, it is likely that the triangle model is overly simplistic and that *K. brevis* uses a combination of C acquisition strategies over the course of bloom development and maintenance. While prey concentration clearly modulates grazing (Fig. 6B, 9B), light and nutrient availability do not. In addition, grazing appears to stimulate primary productivity by *K. brevis*. Understanding the full metabolic capabilities of this nutritionally flexible mixotroph will help gain better insight on *K. brevis* bloom ecology on the WFS.

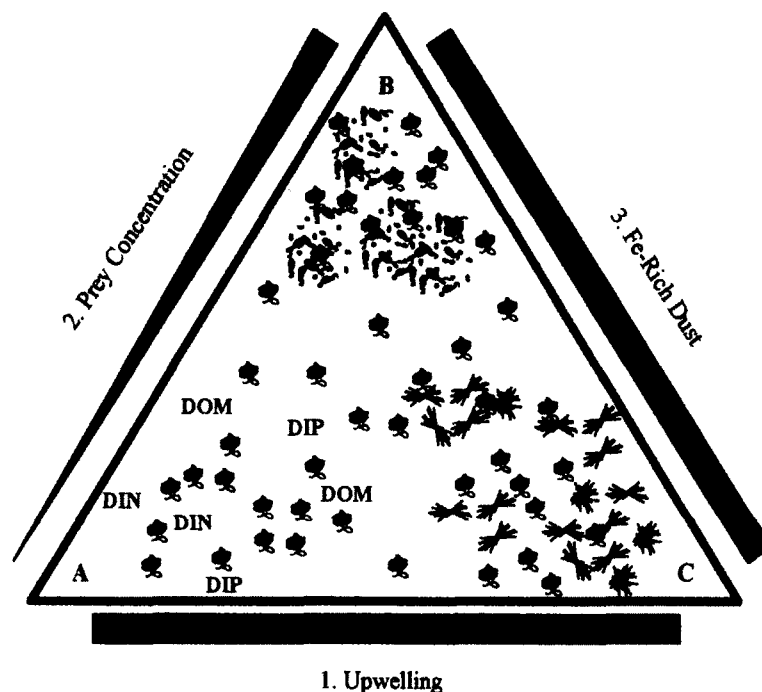


Fig. 18. A theoretical model describing the relative contributions of autotrophic (A), phagotrophic (B), and osmotrophic (C) C acquisition by *Karenia brevis* under different environmental conditions. This triangle includes: upwelling conditions, when inorganic N inputs are likely high and can support autotrophic C uptake; variable prey abundance because this can directly control ingestion rates; and *Trichodesmium*, because N_2 fixation and release from these organisms can provide inorganic and organic nutrients to support autotrophic growth or C acquisition from regenerated organic compounds. I speculate based on culture work (Chapter 2), that ingestion rates (black bar) would not be modulated by upwelling because grazing by *K. brevis* appears insensitive to nutrient concentrations (1). Grazing would increase in response to increasing prey concentration, as found during functional response experiments (Chapter 2) (2). Ingestion rates would also be similar in the presence or absence of *Trichodesmium* because as for upwelling, grazing is insensitive to nutrient status of *K. brevis* (3).

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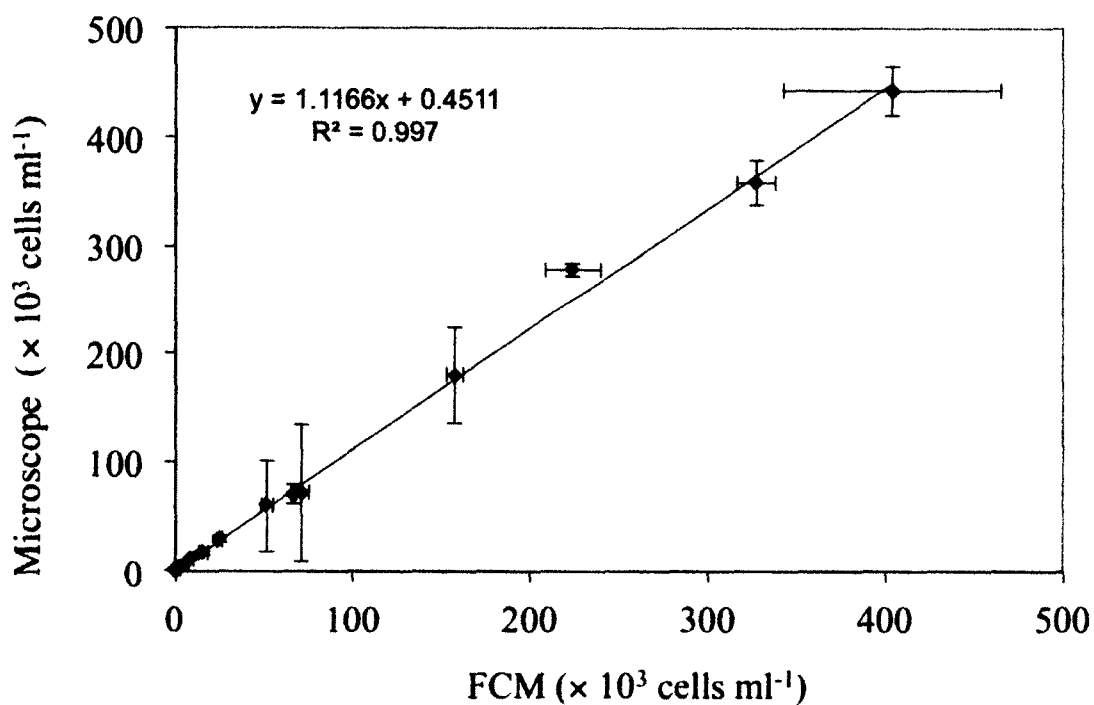
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APPENDIX A
MICROSCOPE AND FLOW CYTOMETRY CELL ABUNDANCE
COMPARISON

Comparison of *Synechococcus*, heterotrophic bacteria, and *Karenia brevis* cell counts made using epifluorescent microscopy and flow cytometry (FCM).



APPENDIX B.

NITRATE AND PHOSPHATE CONCENTRATIONS IN NUTRIENT-REPLETE AND -DEplete EXPERIMENTS

Nitrate (plus nitrite, NO_x^-) and phosphate (PO_4^{-3}) concentrations were measured in f/2 media to ensure that concentrations were effectively reduced in nutrient-deplete f/2 media compared to nutrient-replete f/2 media with full nutrient amendments.

Concentrations of NO_x^- and PO_4^{-3} were measured in the nutrient-deplete (SB3) grazing experiment and the nutrient-deplete DIC vs grazing experiment. The concentration of NO_x^- and PO_4^{-3} were also directly compared using an independent sample t-Test ($p < 0.05$) to determine if concentrations were significantly reduced from nutrient-replete to nutrient-deplete media. NO_x^- (*) and PO_4^{-3} (#) concentrations were significantly lower in the nutrient-deplete f/2 media compared to nutrient-replete f/2 media, suggesting that the method used in Chapter 2 significantly reduced NO_x^- and PO_4^{-3} concentrations.

Media	n	NO_x^- (μM)	T value	P value	PO_4^{-3} (μM)	T value	P value
ND (SB3)	3	2.10 (1.97)			0.13 (0.07)		
DIC vs ND grazing	3	1.35 (0.80)			0.21 (0.08)		
Nutrient-replete	3	306.53* (92.0)	5.754	0.005	8.83# (2.09)	7.277	0.002
Nutrient-deplete	3	0.40* (0.32)			0.04# (0.05)		

VITA

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Publications

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