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Grazing by *Karenia brevis* on *Synechococcus* enhances its growth rate and may help to sustain blooms

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ABSTRACT: Grazing rates of *Karenia brevis* Clones CCMP2228 and CCMP2229 were determined in laboratory experiments using *Synechococcus* sp. Clone CCMP1768 as food. Growth (days to weeks) and uptake rates (hours to days) were assessed. In the growth experiments, *K. brevis*, previously depleted in nitrogen (N), was grown at 2 light intensities in the presence of varying concentrations of *Synechococcus*. Under high irradiance (300 µmol photons m^{-2} s^{-1}; 14 h light:10 h dark cycle), exponential growth rates approximated those of phototrophic growth without *Synechococcus* (0.26 to 0.35 d^{-1}). At this irradiance, *K. brevis* cells in all treatments grew for about 10 d. Under lower irradiance (43 µmol photons m^{-2} s^{-1}), exponential growth rates of *K. brevis* cells varied with the enrichment level of *Synechococcus*, with rates under the highest *Synechococcus* enrichment level being nearly twice (0.58 d^{-1}) those observed for the high irradiance treatments. Short-term uptake experiments (3 experiments, 2 clones) were done to examine the N-specific rates of grazing of ^{15}N-labeled *Synechococcus*. N-specific rates of grazing ranged from 9.28×10^{-4} h^{-1} to 1.22×10^{-2} h^{-1} and varied with the relative proportion of *Synechococcus*:*K. brevis*. These rates represent a range of 0.026 to 2.15 pmol-N *K. brevis*$^{-1}$ d^{-1}, or 0.96 to 83.8 *Synechococcus K. brevis*$^{-1}$ h^{-1}. Evidence of *Synechococcus* inside *K. brevis* was provided by confocal microscopy. Grazing by *K. brevis* thus enhances the range of nutritional substrates available to meet its growth requirements, and may play a substantial role in sustaining natural populations in inorganic N-poor waters.

KEY WORDS: *Karenia brevis* · *Synechococcus* · Mixotrophy · Grazing rate · Irradiance · Nitrogen source · Harmful algal bloom · Red tide

INTRODUCTION

Karenia brevis is a potentially toxic dinoflagellate that causes red tides in the Gulf of Mexico, and is most prevalent along the southwest Florida shelf (e.g. Vargo et al. 2001, 2004). The toxins associated with these blooms, brevetoxins, have been associated with fish and marine mammal kills and also cause respiratory distress and neurotoxic effects in human consumers of contaminated shellfish (Landsberg 2002). Thus, there is considerable management interest in the causes of these blooms. Although there is now consensus that nutrient enrichment from land-based sources has degraded estuarine and coastal marine waters worldwide, leading to alterations in ecosystems and the expansion of many harmful algal blooms (HABs) in many regions (e.g. Glibert et al. 2005, Glibert & Burkholder 2006), the sources of nutrients needed to sustain large *K. brevis* blooms are still not well understood (Walsh & Steidinger 2001, Brand & Compton 2006, Vargo et al. 2008). While some research has supported the premise that nutrient enrichment can contribute

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to long-term increases in *K. brevis* abundance in nearshore areas (Brand & Compton 2007), other studies have reported that blooms of this species generally initiate offshore and are sustained in seemingly oligotrophic waters (e.g. Walsh & Steidinger 2001). Thus, it is unclear whether *K. brevis* blooms require nearshore nutrient enrichment or can be sustained in relatively nutrient-poor waters (Vargo et al. 2004, 2008, Hu et al. 2006).

Relationships between nutrient export from the coastal zone and the marine ecosystem are complex, involving both direct and indirect pathways mediated through nutrient cycling, trophic interactions, and/or biogeochemical transformations (e.g. Glibert et al. 2005, Glibert & Burkholder 2006). While many HABs are related to inorganic nutrient enrichment, some are apparently not; moreover, inorganic nutrients are not the only cause of bloom stimulation (Glibert et al. 2005). An alternative nutritional strategy for some HABs is mixotrophy, in particular phagotrophy from grazing on algae or other prey (Stoecker et al. 2006). Such mixotrophy is observed in many dinoflagellate species (reviewed by Stoecker 1999, Burkholder et al. 2008). Grazing rates by *Karenia brevis* have only been published to date in 1 study (Jeong et al. 2005) and there is much to be learned about its ability to graze under a range of conditions.

Mixotrophic dinoflagellates ingest a wide variety of prey items including bacteria, other algae and protists, and fish, shellfish, and mammalian tissues (Burkholder et al. 2008). The photosynthetic cyanobacterium *Synechococcus* has been suggested to be one such food source, due to its ubiquitous nature in both offshore and coastal waters, its potential for high growth rates (Kana & Glibert 1987), and its co-occurrence with many dinoflagellates (e.g. Murrell & Lores 2004). *Synechococcus* is of particular interest in Florida coastal waters because this species develops large blooms in the Florida Bay region (Phlips & Babylak 1996, Glibert et al. 2004) and is also prevalent along the southwest Florida coast (Heil et al. 2007).

The potential for *Synechococcus* to be grazed by *Karenia brevis* under a range of laboratory conditions was explored in the present study. We hypothesized that grazing has the potential to contribute a significant amount of N to sustain growth of *K. brevis*. The rates of growth with and without this particulate source of nutrients and under varying light regimes were compared to rates under strictly phototrophic growth conditions. The present study provides the first quantitative measurements of grazing over a range of conditions, and shows that grazing can contribute up to 40% of the cellular N requirements h^{-1} for *K. brevis*. With evidence that blooms of *Synechococcus* can be enhanced due to anthropogenic nutrients, the potential importance of this particulate nutrient source for sustaining red tide blooms *in situ* is large and may help to resolve the current uncertainty as to how *K. brevis* blooms are maintained.

MATERIALS AND METHODS

Cultures and methods of growth. All cultures were obtained from the Provasoli-Guillard National Culture Collection maintained at the Bigelow Laboratory for Ocean Sciences, West Boothbay Harbor, Maine, USA. The *Karenia brevis* cultures included Clone CCMP2229, originally isolated from Manasota Key, Florida, and Clone CCMP2228, originally isolated off Sarasota, Florida. *Synechococcus* strain CCMP1768 was isolated from the Gulf of Mexico.

The *Karenia brevis* cultures were grown in L1 medium at a salinity of 30 (Guillard & Hargraves 1993); the N source and prey concentration varied with the experiment, as described below. Maintenance cultures were grown with NO_3^- as the N source at a light intensity of 50 µmol photons m^{-2} s^{-1} on a 14 h light:10 h dark cycle. The seawater used in media preparation was from the Indian River inlet, Delaware, and was autoclaved prior to nutrient amendment. Cultures were maintained at 20°C in walk-in temperature-controlled rooms. Fluorescent fixtures were positioned to provide a range of light intensities, which varied by experiment. Cultures were not axenic, but aseptic techniques were used to minimize additional bacterial contamination during the growth periods.

Experimental design. Six experiments were conducted, including 2 designed to assess the rate of growth of *Karenia brevis* under varying conditions, and 4 designed to measure short-term rate processes.

Expt 1: Phototrophic growth of Karenia brevis with NO_3^-, NH_4^+ or urea at 3 irradiance levels: Clone CCMP2228 of *K. brevis* (initial inoculum: 1.3×10^4 cells ml^{-1}) was grown in clean borosilicate test tubes, with 3 N forms (NO_3^-, NH_4^+, or urea; enriched at concentrations specified for N in L1 media) and at 3 irradiance levels (15, 30, or 115 µmol photons m^{-2} s^{-1}). Growth was monitored approximately daily for 30 d, as described below (see 'Analytical protocols'). Five replicates were maintained for each N source and light level. Growth rates were measured for the exponential period.

Expt 2: Mixotrophic growth of Karenia brevis vs. irradiance and Synechococcus availability: Cells of *K. brevis* Clone CCMP2228, grown on L1 medium (Guillard & Hargraves, 1993) but at the point of N depletion, were used for this experiment. These cells were transferred to 30 clean glass test tubes containing L1 medium minus N. The light exposures for this experiment were 43 and 300 µmol photons m^{-2} s^{-1}, measured

using a Biospherical Instruments QSL-100 with a 4π probe inserted into the tubes. Initial culture densities of *K. brevis* were 450 cells ml^{-1} for the high light treatments and 520 cells ml^{-1} for the low light treatments. The ambient concentration of NO_3^- in the Indian River source water was 26 µmol N, and that of DON was 30 µmol N at the start of the experiment; other N substrates were negligible.

The experiment was initiated with the addition of 5 different densities of *Synechococcus* (final concentrations of 0, 6.1×10^3, 2.1×10^4, 6.2×10^4, and 8.6×10^4 cells ml^{-1}). Each light and treatment level was replicated in triplicate. To compensate for the different volumes of *Synechococcus* that were added (0.1 to 1.0 ml *Synechococcus* stock culture) and nutrients that would have been carried over with the transfer of *Synechococcus*, all cultures were brought to a total volume of 50 ml using filtrate from the *Synechococcus* cultures (filter pore size = 0.22 µm). Growth was assessed approximately daily for 25 d (see 'Analytical protocols').

Expt 3: Karenia brevis uptake of urea: Cultures of both clones in late exponential growth phase were used to assess the rate at which *K. brevis* directly took up dissolved urea. Culture aliquots, previously maintained on NO_3^-, were exposed over short periods (less than 1 h) to a concentration gradient of ^{15}N-labeled urea, from 0.5 to 20.0 µmol N l^{-1}. Cultures were not N-depleted at the time of urea exposure, but they were urea-depleted. Thus, all enrichment levels were ~100 atom percent (atom %). After incubation, the cultures were filtered onto pre-combusted GF/F filters (nominal pore size = 0.7 µm). The filters were dried and later analyzed by mass spectrometry (see 'Analytical protocols').

Expts 4 to 6: Mixotrophy in Karenia brevis. Short-term grazing rates with Synechococcus as prey: Three experiments were conducted using varying proportions of *K. brevis* and *Synechococcus*. Two experiments used both clones of *K. brevis*, while the final experiment used only *K. brevis* Clone CCMP2229. In each experiment, a monoculture of *Synechococcus* (late exponential growth phase) was labeled with 10 µmol l^{-1} ^{15}N-urea and incubated for 24 h to ensure uniform isotope labeling. The amount of isotope label in the *Synechococcus* culture inocula was determined by mass spectrometry. The *K. brevis* cultures (N-starved, in late exponential growth phase) were gently transferred to new 60 ml culture flasks and an inoculum of the labeled *Synechococcus* was added. Depending upon the experiment, 3 to 7 different densities of ^{15}N-labeled *Synechococcus* were used. In all, the treatments yielded ratios of *Synechococcus*: *K. brevis* (cell:cell) of 0.36 to 225.8. The flasks were held in a constant temperature incubator for ~24 h on a 14 h light:10 h dark cycle. Following incubation, a 5 ml subsample was removed and preserved in glutaraldehyde (1% final concentration). The remaining contents of the flasks were filtered through pre-combusted filters (GF/D, nominal pore size = 2.7 µm) that retained the *K. brevis* cells (~20 µm diameter) and possibly some of the *Synechococcus* cells (if attached to *K. brevis*). The filtrate was then re-filtered through a smaller pore pre-combusted filter (GF/F, nominal pore size = 0.7 µm) to retain *Synechococcus* cells that had not been collected with *K. brevis* on the first filter. The filters were rinsed with ultra-filtered Indian River water and dried for later analysis by mass spectrometry to determine the amount of ^{15}N label that was in the *K. brevis* cells versus the *Synechococcus* cells. Considering all 3 experiments collectively, 30% of the treatments of *K. brevis* (by strain) and *Synechococcus* (by density) were tested in duplicate. All ^{15}N samples were processed using a Sercon mass spectrometer.

Analytical protocols. In Expts 1 and 2, growth of *Karenia brevis* and *Synechococcus* was tracked by changes in fluorescence and calibrated by microscopy and flow cytometry. On an approximately daily basis, 5 ml of each culture were removed, of which 1 ml was transferred to a cuvette and fluorescence emission at 682 nm (chlorophyll) and 560 nm (phycoerythrin) from 495 nm excitation were recorded on a Jobin Yvon Fluoro Max-3 spectrofluorometer. The chlorophyll fluorescence of *K. brevis* alone in the mixed species cultures was calculated as: $chl_{K.b.} = Em_{682} - 0.19Em_{560}$, where $chl_{K.b.}$ is the chlorophyll fluorescence signal from *K. brevis*, and Em_{682} and EM_{560} are the 682 and 560 fluorescence emission signals of the mixed culture. The emission at 560 nm was used as a measure of *Synechococcus* in these experiments. The factor, 0.19, is the average fluorescence emission ratio (682:560nm) from *Synechococcus* only. The remaining 4 ml of subsample was preserved in glutaraldehyde (1% final concentration).

Of the 540 samples measured by fluorescence, 66 were analyzed by flow cytometry using a Becton Dickinson FACS-caliber flow cytometer to provide an independent measurement of *Synechococcus* density for cross-confirmation of the fluorescence patterns. Cell numbers were determined by gating by forward and side light scatter. The correlation between fluorometry and flow cytometry readings varied for cells grown at high and low light levels. For high light the correlation was:

$$\text{Fluorometry reading} = 5.578x - 23\,867 \quad (r^2 = 0.87,\ n = 41) \tag{1}$$

For low light, the correlation was:

$$\text{Fluorometry reading} = 30.97x - 51\,935 \quad (r^2 = 0.69;\ n = 25) \tag{2}$$

where x is cell number (cells ml^{-1}) determined by flow cytometry.

Selected aliquots were also examined by confocal microscopy to assess whether *Synechococcus* cells were actually retained within *Karenia brevis* cells. Autofluorescence of *Synechococcus* phycobilisomes was used to detect the cyanobacteria inside *K. brevis* cells. The preserved samples were filtered onto 25 mm GTBP black polycarbonate filters (Millipore, pore size = 3.0 µm). Each filter was placed on a microscope slide, covered by a drop of immersion oil (Cargille type FF) and a cover slip, and examined with a Leica TCS SP1 confocal microscope and a 63×, 1.2 numerical aperture water immersion lens. For these images, the excitation wavelength was 488 nm and the emission windows for *K. brevis* and *Synechococcus* pigments were 660 to 710 and 590 to 630 nm, respectively. Images were captured at consecutive intervals of 0.15 µm along the *z*-axis.

Particulate and dissolved nutrients in the cultures were measured using standard methods. Particulate N in filtered samples (GF/D or GF/F) was determined using a Control Equipment CHN analyzer and dissolved nutrients were analyzed using autoanalysis (Lane et al. 2000), except for urea, which was measured using the protocol of Revilla et al. (2005).

Data analysis. Growth rates in Expts 1 and 2 were calculated for specific time intervals (days) within the exponential growth phase using the equation:

$$r = \ln(N_t/N_0)/t \quad (3)$$

where r is specific growth rate (d^{-1}), N_t is the measure of cells (or units of fluorescence) at time t, N_0 is that at time 0, and t is the interval of growth in days. The mean growth rates for the time interval when the highest observed rates were sustained (i.e. exponential growth phase) are reported. All growth rates are reported as the means of either 5 (Expt 1) or 3 replicates (Expt 2). For literature comparisons, growth rates were also expressed as divisions d^{-1} by dividing r by ln 2. Statistical comparisons between groups were based on 1-way ANOVAs; *t*-tests were used to compare differences within groups. All comparisons were made at a significance level of 0.01.

The N-specific uptake rates of urea by *Karenia brevis* in Expt 3 were calculated as:

$$V = \text{atom \% excess}/(\text{atom \% enrichment} \times \text{time}) \quad (4)$$

where V is the N-specific uptake rate, h^{-1}, atom % excess is the ^{15}N enrichment of the culture (minus natural ^{15}N background) at the end of the incubation period, atom % enrichment is the initial atom % of the substrate added, and time is the duration of the experiment in hours (e.g. Glibert & Capone 1993).

To calculate the N-specific grazing rates of *Synechococcus* by *Karenia brevis* in Expts 4 to 6, a version of equation 4 was used. However, in this case, the atom% enrichment was that of the initial *Synechococcus* culture and the atom% excess was that of the *K. brevis* culture (GF/D fraction) after incubation. Since *Synechococcus* was growing during the period of the 24 h incubation, it was becoming isotopically diluted as the initial ^{15}N-labeled urea had been consumed and the cells were using other regenerated forms of N. Hence, an isotope dilution correction was made to account for the exponentially changing enrichment of the *Synechococcus* cells (e.g. Glibert et al. 1982). To correct the atom % enrichment of the GF/D filters for any contribution by retained *Synechococcus* cells (or bacteria), the residual amount of *Synechococcus* on the GF/D filters was determined from the difference of mass of the cells retained on the GF/Fs and the initial culture. The ^{15}N atom% enrichment due to the *K. brevis* contribution only was then determined according to the following equation:

$$\text{Atom \% } K.\ brevis = [(\text{measured atom \% GF/D})(\text{mass of } K.\ brevis + \text{mass of } Synechococcus) - (\text{atom \% } Synechococcus)(\text{mass of } Synechococcus)]/(\text{mass of } K.\ brevis) \quad (5)$$

where all units of mass are in µmol-N. Grazing rates in terms of amount of N grazed per unit time (N ingestion rates) were calculated by multiplying the N-specific grazing rates by the particulate N (PN) content of the *K. brevis* culture:

$$\text{N ingestion rate} = \text{N-specific grazing rate} \times \text{PN} \quad (6)$$

Lastly, all grazing rates were also corrected for the direct uptake of urea by the cultures by subtracting the urea uptake rates determined above.

Grazing rates were also calculated in terms of cells of *Synechococcus* grazed (*Synechococcus K. brevis*$^{-1}$ h^{-1}) by converting the N ingestion rate to a cell-specific rate, based on a conversion of 1.65 fmol-N $cell^{-1}$ of *Synechococcus elongatis* (Richardson 2004). Clearance rates (CR, µl *K. brevis*$^{-1}$ h^{-1}) were calculated as described by Jeong et al. (2005) as follows:

$$\text{CR} = \text{IR}/\text{prey density} \quad (7)$$

where IR is the cell-specific ingestion rate (cells *Synechococcus K. brevis*$^{-1}$ h^{-1}).

RESULTS

Growth experiments

Expt 1 was designed to provide the benchmark phototrophic growth rates for one of the clones of *Karenia brevis*. For all 3 N substrates, growth was poor at the 2 lowest experimental irradiances (31 and 15 µmol photons m^{-2} s^{-1}; Table 1). Growth at the highest irradiance

on all N substrates was significantly higher than growth at the lower irradiance levels. Growth rates on NH_4^+ and on NO_3^- at the highest irradiance (115 µmol photons m^{-2} s^{-1}) were virtually identical at 0.35 to 0.37 d^{-1} (Table 1), while the average rate of growth on urea at the same irradiance was slightly less, at 0.28 d^{-1} (Table 1), though not statistically different from rates observed using NH_4^+ or NO_3^- as substrates.

Expt 2 explored the potential for growth of *Karenia brevis* on a particulate N source (*Synechococcus*). In this experiment, the only sources of N were the *Synechococcus* cells, the slight quantity of inorganic N that was transferred with the original inoculum, any dissolved N that may have been released by the cultures during the growth cycle, and contaminating bacteria. Exponential growth rates for those cultures maintained at the higher irradiance in this experiment (300 µmol photons m^{-2} s^{-1}) were very similar to those obtained in Expt 1, ranging from 0.26 to 0.35 d^{-1} (Fig. 1). In contrast, a much wider range of exponential growth rates was observed for those cultures grown at the lower irradiance (43 µmol photons m^{-2} s^{-1}), 0.14 to 0.58 d^{-1}, depending on the *Synechococcus* enrichment level (Fig. 1). For the low light-grown culture, the highest exponential growth rates corresponded with those of *K. brevis* cultures that had the highest level of *Synechococcus* added. At both the highest and 2 lowest levels of *Synechococcus* enrichment, mean exponential growth rates of *K. brevis* under the 2 light regimes were significantly different (Fig. 1).

The time interval over which the exponential growth was observed also varied in Expt 2, depending on the irradiance and *Synechococcus* enrichment levels. At the higher irradiance level, all cultures grew rapidly for approximately 10 d, after which the culture growth began to decline (Fig. 2A, C, E, Table 2). There were no significant differences in the average exponential rates of growth, but the absolute cell number attained was related to the amount of *Synechococcus* added; the lowest *Synechococcus* level yielded the highest final biomass of *Karenia brevis* (Fig. 2A). At the lower irradiance, a different pattern was observed. In this case, the culture with the highest *Synechococcus* concentration initially grew fastest, then growth of the culture with the next highest *Synechococcus* level increased a few days later, and again a similar increase occurred a few days later with the third highest *Synechococcus* level, and so on (Fig. 2B, D, F). In contrast to the high light treatments, the maximum biomass of *K. brevis* attained varied much less with amount of *Synechococcus* added (e.g. Fig. 2D, F).

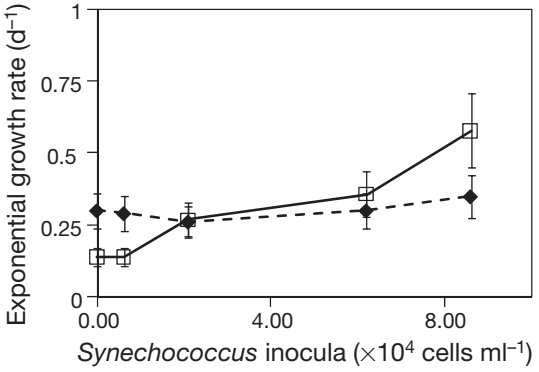

Fig. 1. *Karenia brevis*. Mean exponential growth rates (d^{-1}) of *K. brevis* in the presence of varying concentrations of *Synechococcus* as the only N source at 2 light intensities: 300 (– – –) and 43 µmol photons m^{-2} s^{-1} (——). Each point represents the mean calculated growth rate of triplicate cultures for the exponential period of growth for that experimental condition (see Table 1). Error bars are SD

Table 1. *Karenia brevis*. Mean growth rates of *K. brevis*, Clone CCMP2228, as a function of N source and light intensity for Expt 1. All growth rates <0 are herein reported as 0. The coefficient of variation (CV) of the 5 replicate cultures is also given

Substrate	Light intensity (µmol photons m^{-2} s^{-1})	Mean growth rate (d^{-1})	CV
Ammonium	15	0	0
	31	0.05	0.60
	115	0.37	0.19
Urea	15	0	0
	31	0	0
	115	0.28	0.35
Nitrate	15	0	0
	31	0	0
	115	0.35	0.14

Table 2. *Karenia brevis*. Time period over which exponential growth of *K. brevis* Clone CCMP2228, grown with *Synechococcus* Clone CCMP1768, was sustained for the experimental conditions for Expt 2. See Fig. 2 for the full growth cycle of all cultures

Light intensity (µmol photons m^{-2} s^{-1})	Initial inoculum of *Synechococcus* (cells ml^{-1})	Initial inoculum of *K. brevis* (cells ml^{-1})	Rate calculation time period (d)
300	0	4.5×10^2	1–10
	6.2×10^3	4.5×10^2	1–10
	2.1×10^4	4.5×10^2	1–10
	6.2×10^4	4.5×10^2	1–10
	8.6×10^4	4.5×10^2	1–10
43	0	5.2×10^1	1–10
	6.2×10^3	5.2×10^1	1–10
	2.1×10^4	5.2×10^1	10–16
	6.2×10^4	5.2×10^1	5–11
	8.6×10^4	5.2×10^1	1–5

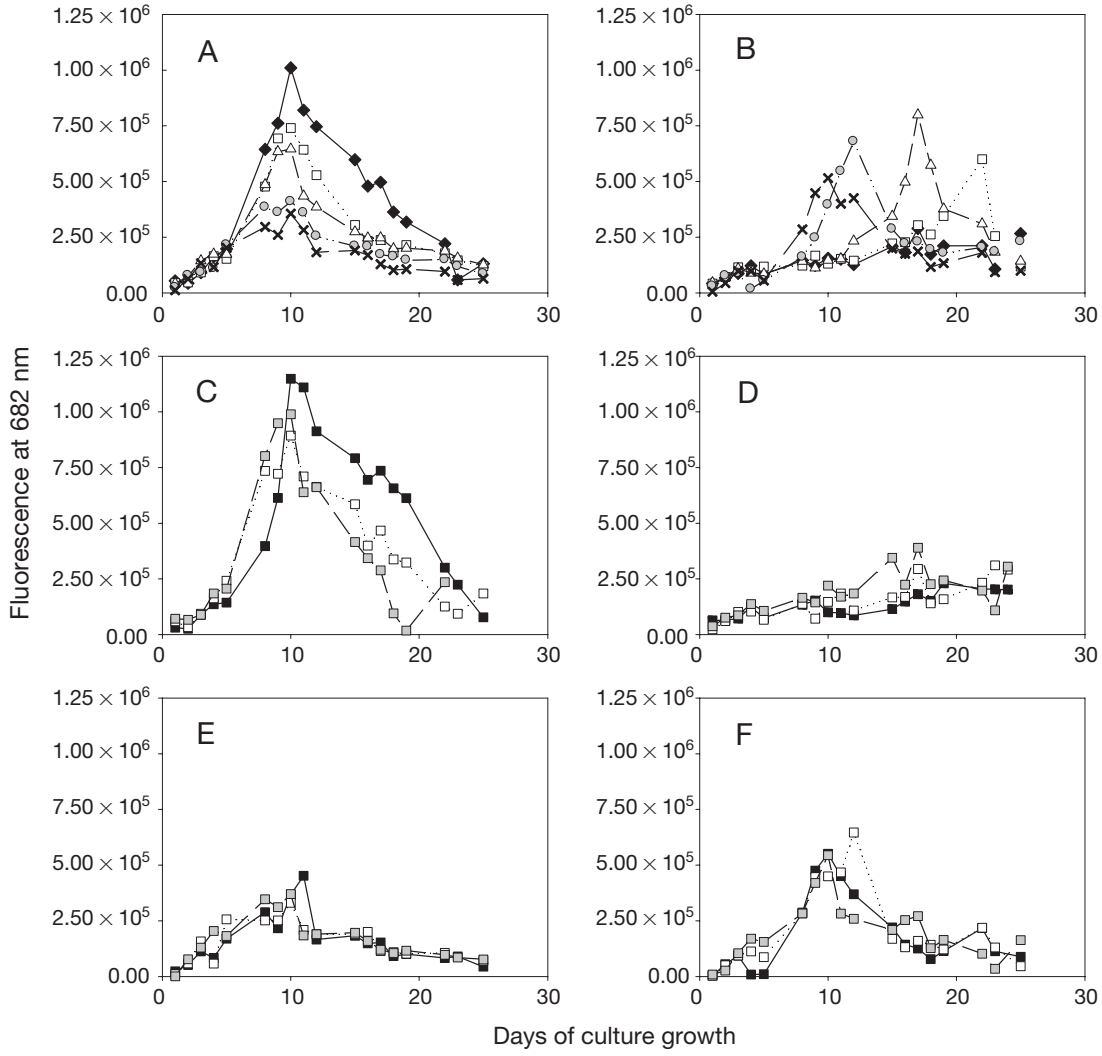

Fig. 2. *Karenia brevis*. Time course of growth of *K. brevis* in mixed *K. brevis* and *Synechococcus* cultures at 2 light intensities as indicated by the chlorophyll *a* fluorescence at 682 nm corrected for the presence of *Synechococcus* chlorophyll (see Expt 2 for details). (A) Comparative growth at 300 µmol photons m^{-2} s^{-1} and 5 levels of initial enrichment of *Synechococcus*: no *Synechococcus* (◆); 6.1×10^3 cell ml^{-1} (□); 2.1×10^4 cells ml^{-1} (△); 6.2×10^4 cell ml^{-1} (○); and 8.6×10^4 cells ml^{-1} (×). (B) As for (A) except at 43 µmol photons m^{-2} s^{-1}. Each point is the mean of 3 individual culture measurements. (C–F) Examples of the triplicate cultures (open, gray and black symbols) at high (C, E) and low (D, F) light for treatments with the lowest (C, D) and highest (E, F) *Synechococcus* enrichment levels

The exponential rates of growth of *Synechococcus* in the mixed cultures differed considerably. At the higher irradiance level, all growth rates of *Synechococcus* were negative (Fig. 3A, C, E). At the lower light intensity, the highest observed growth rates (i.e. exponential growth phase) of all cultures ranged from 0.23 to 0.49 d^{-1}, although the time interval over which the highest growth rate was attained depended on the initial inoculum level (Fig. 3B, D, E, Table 3). Rates of growth of *Karenia brevis* and *Synechococcus* were inversely related in the low light treatment: the highest growth rate of *K. brevis* corresponded with the lowest observed growth rate of *Synechococcus.*

Table 3. *Synechococcus*. Time period over which exponential growth of *Synechococcus* Clone CCMP1768 was observed for the experimental conditions indicated for Expt 2. See Fig. 3 for the full growth cycle of all cultures. Growth rates for all cultures at 300 µmol photons m^{-2} s^{-2} were negative and are not shown

Light intensity (µmol photons m^{-2} s^{-1})	Initial inoculum of *Synechococcus* (cells ml^{-1})	Rate calculation time period (d)
43	6.2×10^3	10–20
	2.1×10^4	12–17
	6.2×10^4	7–11
	8.6×10^4	1–10

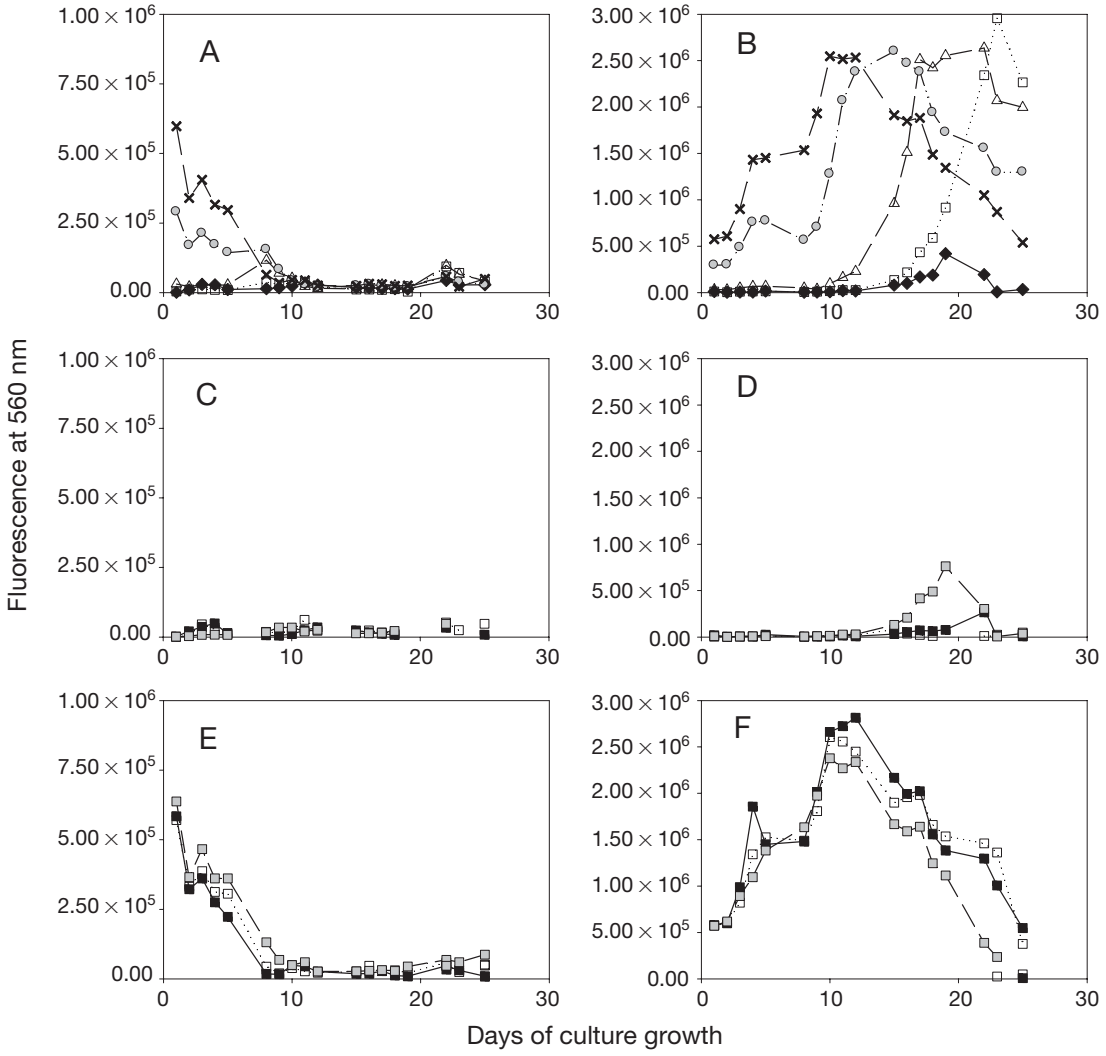

Fig. 3. *Karenia brevis*. Time course of growth of *Synechococcus* in mixed *K. brevis* and *Synechococcus* cultures at 2 light intensities as indicated by phycoerythrin fluorescence at 560 nm. See Fig. 2 for further details

Confocal micrographs from samples taken during the growth experiments confirmed that *Synechococcus* was consumed by *Karenia brevis* (Fig. 4). Up to several *Synechococcus* cells could be distinguished within individual *K. brevis* cells.

Urea uptake rates

The goal of Expt 3 was to obtain direct rates of urea uptake by *Karenia brevis* and to use these data to correct the ^{15}N grazing experiments for ^{15}N urea uptake, i.e. as dissolved substrate controls and as controls for any bacterial uptake (urea was the initial ^{15}N-substrate initially used to label the *Synechococcus* cells). Because these *K. brevis* cells were not acclimated to growth on urea, this experiment can be viewed as yielding baseline or minimal rates. The rate of urea uptake would be expected to be higher after acclimation when the enzyme systems were optimized for maximal urea uptake.

Under these experimental conditions, the N-specific uptake rates (V) of urea for both clones were very low (2×10^{-5} to 1.4×10^{-4} h^{-1}; Fig. 5). Although the data could be fit to a Michaelis-Menten formulation, a linear fit to the data was better.

Grazing rates

Expts 4 to 6 were designed to assess short-term grazing rates by *Karenia brevis* on *Synechococcus*. N-specific rates of grazing on *Synechococcus* by *K. brevis* ranged from 9.28×10^{-4} to 1.22×10^{-2} h^{-1} and varied

with the relative proportion of *Synechococcus*:*K. brevis* (Table 4). These rates also represent a range of 0.026 to 2.15 pmol N *K. brevis*$^{-1}$ d^{-1}, or 0.96 to 83.8 *Synechococcus K. brevis*$^{-1}$ h^{-1} (Table 4, Fig. 6). Both clones of *K. brevis* had comparable rates of N-specific grazing. Expts 4 to 6 used very different cell density ranges of *K. brevis* and *Synechococcus*. When the data from all 3 experiments were considered, it was found that as more *Synechococcus* prey were added relative to *K. brevis*, the grazing rate of *K. brevis* (expressed as

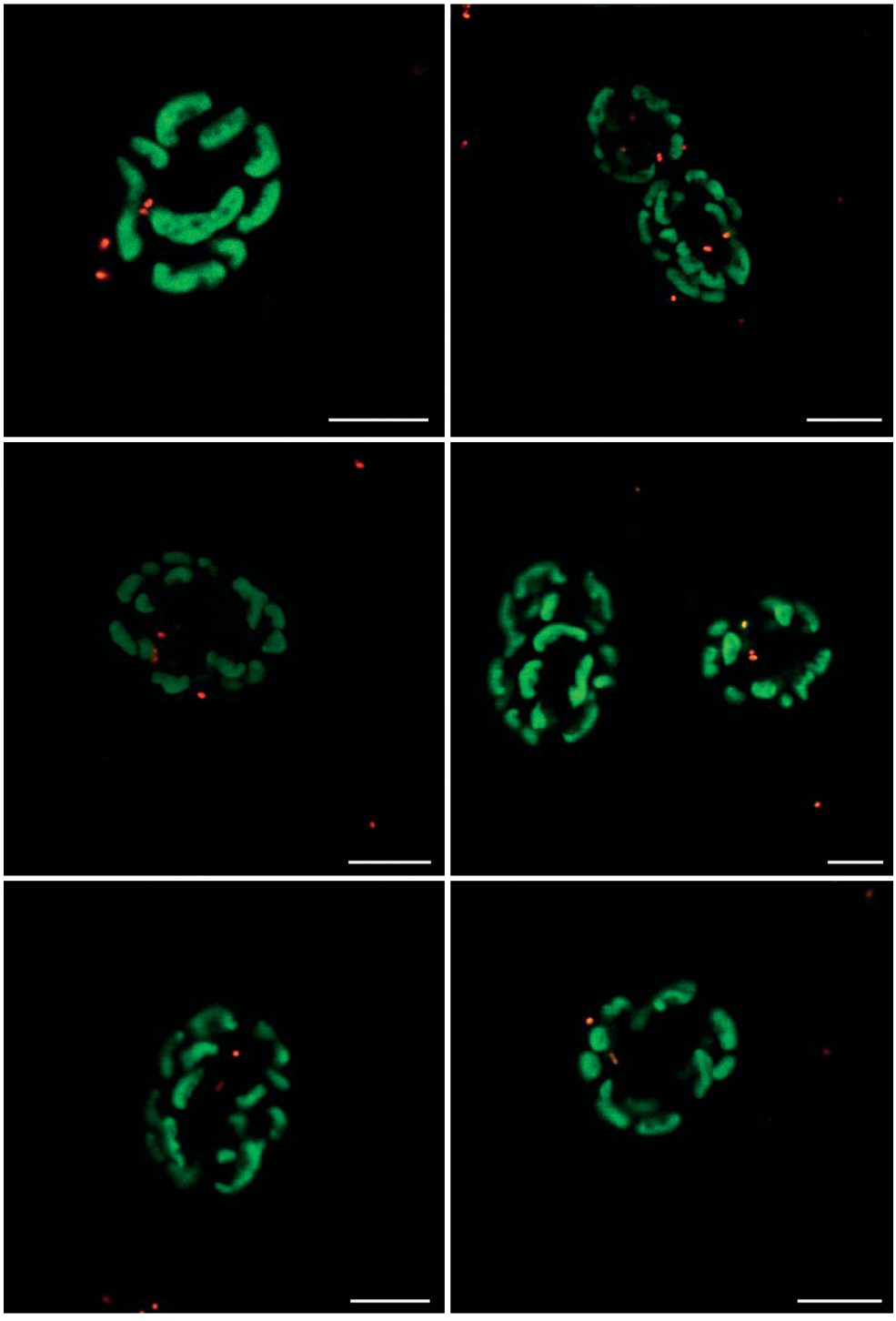

Fig. 4. *Karenia brevis* and *Synechococcus*. Representative confocal micrographs of *K. brevis* cells with retained *Synechococcus*. Chlorophyll fluorescence is shown in green and phycoerythrin fluorescence is shown in red. Scale bars = 10 µm

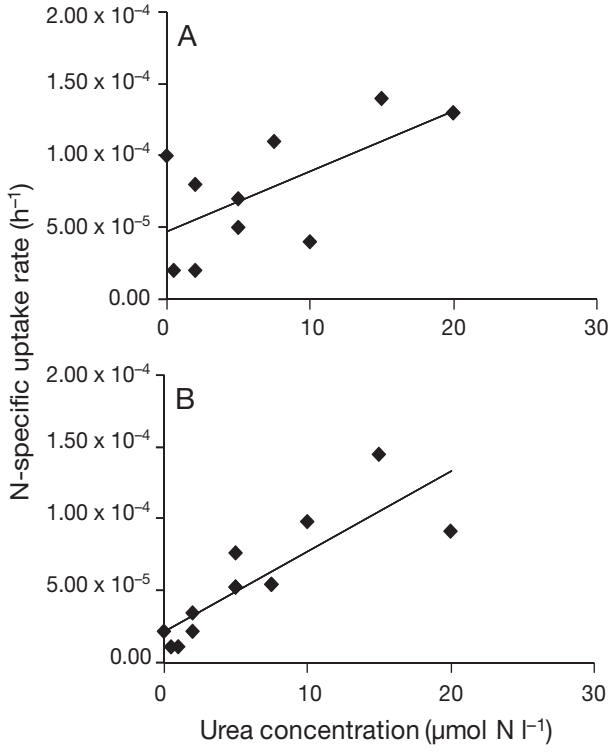

Fig. 5. *Karenia brevis*. Nitrogen-specific uptake rates of urea by *K. brevis* Clones (A) CCMP2229 and (B) CCMP2228 as a function of the concentration of ^{15}N-urea added. The r^2 for the best fit line, in this case linear, is 0.41 (A) and 0.72 (B)

both N-specific grazing and as cell-specific grazing rates) increased but did not saturate (Table 4, Fig. 6). The clearance rates varied from 0.10 to 8.6 µl *K. brevis*$^{-1}$ h^{-1} and were inversely related to the ratio of *Synechococcus*: *K. brevis* (Table 4).

DISCUSSION

Growth rates

The growth rates reported here for *Karenia brevis* cultures that were maintained phototrophically (Table 1) were comparable to those reported by a wide range of investigators. Studies of growth of the same clones used here by Neely (2006) at salinity of 27 and temperatures from 20 to 25°C were, with the exception of one clone, <0.4 d^{-1}; a Texas clone also used in the Neely (2006) study yielded only slightly higher growth rates. Magana & Villareal (2006) reported maximum growth rates for *K. brevis* Clone SP3 of 0.17 to 0.36 divisions d^{-1} (0.12 to 0.25 d^{-1}) over a wide range of salinities and temperatures, with the highest growth rate observed at salinity 30, irradiance 31 µmol photons m^{-2} s^{-1}, and temperature 20°C. They also found little or no growth at irradiances <31 µmol photons m^{-2} s^{-1}, which was comparable to our findings on all substrates investigated. Shanley & Vargo (1993), in studies of the Wilson clone (reported as *Gymnodinium breve*), reported increasing rates of growth with increasing irradiance. The highest growth rates of 0.53 divisions d^{-1} (0.36 d^{-1}) were found when cells were acclimated to the highest experimental growth irradiance of 90 µmol photons m^{-2} s^{-1}. Maier Brown et al. (2006) also reported growth rates of several *K. brevis* clones including a Mexico beach clone, the Wilson clone, and a Charlotte Harbor clone; of these, the growth rates of the Charlotte Harbor clone were 0.25 to 0.30 d^{-1} for salinities above 20, but rates were considerably lower for the other 2 clones.

In the present study, growth rates of cultures maintained on *Synechococcus* (Fig. 1) were in the same range as those grown phototrophically when the cells were maintained at the higher experimental irradiance. At the higher irradiance, growth of *Karenia brevis* on *Synechococcus* was not sustained beyond ~10 d (Fig. 2). After all of the *Synechococcus* cells or other sources of nutrients apparently were consumed, growth of *K. brevis* declined. However, at the lower irradiance level, maximum growth rates of *K. brevis* varied considerably. At the 2 lowest concentrations of *Synechococcus* cells, growth rates were about half of those that were obtained under either phototrophic conditions or in the higher-irradiance mixotrophic conditions. At the highest density of *Synechococcus* cells in low light, the growth rate was significantly enhanced, up to 0.58 d^{-1}. This growth rate was ~60% higher than the rate at the next highest *Synechococcus* treatment level. Given the fact that growth rates increased linearly with the *Synechococcus* levels added and showed no evidence of growth rate saturation, it is possible that even higher growth rates are attainable with higher densities of *Synechococcus*.

In the present study, the potential for heterotrophic bacteria to also contribute to the nutrition of *Karenia brevis* cannot be discounted (e.g. Seong et al. 2006), nor can the use of dissolved substrates from disruption of the *Synechococcus* or *K. brevis* cells. If bacteria were being grazed in these experiments, it would only underscore that mixotrophy can help to sustain growth. However, the observed presence of *Synechococcus* in the *K. brevis* cells based on confocal microscopy (Fig. 4) confirms that *Synechococcus* cells were indeed grazed.

Urea uptake rates

Karenia brevis has previously been found to use urea (e.g. Bronk et al. 2004, Sinclair 2008), but the rates of urea uptake for our experiments were very low (Fig. 5). In fact, they are roughly 2 orders of magnitude lower

Table 4. *Karenia brevis*. Initial conditions and calculated grazing and ingestion rates of *K. brevis* (Clones CCMP2228 and CCMP2229) on *Synechococcus* (Clone CCMP1768; *Syn*) based on ^{15}N experiments. All experiments also included controls with no *Synechococcus* added (not shown). *: replicated treatments. SE of the N-specific grazing rates are given in parentheses

Expt no. and replication	*K. brevis* clone no. (CCMP)	Initial Sy*necho-coccus* concentration (cells l^{-1})	*Syn*:*K. brevis* (cell:cell)	*Syn*:*K. brevis* (pmol-N: pmol- N)	N-specific grazing rates (*V*, h^{-1})	Clearance rate (µl *K. brevis*$^{-1}$ h^{-1})	N-ingestion rates (pmol-N *K. brevis*$^{-1}$ d^{-1})	Cell-specific grazing rate (*Syn K. brevis*$^{-1}$ h^{-1})	*K. brevis* cell N ingested (% h^{-1})
4*	2229	1.94×10^5	0.77	4.96×10^{-4}	2.92×10^{-4} (3.2×10^{-5})	4.9	0.026	0.96	0.73
*		5.83×10^5	2.38	1.54×10^{-3}	9.28×10^{-4} (3.9×10^{-5})	5.2	0.084	3.04	2.32
*		9.67×10^5	4.08	2.65×10^{-3}	1.49×10^{-3} (5.0×10^{-5})	5.0	0.14	4.87	3.73
		1.94×10^6	9.03	5.85×10^{-3}	2.93×10^{-3}	4.9	0.26	9.58	7.36
		5.83×10^6	46.4	3.00×10^{-2}	1.22×10^{-2}	6.9	1.10	40.0	30.7
		9.72×10^6	225.8	1.46×10^{-1}	2.56×10^{-2}	8.6	2.15	83.8	65.1
*	2228	1.94×10^5	0.72	4.63×10^{-4}	3.17×10^{-4} (2.8×10^{-5})	5.4	0.028	1.04	0.78
*		5.83×10^5	2.22	1.44×10^{-3}	7.02×10^{-3} (8.4×10^{-5})	3.9	0.063	2.30	1.76
*		9.67×10^5	3.81	2.47×10^{-3}	1.20×10^{-3} (3.5×10^{-5})	4.0	0.107	3.90	3.01
		1.94×10^6	8.43	5.47×10^{-3}	2.75×10^{-3}	4.6	0.25	9.01	6.92
		5.83×10^6	41.7	2.70×10^{-2}	1.44×10^{-2}	8.1	1.29	47.0	35.6
		9.72×10^6	210.9	1.37×10^{-1}	2.42×10^{-2}	8.2	1.99	79.3	61.8
5	2229	3.11×10^6	0.36	2.35×10^{-4}	8.17×10^{-4}	0.6	0.13	1.90	1.40
		1.56×10^7	1.82	1.18×10^{-3}	1.43×10^{-3}	0.2	0.22	3.40	2.50
		3.11×10^7	3.63	2.35×10^{-3}	2.50×10^{-3}	0.2	0.39	6.10	4.48
		7.80×10^7	9.08	5.89×10^{-3}	3.92×10^{-3}	0.1	0.60	10.1	7.42
		1.56×10^8	18.17	1.18×10^{-2}	5.38×10^{-3}	0.1	0.83	15.2	11.1
		3.11×10^8	36.33	2.35×10^{-2}	7.31×10^{-3}	0.1	1.13	24.1	17.7
	2228	3.11×10^6	0.49	3.17×10^{-4}	6.13×10^{-4}	0.5	0.1	1.43	1.05
		1.56×10^7	2.45	1.58×10^{-3}	1.90×10^{-3}	0.3	0.29	4.54	3.33
		3.11×10^7	4.90	3.17×10^{-3}	2.97×10^{-3}	0.2	0.46	7.26	5.32
		7.80×10^7	12.25	7.92×10^{-3}	5.41×10^{-3}	0.2	0.84	14.0	10.2
		1.56×10^8	24.49	1.58×10^{-2}	7.21×10^{-3}	0.1	1.11	20.3	14.9
		3.11×10^8	48.99	3.17×10^{-2}	8.73×10^{-3}	0.1	1.35	28.8	21.1
6*	2229	2.84×10^7	0.86	5.58×10^{-4}	9.1×10^{-4} (0)	0.1	0.16	21.9	1.46
*		7.10×10^7	2.15	1.39×10^{-3}	1.87×10^{-3} (1.6×10^{-4})	0.1	0.34	4.10	3.00
*		1.42×10^8	4.30	2.79×10^{-3}	5.29×10^{-3} (1.9×10^{-4})	0.1	0.95	11.6	8.50

than rates reported by Bronk et al. (2004) and Heil et al. (2007) for natural Florida blooms, but are comparable to rates reported by Sinclair (2008) for cultures grown under low light. Uptake rates of urea by *K. brevis* from the southwestern Florida shelf, reported by Heil et al. (2007), were comparable to N-specific uptake rates of urea for a related *Karenia* species, *K. mikimotoi*, during a bloom off the East China Sea (Li et al. 2009). The higher rates in the field relative to those determined in the present study are likely a consequence of up-regulation of urea metabolism in the field populations when other sources of N were limiting. As noted above, our laboratory experiments were conducted under low urea concentrations, but abundant NO_3^-.

The rates of urea uptake reported here were about 100-fold lower than the grazing rates when compared on the basis of N taken up. Although our reported rates of urea uptake are low compared to other reports of *in situ* rates when cells were preconditioned with urea, urea uptake and grazing rates may be more comparable under natural conditions.

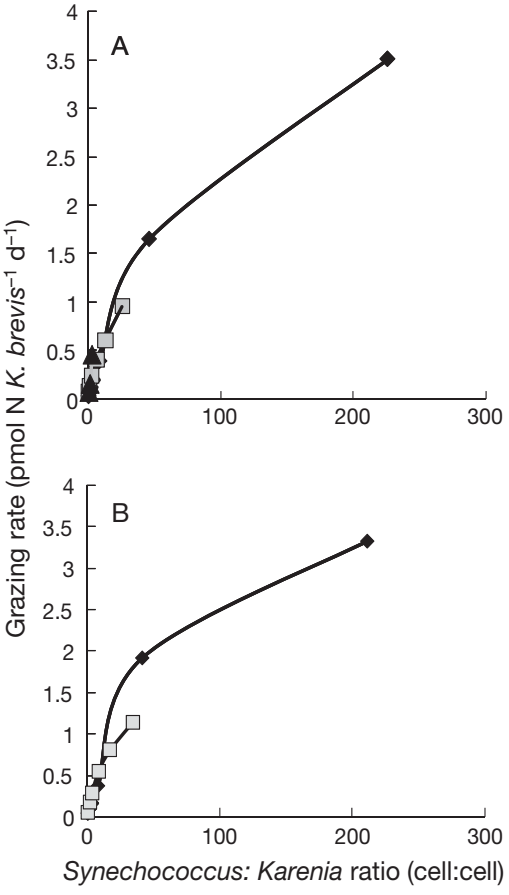

Fig. 6. *Karenia brevis*. Rate of grazing by *K. brevis* Clones (A) CCMP2229 and (B) CCMP2228, expressed as pmol-N dinoflagellate^{-1} d^{-1}, as a function of the ratio of prey (*Synechococcus*) to predators (*K. brevis*) on a cell:cell basis. Results are from Expt 4 (◆), Expt 5 (□), and Expt 6 (▲, panel A only)

Grazing rates

Grazing by *Karenia brevis* on *Synechococcus* was previously reported by Jeong et al. (2005), who compared the ability of 18 red tide species to graze *Synechoccocus*. They reported that, for an initial *Synechococcus* concentration of 1.25×10^6 cells ml^{-1}, the ingestion rate was 5 *Synechococcus K. brevis*$^{-1}$ h^{-1}. Our results (0.96 to 83.8 *Synechococcus K. brevis*$^{-1}$ h^{-1}), using different methodology, compare favorably with, and fall either side of, the values of Jeong et al. (2005). Grazing by *K. brevis* on *Synechococcus* has also been observed elsewhere (L. A. Procise & M. M Mulholland unpubl. data), but at rates apparently much lower than those of Jeong et al. (2005) or the present study. The clearance rates reported herein (Table 4) also compare favorably with the range reported by Jeong et al. (2005) for several harmful algal species, although a clearance rate for *K. brevis* was not reported specifically. The confocal microscopy results also suggest that ingestion of *Synechococcus* is on the order of a few cells at a time, as no image yielded more than a few fluorescently visible cells inside the *K. brevis* cells.

The N-specific grazing rates for the highest prey: predator ratio were comparable to the specific growth rates reported here when both rates were normalized on a daily basis, suggesting that this source of nutrition can be important under specific environmental conditions. Such a conclusion can be verified using mass balance calculations. The cellular N quota of *Synechococcus* has been reported to range from 1.65 fmol-N cell^{-1} (Richardson 2004) to 3.5 fmol-N cell^{-1} (Kana & Glibert 1987) and that of *Karenia brevis* has been estimated to be 5.4 pmol-N cell^{-1} (Shanley & Vargo 1993, Sinclair et al. 2006, present study). Thus, it would require roughly 1.5×10^3 to 3.3×10^3 *Synechococcus* cells to double the biomass of *K. brevis* if it is assumed (for the basis of mass balance calculations only) that all N were retained for growth. At a *K. brevis* growth rate of 0.3 d^{-1} (0.43 divisions d^{-1}), the cellular N quota would be obtained from ingestion of 20 to 40 *Synechococcus* cells h^{-1}. At a growth rate of 0.58 d^{-1} (0.84 divisions d^{-1}), as reported here for low-light growth on *Synechococcus* (Table 2), the cellular N quota would be obtained from ingestion of 50 to 100 *Synechococcus K. brevis*$^{-1}$ h^{-1}. The rates reported here are in this general range, 0.96 to 83.8 *Synechococcus K. brevis*$^{-1}$ h^{-1} (Table 4), indicating that grazing on picoplankton can potentially account for a major fraction of the N demand for growth of *K. brevis*.

Clonal differences

No significant differences were found between the 2 *Karenia brevis* clones studied here. Intraspecific differences in various traits of many clones of dinoflagellates, including N uptake rates by *K. brevis*, have been observed (e.g. Burkholder & Glibert 2006, Sinclair 2008), but in this case, the ability to feed may be a characteristic of both clones. The clone used by Jeong et al. (2005) was different from the clones used in the present study, further suggesting that feeding may be a common trait of *K. brevis.*

Clonal differences in the growth and N nutrition of *Synechococcus* spp. are well known. Some clones arrest their cell division upon N limitation, while others continued to divide at slower rates during N depletion (Glibert et al. 1986). Clonal differences in the ability of *Synechococcus* to take up NH_4^+ and NO_3^- have also been shown (Glibert & Ray 1990), but very little is known about the clonal differences in their ability to grow on urea. The extent to which growth and nutrient

uptake rates of the Florida clone varies with N growth status is not well understood (but see Richardson 2004), but will affect its ability to maintain populations that may be available as food for *Karenia brevis in situ.*

Implications for natural blooms

The sources of N supporting *Karenia brevis* have long been in debate for the southwestern Florida shelf and, more generally, the Gulf of Mexico. Blooms have been hypothesized to initiate in response to organic N that becomes available following outbreaks of the cyanobacterium *Trichodesmium* (e.g. Walsh & Steidinger 2001, Mulholland et al. 2006). *Trichodesmium* may release a significant fraction of its newly fixed N in the form of dissolved organic nitrogen (DON) (Glibert & Bronk 1994), and estimates of this contribution in natural blooms suggest that DON from *Trichodesmium* may be sufficient to support moderately dense ($\leq 10^5$ cells l^{-1}) *K. brevis* blooms (Mulholland et al. 2006). Other sources of nutrients that may sustain moderate to dense blooms ($>10^5$ cells l^{-1}) have been proposed. Vargo et al. (2004) estimated that estuarine (land-based) sources of N and P could help to sustain moderate nearshore blooms of *K. brevis*. Vargo et al. (2008) evaluated potential dissolved nutrient sources and fluxes and suggested that in addition to estuarine inputs, sources such as release of N from dead and decaying fish, atmospheric deposition, benthic flux, and release from zooplankton may all be important, but no single factor seemed to be sufficient to sustain blooms.

Sinclair et al. (2006) proposed another mechanism for obtaining nutrients. They found that *Karenia brevis* cells can migrate into the sediments on a daily basis and potentially acquire NO_3^- originating from the pore waters. They reported N-specific NO_3^- uptake rates that were not only higher than the urea uptake rates reported herein, but also were higher than the field measurements reported by Heil et al. (2007), ranging from 0.2 h^{-1} at a 0.5 µmol N^{-1} addition to 0.52 h^{-1} at an addition of 11 µmol N^{-1}. The NO_3^- uptake rates in the Sinclair et al. (2006) study were also found to vary as a function of time of day and extent to which the cells had been previously exposed to or conditioned on NO_3^-. Ambient concentrations of NO_3^- in the water column off the southwestern Florida shelf are typically low (<1 µM N; Heil et al. 2007), and Sinclair et al. (2006) found that those cells that were comparatively NO_3^--depleted for the 12 h prior to descending in the sediments had a higher rate of NO_3^- uptake.

With the specific rates of N-specific grazing reported here, we can add grazing as a potentially important nutritional strategy of *Karenia brevis*. The growth experiments demonstrated that in the presence of limiting dissolved N, *K. brevis* could grow and survive for a period of at least days to weeks. Indirect evidence that grazing on *Synechococcus* may be important during blooms comes from a study of the southwestern Florida shelf in 2003 when the phytoplankton community varied in composition along the north–south gradient (Heil et al. 2007). In that study, phytoplankton composition was tracked using pigment signatures, including gyroxanthin diester as an indicator of *K. brevis* (e.g. Millie et al. 1997) and zeaxanthin as an indicator of *Synechococcus* (e.g. Kana et al. 1988). The region where *K. brevis* was detected, off the Peace River, was the region where zeaxanthin was virtually absent despite its presence in surrounding waters (Heil et al. 2007), implying that *Synechococcus* was controlled by *K. brevis* grazing. In the same study, urea was found to be significantly associated with the abundance of *Synechococcus*. It was suggested therein that as anthropogenic sources of N from urea applications in agriculture increase, one possible outcome will be increases in cyanobacterial blooms (Heil et al. 2007).

It can now be hypothesized that as cyanobacterial blooms increase, so too does the potential for *Karenia brevis* growth to be enhanced and for blooms to be sustained through grazing, especially under the low light conditions associated with bloom self-shading. Recognition of this pathway is at least one step toward reconciling the long-term reported increase in *K. brevis* blooms (e.g. Brand & Compton 2007) and the tendency for blooms of this species to develop offshore in seemingly oligotrophic waters (e.g. Vargo et al. 2004, 2008). Many important questions remain, including whether the rates reported in the laboratory are comparable to those in the field, whether all clones of *Synechococcus* are grazed similarly or whether all clones of *K. brevis* have comparable grazing behavior, whether other algae or bacteria are also grazed and at what rate, how grazing rates may change on a diel basis or with physiological status, and whether toxicity of *K. brevis* varies with nutrient uptake and feeding strategies.

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