Feeding by raphidophytes on the cyanobacterium *Synechococcus* sp.

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ABSTRACT: We investigated feeding by the raphidophytes *Chattonella ovata*, *C. subsalsa*, *Fibrocapsa japonica*, and *Heterosigma akashiwo* on the cyanobacterium *Synechococcus* sp. To explore whether each species is able to feed on *Synechococcus* sp., we carefully observed inside target grazer cells using an epifluorescence microscope and tranbsmission electron microscope (TEM). We also explored the feeding behaviors of *C. ovata* and *H. akashiwo* on *Synechococcus* using high-resolution video microscopy. In addition, we measured ingestion rates of *C. ovata*, *C. subsalsa* and *H. akashiwo* on *Synechococcus* sp. as a function of prey concentration. We calculated grazing coefficients by combining the field data on abundances of *H. akashiwo* and co-occurring *Synechococcus* spp. with laboratory data on ingestion rates. Both *C. ovata* and *H. akashiwo* were able to ingest single *Synechococcus* cells. However, neither TEM nor video microscopy showed any *Synechococcus* cells inside or ingested by *F. japonica*. One to two ingested *Synechococcus* cells inside the protoplasm of *F. japonica* cells were very rarely observed. *C. ovata* and *H. akashiwo* engulfed a single *Synechococcus* cell captured by the mucus excreted from mucocysts. The ingestion rates of *C. ovata*, *C. subsalsa*, or *H. akashiwo* on *Synechococcus* increased continuously with increasing prey concentration at prey concentrations $\leq 4 \times 10^6$ to 5.5×10^6 cells ml^{-1}. At a given prey concentration, the highest ingestion rates of the raphidophytes on *Synechococcus* were 18.6 cells raphidophyte^{-1} h^{-1} for *C. ovata*, 20.5 cells raphidophyte^{-1} h^{-1} for *C. subsalsa*, and 3.9 cells raphidophyte^{-1} h^{-1} for *H. akashiwo.* The calculated grazing coefficients attributable to *H. akashiwo* on co-occurring *Synechococcus* spp. were up to 1.24 d^{-1}. The results of the present study suggest that raphidophytes sometimes have a considerable grazing impact on populations of *Synechococcus*.

KEY WORDS: *Chattonella* spp. · *Fibrocapsa japonica* · Graze · *Heterosigma akashiwo* · Harmful algal bloom · HAB · Ingestion · Red tide

INTRODUCTION

The raphidophytes *Chattonella* spp., *Fibrocapsa japonica*, and *Heterosigma* spp. are common red-tide organisms with worldwide distribution, excluding arctic waters (Smayda 1998, Edvardsen & Imai 2006, Demir et al. 2008, Menden-Deuer et al. 2008). They are known to be harmful to other plankton (e.g. Clough & Strom 2005) and fish (e.g. Hiroishi et al. 2005) and have often caused large-scale fish mortality rates in the

waters of many countries (MacKenzie 1991, Honjo 1993, Imai et al. 1996, Bourdelais et al. 2002). The density of *H. akashiwo* sometimes exceeds 100 000 cells ml^{-1} during red tides (Nagasaki et al. 1996, Jeong et al. 2005d), while that of *Chattonella* spp. or *F. japonica* sometimes exceeds 10 000 cells ml^{-1}. Gill tissue damage is known to be the ultimate cause of fish death (Edvardsen & Imai 2006). Reactive oxygen species, neurotoxins, and mucus have been suggested to be responsible for the gill tissue damage (Oda et al. 1997, Marshall et al. 2003, Bowers et al. 2006). However, *Chattonella* spp., *F. japonica*, and *Heterosigma* spp. are known to be important prey for mixotrophic and heterotrophic dinoflagellates (Tillmann & Reckermann 2002, Jeong et al. 2003, Demir et al. 2008). Therefore, they play diverse roles in marine ecosystems.

The raphidophytes were previously considered to be exclusively autotrophic algae and thus were treated as phytoplankton. Therefore, there have been only a few studies on feeding by raphidophytes; Nygaard & Tobiesen (1993) used isotope-labeled bacteria to show that *Heterosigma akashiwo* was able to ingest heterotrophic bacteria. Recently, Seong et al. (2006) used fluorescent-labeled bacteria (FLBs) and confocal microscopy to show that *Chattonella ovata* and *H. akashiwo* were able to feed on heterotrophic bacteria. However, in these studies the location through which the raphidophytes engulfed prey cells and feeding behaviors of raphidophytes were not explored. Raphidophytes have a very high number of chloroplasts (e.g. Hara & Chihara 1987) and thus under light or epifluorescence microscopy, it is difficult to find openings on the raphidophyte cell body. Using a transmission electron microscope (TEM), raphidophytes have been observed to have 2 types of possible openings: (1) a funnel-shaped groove with 2 flagellae, and (2) mucocysts, which look like small openings when viewed from outside the cell surface (Vesk & Moestrup 1987). The mucocysts secrete large amounts of mucus that sometimes kill fish (Imai et al. 1993). Thus, the mucocysts are candidates to be the location through which the raphidophytes engulf prey cells, and the roles of the mucocysts in raphidophyte feeding should be explored. To explore these topics, TEM and high-resolution video microscopy are necessary. In addition, what other prey raphidophytes feed on besides heterotrophic bacteria is also very important for understanding raphidophyte ecophysiology.

The photosynthetic cyanobacterium *Synechococcus* spp. is a ubiquitous prokaryote in marine environments (Landry et al. 1996, Maranon et al. 2003). It often dominates the abundance and/or the primary production of phytoplankton in both coastal and open ocean waters (Stal et al. 2003, Nielsen et al. 2004). The abundance of *Synechococcus* spp. often exceeds 10^5 cells ml^{-1} and sometimes forms blooms (Glibert et al. 2004, Murrell &

Lores 2004, Jeong et al. 2005d). Sunda et al. (2006) reported that some blooms dominated by *Synechococcus* spp. were harmful to diverse marine organisms such as seagrasses, spiny lobsters, and multiple sponge species. In ocean waters, *Synechococcus* spp. is thought to be one of the major contributors to CO_2 and nutrient uptake from the ocean waters and in turn eventually from the atmosphere (Maranon et al. 2003). In addition, some *Synechococcus* spp. are known to conduct nitrogen fixation (Phlips et al. 1989, Herrero et al. 2001) and thus are able to survive in environments where the concentration of inorganic nitrogen is very low (Glibert et al. 2004, Sunda et al. 2006). In turn, they may play an important role as a nitrogen source for their predators. Therefore, the growth and mortality of *Synechococcus* spp. are important factors in understanding the cycling of materials in marine planktonic food webs.

Heterotrophic nanoflagellates and ciliates have long been known to be major grazers on *Synechococcus* spp. (Christaki et al. 1999, 2002, Agawin et al. 2004). However, many mixotrophic dinoflagellates have recently been shown to feed on *Synechococcus* spp. (Jeong et al. 2005a, Glibert et al. 2009). Raphidophytes have been reported to often co-occur with *Synechococcus* spp. and/or other cyanobacteria (Hayes & Lewitus 2003, Livingston 2007, H. J. Jeong et al. unpubl. data). Therefore, there is a possibility that raphidophytes feed on *Synechococcus* spp. However, the interactions between raphidophytes and *Synechococcus* spp, and in particular the possible predator-prey relationships, are still poorly understood.

The goal of the present study was to understand the interactions between raphidophytes and *Synechococcus* spp. Our questions were: (1) Are raphidophytes able to feed on *Synechococcus* spp.? (2) If so, what are the feeding behaviors (mechanisms) they use? (3) What is the functional response of raphidophytes to the concentrations of *Synechococcus* spp.? (4) What is the grazing impact of raphidophytes on the population of *Synechococcus* spp. in natural environments?

To answer these questions we (1) investigated whether or not the common raphidophytes *Chattonella ovata*, *Fibrocapsa japonica*, and *Heterosigma akashiwo* are able to feed on *Synechococcus* sp. We observed inside the protoplasm of target raphidophyte cells using epifluorescence microscopy and TEM after adding living *Synechococcus* sp. cells; (2) explored the feeding behaviors of *C. ovata* and *H. akashiwo* on *Synechococcus* sp. using high-resolution video microscopy and several different types of microscope; (3) examined the functional responses of *C. ovata* and *H. akashiwo* to *Synechococcus* sp. as a function of *Synechococcus* sp. concentration in the laboratory; (4) estimated the grazing coefficients attributable to *H. akashiwo* on co-occurring *Synechococcus* sp. using our data for ingestion rates obtained from laboratory experiments and the abundances of

predators and prey in the field. The results of the present study provide a basis for understanding the tropho-dynamics of raphidophytes, the interactions between raphidophytes and *Synechococcus* spp., and the bloom dynamics of these 2 components.

MATERIALS AND METHODS

Preparation of experimental organisms. *Chattonella ovata* (equivalent spherical diameter [ESD] = 40.0 µm), *Fibrocapsa japonica* (20.5 µm), and *Heterosigma akashiwo* (11.0 µm) used in the experiments on feeding occurrence (Expt 1), feeding behavior (Expt 2), and effects of prey concentration (bottle incubation, Expt 3) were grown at 20°C and 30 to 31 salinity in enriched f/2 seawater medium (Guillard & Ryther 1962) without silicate under a 14 h light:10 h dark cycle of 30 µE m^{-2} s^{-1} (Table 1). *Synechococcus* sp. (GenBank accession no. DQ023295; ESD = ca. 1 µm) were grown at 20°C and 30 to 31 salinity in enriched f/2 seawater medium under a 14 h light:10 h dark cycle of 30 µE m^{-2} s^{-1} of cool white fluorescent light. ESDs were measured with an electronic particle counter (Coulter Multisizer II). Cultures in their exponential growth phase were used for these feeding experiments.

The *Chattonella subsalsa* (strain CCMP 2191; ESD = 36.5 µm) cultures used in the ^{15}N studies (Expt 4) were obtained from the Provasoli-Guillard National Center for Culture of Marine Phytoplankton, Bigelow Laboratory, West Boothbay Harbor, Maine, USA. Maintenance cultures were grown in 32 salinity and enrichment solution artificial seawater (ESAW) medium (Harrison et al. 1980) under a 14 h light:10 h dark cycle of 200 µE m^{-2} s^{-1}. A monoculture of *Synechococcus* sp. in late exponential growth phase (strain CCMP 1768 originally isolated from the Gulf of Mexico and obtained from Bigelow Laboratory) was used for Expt 4.

Feeding occurrence. Expt 1 was designed to investigate whether or not each raphidophyte species was able to feed on *Synechococcus* sp. We observed *Syne-chococcus* sp. cells inside the predators using epifluorescence microscopy and TEM after adding living *Synechococcus* sp.

A dense culture of each raphidophyte species, maintained in f/2 medium and growing photosynthetically in the exponential growth phase and incubated under a 14 h light:10 h dark cycle of 30 µE m^{-2} s^{-1}, was transferred to a 1 l polycarbonate (PC) bottle containing freshly filtered seawater. Three 1 ml aliquots were then removed from the bottle and examined using a compound microscope to determine the raphidophyte concentration.

For observation of *Synechococcus* sp. under an epifluorescence microscope, the initial concentrations of each raphidophyte predator (ca. 2000 to 10 000 cells ml^{-1}) and *Synechococcus* sp. (ca. 6×10^6 to 7×10^6 cells ml^{-1}) were established using an autopipette to deliver a predetermined volume of culture with a known cell density to the experimental bottles. Triplicate 42 ml PC bottles (mixtures of raphidophyte predator and *Syne-chococcus* sp.) and triplicate predator control bottles (containing only the raphidophyte) were set up at a single prey concentration for each raphidophyte predator. The bottles were filled to capacity with freshly filtered seawater, capped, and then placed on a plankton wheel rotating at 0.9 rpm at 20°C under continuous illumination of 30 µE m^{-2} s^{-1} for incubation <12 h (*Chattonella ovata* and *Heterosigma akashiwo*) or a 14 h light:10 h dark cycle of 30 µE m^{-2}s^{-1} for incubation >12 h (*Fibrocapsa japonica*). After 5, 10, 30, and 60 min and 4 h incubation for *C. ovata* and *H. akashiwo*, which fed on prey cells very well, and 5, 10, 30, and 60 min and 4, 12, 24, 48, and 72 h incubation for *F. japonica*, which rarely fed on *Synechococcus* sp. cells, a 10 ml aliquot was removed from each bottle and then fixed with formalin (final conc. = 4%). The fixed aliquots were filtered onto 5 µm pore-sized, 25 mm PC white membrane filters, and the concentrated cells on the membranes were then observed under an epifluorescence microscope (Zeiss-Axiovert 200M) with green-light excitation at a magnification of 1000× to determine whether or not each raphidophyte predator

Table 1. Sampling location and date and water temperature (Temp.) and salinity for isolation of each species. ESD: equivalent spherical diameter, na: not available, DE: Delaware, USA

Organism	ESD (µm)	Location	Date	Temp. (°C)	Salinity	Source/strain
Chattonella ovata	40.0	Off Saemankeum, Korea	Sep 99	25.5	31.3	Seong et al. (2006)
C. subsalsa	36.5	Indian River Bay, DE, USA	Aug 01	na	na	CCMP 2191
Fibrocapsa japonica	20.5	Hobson's Bay, Melbourne, Australia	Jul 98	na	na	CCMP 1661
Heterosigma akashiwo	11.0	Keum Estuary, Korea	May 01	16.0	27.7	Jeong et al. (2003)
Synechococcus sp. (GenBank accession no. DQ023295)	1.0	East China Sea	Oct 03	22.6	31.9	Jeong et al. (2005a)
Synechococcus sp.	1.0	Gulf of Mexico	Jun 95	na	na	CCMP 1768

was able to feed on *Synechococcus* sp. Pictures showing ingested *Synechococcus* sp. cells inside each raphidophyte predator cell were taken using a digital camera (Zeiss AxioCam MRc5) on the microscope at a magnification of 1000×.

For observations using TEM, living *Synechococcus* sp. cells were added into each of three 270 ml PC bottles (final conc. = ca. 7×10^6 cells ml^{-1}); each contained a target raphidophyte of concentration 2000 to 10 000 cells ml^{-1}. One 'target prey only' control bottle and 1 raphidophyte control bottle (without added prey) were set up for each experiment. The bottles were filled to capacity with freshly filtered seawater, capped, and then placed on a plankton wheel rotating at 20°C under a 14 h light:10 h dark cycle of 30 µE m^{-2} s^{-1}. At the beginning, and after 1, 3, and 5 d incubation periods, the contents of 1 experimental bottle from each interval were distributed into five 50 ml centrifugal tubes and then concentrated at $1610 \times g$ for 10 min using a centrifuge (Vision Centrifuge VS-5500). Five pellets from the 5 centrifugal tubes were then transferred into 1.5 ml tubes and fixed for 1.5 h in 4% (w/v) glutaraldehyde in a culture medium. Afterwards, the fixative was removed and the pellets were rinsed using a 0.2 M cacodylic acid/sodium salt solution (pH 7.4). The pellet was then embedded in agar. After several rinses with the medium, the cells were post-fixed in 1% (v/v) osmium tetroxide in deionized water. Dehydration was accomplished using a graded ethanol series (50, 60, 70, 80, 90, and 100% ethanol, followed by two 100% ethanol steps). The material was embedded in Spurr's low-viscosity resin (Spurr 1969). Sections were obtained with an RMC MT-XL ultramicrotome (Boeckeler Instruments) and post-stained with 3% (w/v) aqueous uranyl acetate followed by lead citrate. The stained sections were viewed with a JEOL-1010 electron microscope.

Feeding behavior. Expt 2 was designed to investigate the feeding behavior of *Chattonella ovata* and *Heterosigma akashiwo* on *Synechococcus* sp. We did not conduct this experiment with *Fibrocapsa japonica* because in Expt 1, this species was revealed to very rarely feed on *Synechococcus* sp. The feeding behavior of *C. ovata* and *H. akashiwo* on living *Synechococcus* sp. was observed under light and epifluorescence microscopes with a high-resolution video system. In particular, we explored how each raphidophyte captures prey cells, how many prey cells are captured simultaneously, and where the raphidophytes engulf the prey.

A dense culture of *Chattonella ovata*, *Heterosigma akashiwo*, and living *Synechococcus* sp. was added to triplicate 42 ml PC bottles (final conc. of *C. ovata* / *Synechococcus* sp. = 2000 / 7×10^6; final conc. of *H. akashiwo* / *Synechococcus* sp. = 10 000 / 7×10^6). The bottles were filled to capacity with freshly filtered seawater, capped, and then placed on a plankton wheel

rotating at 20°C under continuous illumination of 30 µE m^{-2} s^{-1}. The bottles were incubated for 1, 2, 4, and 6 h. A 0.1 ml aliquot was transferred onto a microscope slide and a cover glass was then placed. We monitored the behavior of >300 raphidophyte cells for each predator with respect to the living prey, using differential interference contrast (DIC) optics under an inverted epifluorescence microscope at a magnification of 400 to 1000×. The feeding process of the raphidophyte was recorded using a video analyzing system (Sony DXC-C33) and also a digital camera (Zeiss AxioCam MRc5).

Effects of prey concentration. Expts 3 and 4 were designed to investigate the ingestion rates of *Heterosigma akashiwo* and *Chattonella ovata* (or *C. subsalsa*) on *Synechococcus* sp. as a function of prey concentration. Two different methods were used for these experiments; the first method (Expt 3) was measuring ingestion rates by comparing the concentrations of the raphidophyte predator and *Synechococcus* sp. between the experimental and control bottles. This method was used for *H. akashiwo* and *C. ovata*. The second method (Expt 4) was measuring ingestion rates using the ^{15}N isotope method as in Glibert et al. (2009). This method was used for *C. subsalsa*.

In Expt 3, a dense culture of each raphidophyte predator (ca. 5000 cells ml^{-1} for *Chattonella ovata* and ca. 100 000 cells ml^{-1} for *Heterosigma akashiwo*) maintained in f/2 medium and growing photosynthetically in the exponential phase under a 14 h light:10 h dark cycle of 30 µE m^{-2} s^{-1} was transferred into a 1 l PC bottle. Three 1 ml aliquots from the bottle were counted using a compound microscope to determine the cell concentrations of the raphidophyte predator, and the cultures were then used to conduct the experiments.

The initial concentrations of *Chattonella ovata* (or *Heterosigma akashiwo*) and live *Synechococcus* sp. were established using an autopipette to deliver predetermined volumes of known cell concentrations to the bottles. Triplicate 42 ml PC experimental bottles (containing mixtures of predator and prey), prey control bottles (containing prey only), and predator control bottles (containing predator only) were also established. Ten ml of f/2 medium were added to all the bottles, which were then filled to capacity with freshly filtered seawater, capped, placed on plankton wheels rotating, and incubated at 20°C under a 14 h light:10 h dark cycle of 30 µE m^{-2} s^{-1}. To determine the actual initial predator and prey densities (cells ml^{-1}) at the beginning of the experiment and after 24 h incubation, a 4 ml aliquot was removed from each bottle and fixed with 5% Lugol's solution, while another 4 ml aliquot was fixed with 4% formalin. All or >300 predator cells, fixed in Lugol's solution, in three 1 ml Sedgwick-Rafter counting chambers were enumerated. The aliquots fixed with formalin were filtered onto 0.2 µm pore

sized, 25 mm PC black membrane filters and then the concentrated cells on the membranes were observed under an epifluorescence microscope (Olympus BX51) with green-light excitation at a magnification of 1000× to determine the concentration of *Synechococcus* sp. The actual initial predator (and prey) concentrations were 22 to 2330 cells ml^{-1} (4220 to 3 880 680 cells ml^{-1}; 8 prey concentrations) for *C. ovata* and 102 to 12 780 cells ml^{-1} (6360 to 4 608 780 cells ml^{-1}; 7 prey concentrations) for *H. akashiwo*. We tried to minimize the concentration of heterotrophic bacteria in the *C. ovata* and *H. akashiwo* cultures using diverse antibiotics and/or mechanical filtering. Among the antibiotics (chloramphenicol, gentamicin, kanamycin, cephalothin, norfloxacin, erythromycin, ciprofloxacin), chloramphenicol was the most effective. The final concentration of 15 µg ml^{-1} was an optimal concentration in which >50% of the heterotrophic bacteria were killed, while there was no apparent harmful effect on the swimming pattern, morphology, and abundance of *C. ovata*. We also reduced the abundance of heterotrophic bacteria inside a culture of *H. akashiwo* by removing waters from the culture of the raphidophyte using a siphon and 5 or 10 µm meshed net and then adding autoclaved seawater to the culture. However, there were still some heterotrophic bacteria in the culture. We counted the actual initial concentration of heterotrophic bacteria using the same method as Seong et al. (2006). The actual initial concentrations of heterotrophic bacteria in the *C. ovata* and *H. akashiwo* incubation bottles were <13% and <18% of *Synechococcus* sp., respectively.

Ingestion and clearance rates were calculated using the equations of Frost (1972) and Heinbokel (1978).

In Expt 4, 1 d prior to the grazing experiments, a monoculture of *Synechococcus* sp. in late exponential growth phase (strain CCMP 1768) was labeled with 10 µmol ^{15}N-urea and incubated for 24 h to ensure uniform isotope labeling. The amount of isotope label in the *Synechococcus* sp. culture was subsequently determined by mass spectrometry. The *Chattonella subsalsa* cultures (N-starved, in late exponential growth phase) were gently transferred to new 60 ml culture flasks and an inoculum of the labeled *Synechococcus* sp. was added. The initial concentration of *C. subsalsa* was 3.8×10^4 cell ml^{-1}. Six different cell concentrations of ^{15}N-labeled *Synechococcus* sp. were used, from 1.09×10^5 to 5.4×10^6 cells ml^{-1} and no prey (prey control). The flasks were held in a constant temperature incubator for ~24 h, on a 14 h light:10 h dark cycle. After incubation, the contents of the flasks were filtered through pre-combusted filters (GF/D, nominal pore size 2.7 µm) that retained the raphidophyte cells and possibly some of the *Synechococcus* sp. cells (if attached to the raphidophytes). The filtrate was then re-filtered through a smaller pre-combusted filter (GF/F, nominal pore size 0.7 µm) to retain *Synechococcus* sp. cells that had not been collected 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 raphidophyte cells versus the *Synechococcus* sp. cells. As this experiment was conducted as a pilot experiment as part of a larger effort (Glibert et al. 2009), these particular treatments were not replicated. All ^{15}N samples were processed using a Sercon mass spectrometer.

To calculate the ingestion rates of *Chattonella subsalsa* on *Synechococcus* sp., the specific N-uptake rate (V, time^{-1}) was first calculated using the following equation:

$$V = \text{atom \% excess}/(\text{atom \% enrichment} \times \text{time}) \quad (1)$$

in which the atom % enrichment was that of the initial *Synechococcus* sp. culture and the atom % excess was that of the raphidophyte culture (GF/D fraction) after incubation. To correct the atom % enrichment of the GF/D filters for any contribution by retained *Synechococcus* sp. cells (or bacteria), the residual amount of *Synechococcus* sp. 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 raphidophyte only contribution was then determined:

$$\text{Atom \% raphidophyte} = [(\text{measured atom \% GF/D})$$
(mass of raphidophyte + mass of *Synechococcus* sp.)
– (atom % *Synechococcus* sp.)
(mass of *Synechococcus* sp.)]
$$/ (\text{mass of raphidophyte}) \quad (2)$$

where all units of mass are in µmol-N. Grazing rates in terms of amount of N grazed per time (N ingestion rates) were calculated by multiplying the N-specific ingestion rates times the particulate N (PN) content of the raphidophyte cultures:

$$\text{N ingestion rate} = \text{N specific ingestion rate} \times \text{PN} \quad (3)$$

Lastly, all ingestion rates were also corrected for the direct uptake of urea by the cultures by subtracting urea uptake rates estimated in parallel experiments as reported in Glibert et al. (2009).

Ingestion rates were also calculated in terms of cells of *Synechococcus* sp. grazed (cells raphidophyte^{-1} h^{-1}) by converting the N ingestion rate to a cell-specific rate, based on a conversion of 3.5 fmol-N cell^{-1} of *Synechococcus elongatis* (Kana & Glibert 1987).

Potential grazing impact. We estimated the grazing coefficients (mortality rate due to predation) attributable to *Heterosigma akashiwo* on *Synechococcus* spp. by combining field data on the abundances of *H. akashiwo* and *Synechococcus* spp. with the ingestion

rates of the predator on *Synechococcus* sp. obtained in the present study. Data on the abundances of *H. akashiwo* and co-occurring *Synechococcus* spp. used in this estimation were obtained using the water samples from Masan Bay (in 2004) and Shiwha Bay (2008), Korea. Field data on *Chattonella* spp. and co-occurring *Synechococcus* spp. were not available.

The grazing coefficient (g, d^{-1}) was calculated as:

$$g = CR \times GC \times 24 \qquad (4)$$

where *CR* (ml raphidophyte^{-1}h^{-1}) is the clearance rate of an raphidophyte predator on *Synechococcus* sp. prey at a given prey concentration and *GC* is a grazer concentration (cells ml^{-1}). *CR* was calculated as:

$$CR = IR/pc \qquad (5)$$

where *IR* (cells eaten raphidophyte^{-1} h^{-1}) is the ingestion rate of the algal predator on the target prey and *pc* (cells ml^{-1}) is the prey concentration. *CR* was corrected using $Q_{10} = 2.8$ (Hansen et al. 1997) because *in situ* water temperatures and the temperature used in the laboratory for this experiment (20°C) were sometimes different.

RESULTS

Feeding occurrences

Under TEM, *Synechococcus* sp. had 2 to 3 distinctive thylakoid layers (Fig. 1A). Unfed *Chattonella ovata*, *Fibrocapsa japonica*, and *Heterosigma akashiwo* had dense chloroplasts near the cell surface (Fig. 1B–D). They also had numerous mucocysts near the surface.

Under TEM and high-resolution video microscopy, *Chattonella ovata* and *Heterosigma akashiwo* were observed to feed readily on *Synechococcus* sp. (see Figs. 2 to 6). Under TEM, various numbers of *Synechococcus* sp., ranging from 1 to 100s, were observed in food vacuoles inside the protoplasm of *C. ovata* (Fig. 2). When *C. ovata* cells with 1 or 2 single ingested *Synechococcus* sp. cells were examined, the *Synechococcus* sp. cells were observed inside food vacuoles in mucocysts of *C. ovata*. This implies that *C. ovata* engulfed the single prey cell through its mucocysts. The sizes of the mucocysts were 1.6 to 3.7 μm long by 1.5 to 3.0 μm wide (n = 15). The size of the mucocyst openings of *C. ovata* was ca. 2 to 3 μm. A large food vacuole containing 100s of *Synechococcus* sp. cells was observed in the center of *C. ovata*. Small food vacuoles containing 1 or several *Synechococcus* sp. cells may have merged to form a larger package (Fig. 2).

Under TEM, *Heterosigma akashiwo* also had food vacuoles containing various numbers of *Synechococcus* sp. (Fig. 3). When ca. 160 transversal TEM serial

sections (70 nm layer serial sections, i.e. ca. 11 μm) from the top to the bottom of 1 *H. akashiwo* cell were examined, 20 to 30 mucocysts and 15 to 20 chloroplasts were observed. The mucocysts were scattered in the cell body of *H. akashiwo* and some of them contained prey cells. The mucocysts look like sacks 0.7 to 2.1 μm long by 1.2 to 2.1 μm wide by 0.6 to 2.5 μm thick (n = 20). The size of the mucocyst openings was ca. 2 μm. The mucocyst size seemed to limit the upper limit of the prey size that the raphidophyte was able to feed on. No prey cells were observed inside the funnel-shaped groove from which 2 flagella arose.

Under TEM, no *Synechococcus* sp. cells were observed inside the protoplasm of *Fibrocapsa japonica* cells (n > 100; Fig. 4). However, very rarely (<1%) 1 to 2 *Synechococcus* sp. cells (bright orange-colored inclusions) were observed in the protoplasms of *F. japonica* cells under an epifluorescence microscope (Fig. 4). In conclusion, all 3 raphidophytes are able to feed on *Synechococcus* sp., but the frequency of ingestion of *Synechococcus* sp. by *F. japonica* was very low.

Feeding behavior

On high-resolution video microscopy, *Chattonella ovata* (and *Heterosigma akashiwo*) displayed 2 undulating flagella, but did not generate detectable feeding currents. *C. ovata* (and *H. akashiwo*) excreted mucus from several mucocysts along the cell body. As *C. ovata* (and *H. akashiwo*) swam in rotation, up to 12 *Synechococcus* sp. cells (and up to 4 cells) were observed to attach to the mucus excreted from many mucocysts along the cell body (Fig. 5). *C. ovata* engulfed a single *Synechococcus* sp. cell through a mucocyst within 7 to 20 s after the prey initially attached to the mucus (Fig. 6A–F and video available at www.int-res.com/articles/suppl/a058p181_app/), while *H. akashiwo* engulfed a single *Synechococcus* sp. cell within 40 to 75 s (Fig. 6G–L).

Effects of prey concentration

The mean prey concentrations in the experiment on the feeding by *Chattonella ovata* on *Synechococcus* sp. were 4.4×10^3 to 3.2×10^6 cells ml^{-1}. With increasing prey concentration, the ingestion rate of *C. ovata* on *Synechococcus* sp. continuously increased (Fig. 7). At a given prey concentration, the highest ingestion rate of *C. ovata* on *Synechococcus* sp. was 18.6 cells raphidophyte^{-1} h^{-1}. The maximum clearance rate of *C. ovata* on *Synechococcus* sp. was 0.1 μl raphidophyte^{-1} h^{-1}.

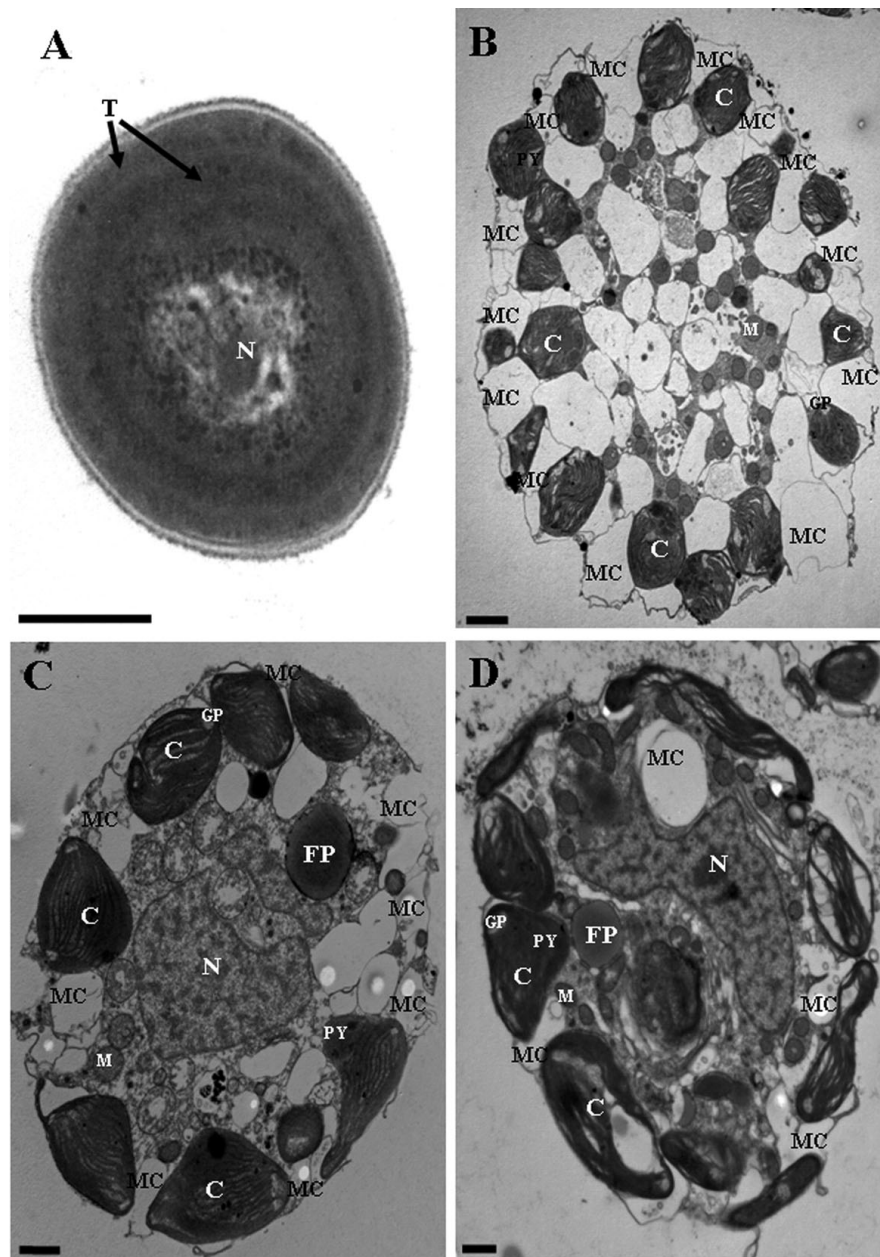The initial prey concentrations in the experiment on the feeding by *Chattonella subsalsa* on *Synechococcus*

Fig. 1. Transmission electron micrographs of the cyanobacterium and raphidophytes growing photosynthetically. (A) *Synechococcus* sp. with 2 distinct layers of thylakoid (T). Unfed (B) *Chattonella ovata*, (C) *Heterosigma akashiwo*, and (D) *Fibrocapsa japonica*. C: chloroplast, FP: fatty particle, GP: genophore, M: mitochondrion, MC: mucocyst, N: nucleus, PY: pyrenoid. Scale bars = 0.2 μm for (A), 2 μm for (B), and 1 μm for (C,D)

sp. were 1.1×10^5 to 5.5×10^6 cells ml^{-1}. With increasing prey concentration, the ingestion rate of *C. subsalsa* on *Synechococcus* sp. continuously increased (Fig. 7). At the given prey concentration, the highest ingestion rate of *C. subsalsa* on *Synechococcus* sp. was 20.5 cells raphidophyte^{-1} h^{-1}.

The initial concentrations of *Synechococcus* sp. in the experiment on the feeding by *Heterosigma*

akashiwo on *Synechococcus* sp. were 6.4×10^3 to 4.6×10^6 cells ml^{-1}. With increasing prey concentration, the ingestion rate of *H. akashiwo* on *Synechococcus* sp. continuously increased (Fig. 8). At the given prey concentration, the highest ingestion rate of *H. akashiwo* on *Synechococcus* sp. was 3.9 cells raphidophyte^{-1} h^{-1}. The maximum clearance rate of *H. akashiwo* on *Synechococcus* sp. was 0.3 μl raphidophyte^{-1} h^{-1}.
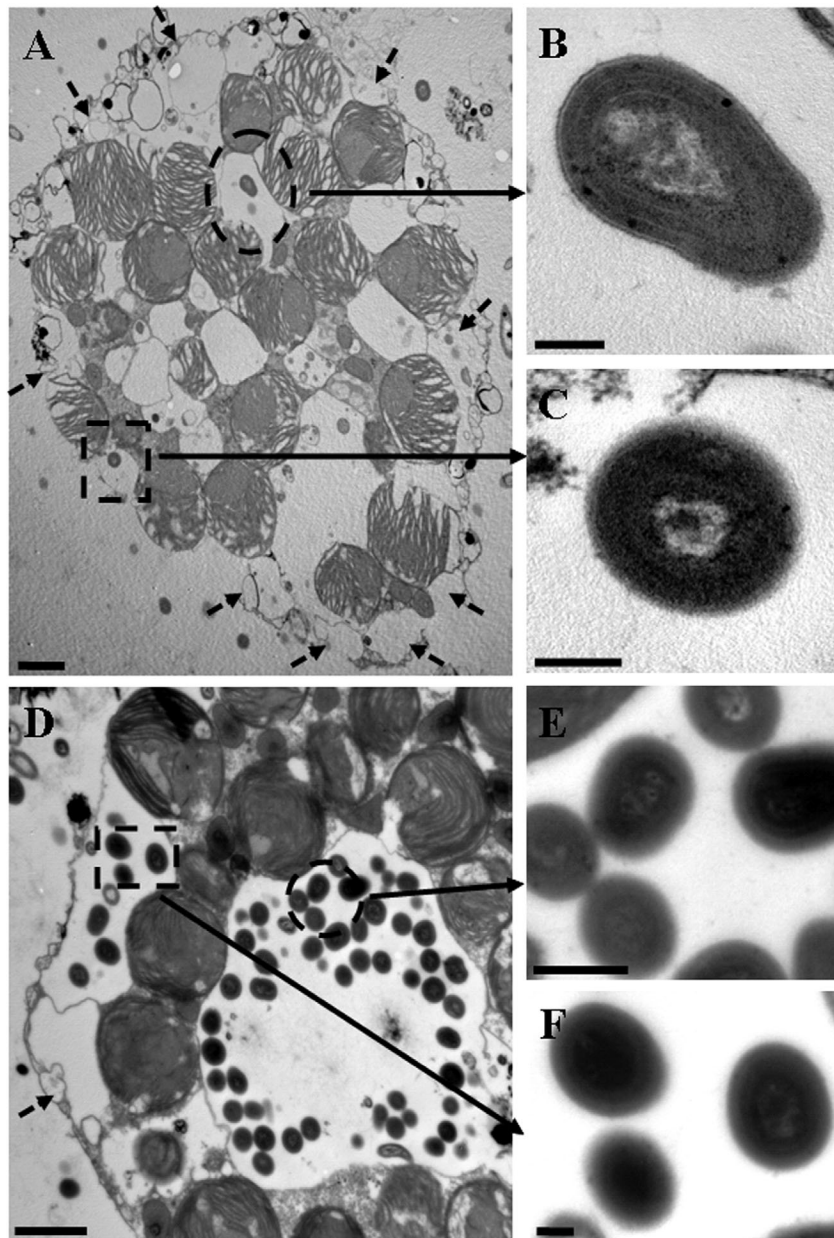
Fig. 2. Transmission electron micrographs of *Chattonella ovata* fed single *Synechococcus* sp. cells. (A) *C. ovata* cell with 2 single *Synechococcus* sp. cells (inside the dashed circle and square). (B,C) Enlarged from (A): single *Synechococcus* sp. cells inside food vacuoles. (D) *C. ovata* cell with a relatively small food vacuole containing 6 single *Synechococcus* sp. cells (3 inside the dashed square) and a large food vacuole containing tens of single *Synechococcus* sp. cells (5 inside the dashed circle). (E,F) Enlarged from (D): single *Synechococcus* sp. cells inside food vacuoles. Dashed arrows indicate mucocysts. Scale bars = 2 μm for (A,D), 0.2 μm for (B,C,E), and 0.5 μm for (F)

Grazing impact

The grazing coefficients attributable to *Heterosigma akashiwo* on co-occurring *Synechococcus* spp. in Masan Bay and Shiwha Bay (n = 23) were 0.001 to 1.238 d^{-1} when the abundances of *H. akashiwo* and *Synechococcus* spp. were 22 to 228 000 cells ml^{-1} and 51 to 194 460 cells ml^{-1}, respectively (Fig. 9).

DISCUSSION

Raphidophyte predators of *Synechococcus* sp. and their feeding behavior

This is the first report on feeding by raphidophytes on cyanobacteria. All 4 raphidophytes tested in the present study were able to feed on *Synechococcus* sp.,
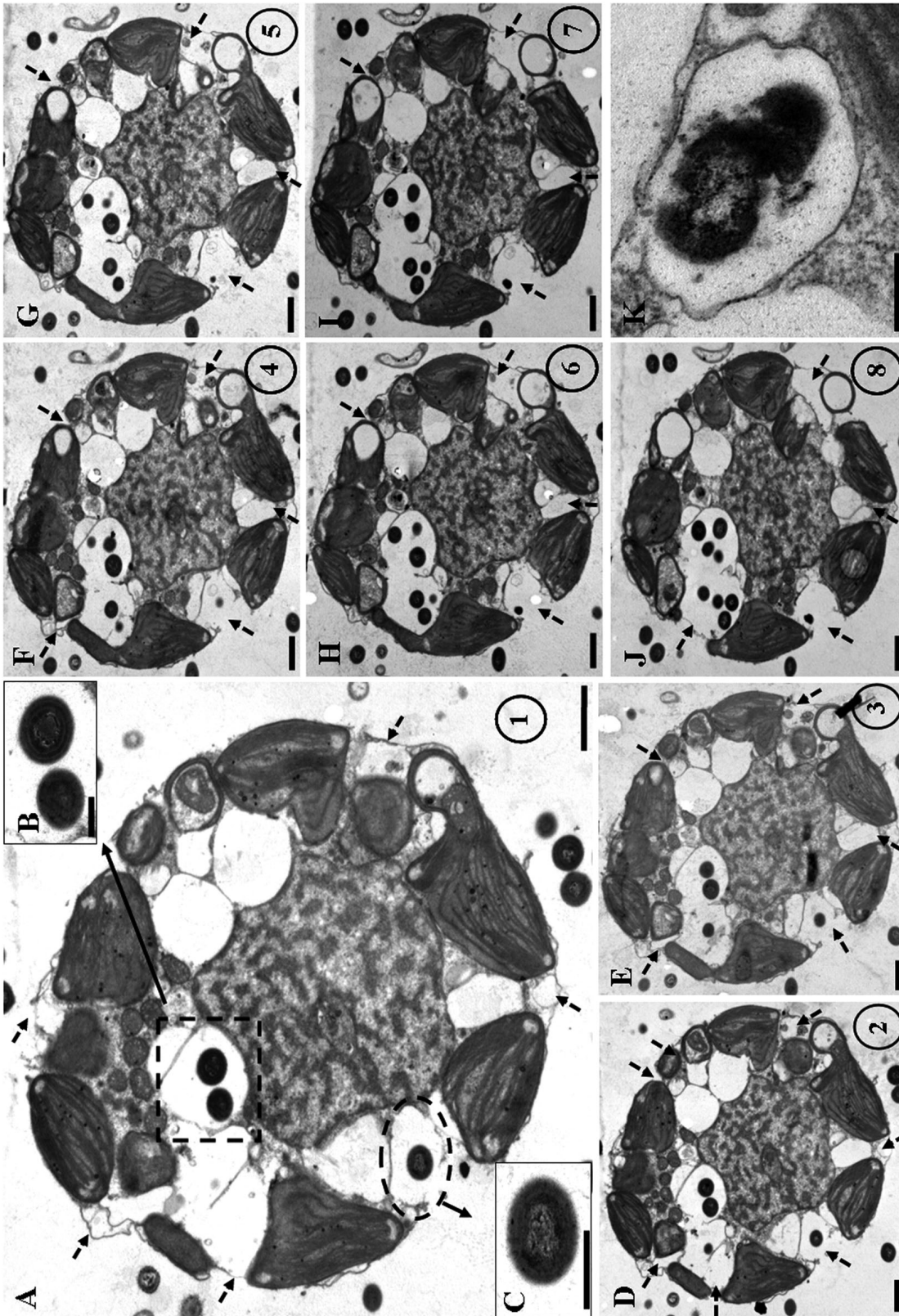
Fig. 3. Transmission electron micrographs of *Heterosigma akashiwo* fed single *Synechococcus* sp. cells. One *H. akashiwo* cell was serially sectioned by 70 nm. (A) One food vacuole containing *Synechococcus* sp. cell (inside dashed circle) and the other food vacuole containing 2 single *Synechococcus* sp. cells (inside dashed square) were observed inside the protoplasm of a *H. akashiwo* cell. (B,C) Enlarged from (A): single *Synechococcus* sp. cells inside food vacuoles. (D–J) Serial sectioned layers showing the variation in the number and shape of *Synechococcus* sp. cells inside the predator cell. All predator cells in (A–J) were the same cell. Circled numbers represent the number of layers serially sectioned by 70 nm. Dashed arrows indicate mucocysts. (K) Two semi-decomposed *Synechococcus* sp. cells inside a food vacuole of another *H. akashiwo* cell. Scale bars = 1 μm for (A,D–J) and 0.5 μm for (B,C,K)
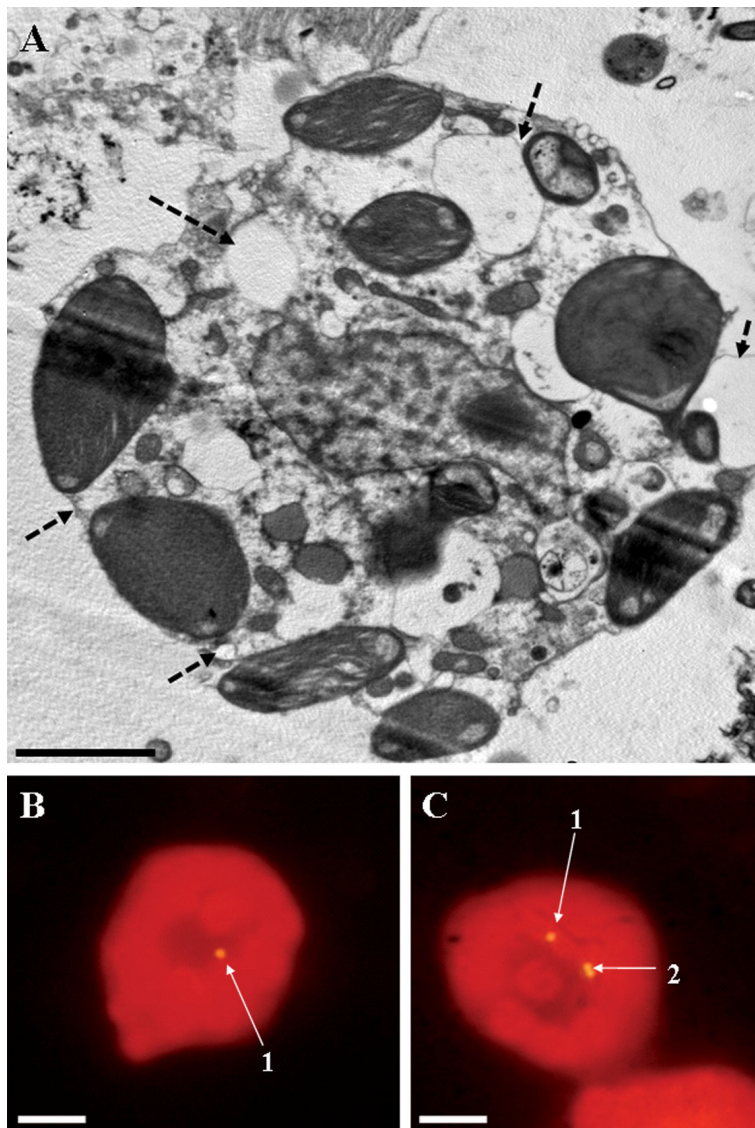
Fig. 4. *Fibrocapsa japonica* observed under (A) transmission electron microscopy and (B,C) epifluorescence microscopy after incubation with live *Synechococcus* sp. cells. (A) No *Synechococcus* sp. cell was observed inside the protoplasm of the predator; dashed arrows indicate mucocysts. *F. japonica* cells containing (B) 1 and (C) 2 *Synechococcus* sp. cells (colored orange). Scale bars = 2 µm

but ingestion of *Synechococcus* sp. by *Fibrocapsa japonica* was very rare. The size of *F. japonica* is between that of *Chattonella ovata* and *Heterosigma akashiwo*. Therefore, the predator sizes may not be the cause of the lack in feeding. On the phylogeny tree based on small subunit ribosomal RNA of the Raphidophyceae, *F. japonica* is an ancestor of *Chattonella* spp. and *Heterosigma* spp. (Bowers et al. 2006). *F. japonica* may have fewer enzymes involved in prey recognition and/or digestion of *Synechococcus* sp. compared to *Chattonella* spp. and *H. akashiwo*. It

would be worthwhile to investigate the genomes and proteomes of these 3 raphidophytes. In natural environments, *Chattonella* spp. may compete with *H. akashiwo* for *Synechococcus* spp. prey, but not with *F. japonica*.

In our preliminary experiments, *Chattonella ovata* and *Heterosigma akashiwo* fed very well on heterotrophic bacteria, *Synechococcus* sp. and beads ≤2 µm, but they did not feed on *Isochrysis galbana* (ca. 5 µm ESD) and an unidentified cryptophyte (5.6 µm), diverse mixotrophic dinoflagellates (≥6 µm), and beads sized 3 to 12 µm. Therefore, the upper prey size limit for both *C. ovata* and *H. akashiwo* seems to be ca. 2 µm. Based on the TEM serial sectioning, the size of the mucocyst openings of *C. ovata* is ca. 3 µm, while that of *H. akashiwo* is ca. 2 µm. Thus, the size of the mucocysts of these raphidophytes may be a critical factor affecting the upper size limit of edible prey. Meanwhile, many mixotrophic dinoflagellates have been reported to feed on *I. galbana*, cryptophytes, diverse mixotrophic dinoflagellates, and/or heterotrophic protists (Stoecker 1999, Jeong et al. 2005b, Burkholder et al. 2008). The mixotrophic dinoflagellates feed on prey cells by peduncles (Hansen & Calado 1999, Berge et al. 2008) or engulfment (Skovgaard 1996, Jeong et al. 2004). In engulfment feeding, they engulf a prey cell through the sulcus, apical horn, or body suture (Jeong et al. 2005b,c). These diverse feeding behaviors and larger feeding openings enable the mixotrophic dinoflagellates to feed on diverse prey, ranging from heterotrophic bacteria to much larger heterotrophic prey. Therefore, in terms of prey items and feeding behaviors, the raphidophytes may be less-flexible red-tide organisms compared to mixotrophic dinoflagellates.

Heterosigma akashiwo is known to be present in 3 major locations where nutrient concentrations are low (Taylor et al. 1994) or high (Han et al. 1989), and in an upwelling area (Tilstone et al. 1994). Taylor et al. (1994) reported that the abundance of *H. akashiwo* had negative correlations with the concentrations of nitrate and phosphate. Thus, in the waters where the nutrient concentrations are low, *H. akashiwo* may maintain its populations by taking up dissolved organic materials and/or feeding on heterotrophic bacteria and *Synechococcus* sp.
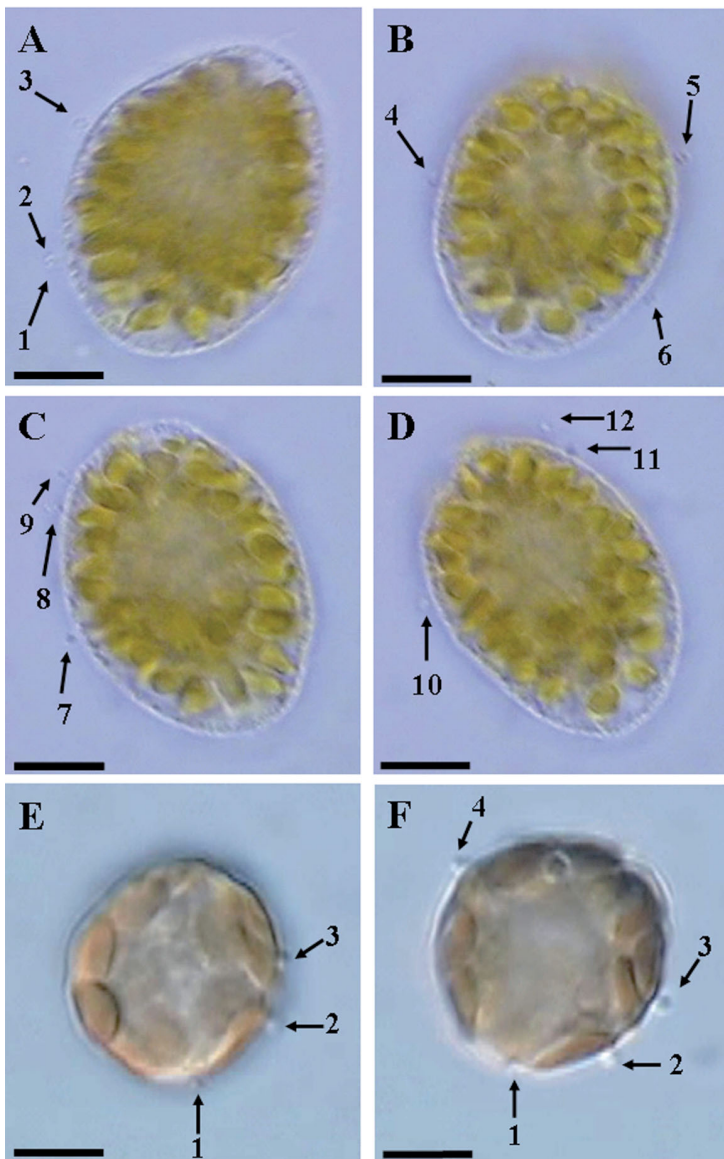
Fig. 5. (A–D) Twelve living *Synechococcus* sp. cells (arrows) captured by the mucus excreted from mucocysts along the cell body of a *Chattonella ovata* cell. As the *C. ovata* cell rotated, all *Synechococcus* sp. cells attached to the cell body of the predator cell were counted. All predator cells in (A–D) were the same cell. Scale bars = 10 µm. (E,F) Four living *Synechococcus* sp. cells (arrows) captured by the mucus excreted from mucocysts along the cell body of *Heterosigma akashiwo*. Both predator cells in (E,F) were the same cell. Scale bar = 5 µm. All images were observed with epifluorescence microscopy and recorded using high-resolution video microscopy

The feeding behavior of the raphidophytes, which engulfed *Synechococcus* sp. cells captured in mucus excreted by their mucocysts, is somewhat different from most heterotrophic nanoflagellates or the heterotrophic dinoflagellates *Oxyrrhis marina* and *Gyrodinium* spp.; these intercept and then ingest a single heterotrophic bacterial cell in feeding currents generally

generated by the flagella of the predators (Boenigk & Arndt 2000, Jeong et al. 2008). Cells of *Heterosigma akashiwo* and *Chattonella* spp. have been reported to be enveloped by glycocalyx (Edvardsen & Imai 2006). Before the present study, the function of the mucus cover had been unclear, but it was suggested to have an affinity to iron (Honjo 1993). The mucus was also suggested to cause fish death (Bourdelais et al. 2002). The results of the present study clearly show that one of the roles of the mucus is to capture small prey. In geological scales, fish appeared ca. 390 million years ago (Shubin et al. 2006). Cyanobacteria and heterotrophic bacteria appeared much earlier than fish. Thus, mucus may have originally developed to capture bacteria, and fish suffocation due to the mucus may be a side effect. Also, a function of the mucocysts of the raphidophytes had been thought to be just excreting mucus (Hallegraeff & Hara 1995). The present study reveals that the mucocysts are used as the location where the raphidophytes engulf prey cells.

Ingestion rates and grazing impact

The highest ingestion rate of *Chattonella ovata* or *C. subsalsa* on *Synechococcus* sp. under the conditions provided in the present study (ca. 18.6 to 20.5 cells grazer^{-1} h^{-1}) is comparable to that of the mixotrophic dinoflagellates *Gymnodinium impudicum* and *Gonyaulax spinifera* on the same prey (ca. 15 to 24 cells grazer^{-1} h^{-1}), while the highest ingestion rate of *Heterosigma akashiwo* on *Synechococcus* sp. (ca. 3.9 cells grazer^{-1} h^{-1}) is also similar to that of the mixotrophic dinoflagellates *Alexandrium minutum* and *Heterocapsa triquetra* (ca. 3 to 4 cells grazer^{-1} h^{-1}). Therefore, *C. ovata* and *C. subsalsa* may compete with *Gymnodinium impudicum* and *Gonyaulax spinifera* for *Synechococcus* sp. prey if they co-occur and the prey concentration limits growth of these predators, while *Heterosigma akashiwo* may do the same with *A. minutum* and *Heterocapsa triquetra*.

The highest ingestion rate of *Chattonella ovata* or *C. subsalsa* on *Synechococcus* sp. under the conditions provided in the present study was higher than the maximum ingestion rates of the small heterotrophic nanoflagellates *Picophagus flagellatus*, *Pseudobodo* sp., *Cafeteria roenbergensis*, and *Bodo saltans* on
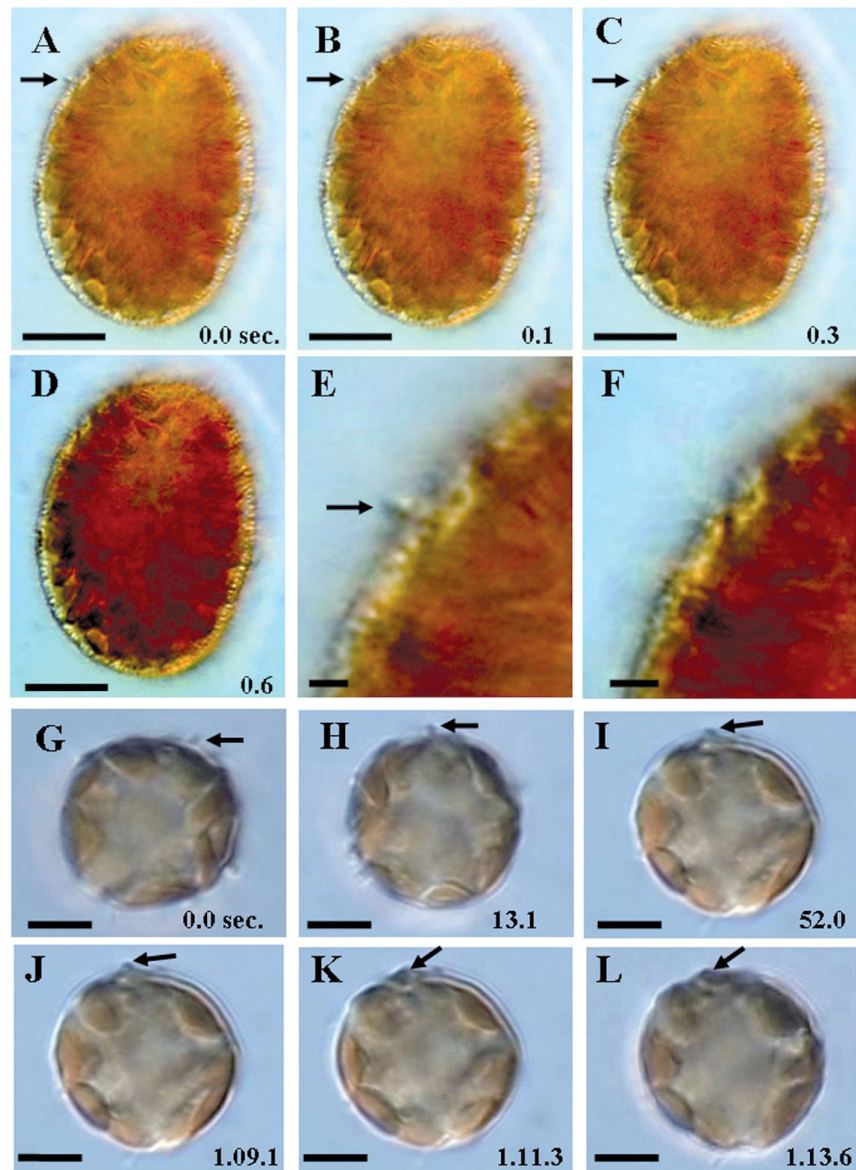
Fig. 6. Feeding processes of raphidophyte on a living *Synechococcus* sp. cell observed with epifluorescence microscopy, recorded using high-resolution video microscopy. Serial photos showed the ingestion of the single *Synechococcus* sp. cell (arrow) by the raphidophyte at the cell surface near a mucocyst. (A–F) A single *Synechococcus* sp. cell captured by a *Chattonella ovata* cell; a video is also available at www.int-res.com/articles/suppl/a058p181_app/. (B,C) Half or more of the *Synechococcus* sp. cell was engulfed by *C. ovata.* (D) *Synechococcus* sp. cell was completely engulfed by *C. ovata.* (E,F) Enlarged from (A) and (D), respectively. (G–L) Feeding processes of *Heterosigma akashiwo* on a living *Synechococcus* sp. cell. Serial photos showed the ingestion of the single *Synechococcus* sp. cell (arrow) by the raphidophyte at the cell surface near a mucocyst. All raphidophyte cells were the same cell. Scale bars = 10 µm for (A–D), 2 µm for (E,F), and 5 µm for (G–K)

Synechococcus sp. (Dolan & Simek 1998, Boenigk et al. 2001, Guillou et al. 2001, Christaki et al. 2002) and comparable to that of the ciliate *Uronema* sp. (Christaki et al. 1999), when corrected to 20°C using Q_{10} = 2.8 (Hansen et al. 1997). However, the highest ingestion rate of *Heterosigma akashiwo* on *Synechococcus* sp. under the conditions provided in the present study is between the maximum ingestion rates of *Pseudobodo* sp. and *Cafeteria roenbergensis*. Therefore, *Chattonella ovata*, *Chattonella subsalsa*, and *H. akashiwo* may sometimes compete with the heterotrophic nanoflagellates and ciliates for *Synechococcus* sp. as well if they co-occur.

The actual initial concentrations of heterotrophic bacteria in the *Chattonella ovata* and *Heterosigma akashiwo* feeding experiments were <13% and <18% of *Synechococcus* sp., respectively. The presence of these heterotrophic bacteria may have somewhat lowered the ingestion rates of *C. ovata* and *H. akashiwo* on *Synechococcus* sp. Also, different strains of these raphidophytes may have somewhat different rates and thus it would be worthwhile to measure ingestion rates of different strains of the raphidophytes.

Interestingly, *Chattonella ovata* and *Heterosigma akashiwo* had much lower maximum clearance rates than the heterotrophic nanoflagellates and ciliates. Capturing and engulfing prey cells using mucus excreted from the mucocysts (*C. ovata* and *H. akashiwo*) may be a less efficient feeding mechanism than intercepting prey cells in feeding currents or filter
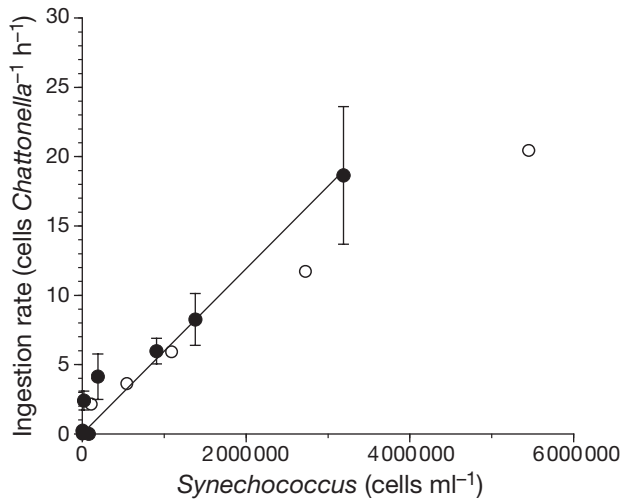
Fig. 7. Ingestion rate (*IR*) of *Chattonella ovata* (●) and *C. subsalsa* (○) on *Synechococcus* sp. as a function of initial prey concentration (*pc*). Data are mean ± SE for *C. ovata* (n = 3 for each prey concentration) and single treatments for *C. subsalsa* (n = 1 for each prey concentration). The curve was fitted by a linear equation using all treatments in Expt 3 (for *C. ovata*): $IR = 5.66 \times 10^{-6} \times pc$, $r^2 = 0.668$

Fig. 9. Calculated grazing coefficients (*g*) of *Heterosigma akashiwo* (n = 23) in relation to the concentration of co-occurring *Synechococcus* sp. (see Eqs. 4 & 5). Clearance rates, measured under the conditions provided in the present study, were corrected using $Q_{10} = 2.8$ (Hansen et al. 1997) because *in situ* water temperatures and the temperature used in the laboratory for this experiment (20°C) were sometimes different. The scales of the circles in the inset boxes are g (d^{-1})

feeding (the heterotrophic nanoflagellates and ciliates). As described in 'Feeding behaviour', *C. ovata* and *H. akashiwo* do not generate detectable feeding currents similar to those created by the heterotrophic nanoflagellates and ciliates (Fenchel 1987, Boenigk & Arndt 2000). The heterotrophic nanoflagellates and ciliates may draw feeding currents and then capture prey cells carried inside the feeding currents. Thus,
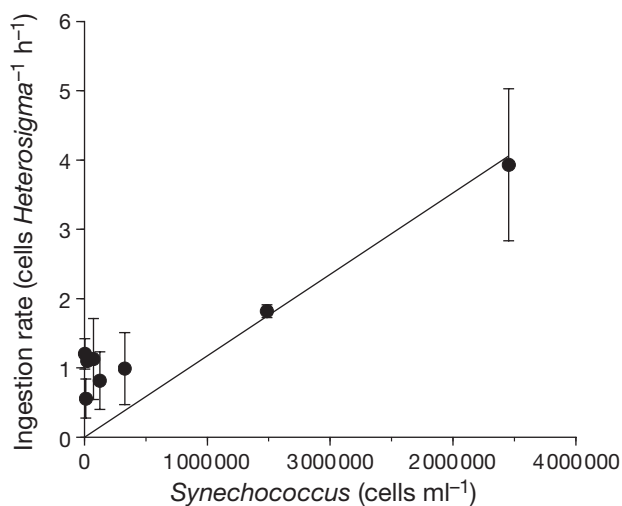
they have relatively high clearance rates. However, *C. ovata* and *H. akashiwo* may need to swim through *Synechococcus* sp. cells to capture prey cells without filtering, which may be responsible for their relatively lower maximum clearance rates.

The grazing coefficients attributable to *Heterosigma akashiwo* on co-occurring *Synechococcus* spp. in Masan Bay and Shiwha Bay were 0.001 to 1.238 d^{-1} (i.e. up to 71% of a *Synechococcus* spp. population was removed by a population of *H. akashiwo* in 1 d) when the abundances of *H. akashiwo* and *Synechococcus* sp. were 22 to 87 680 and 51 to 194 460 cells ml^{-1}, respectively. Therefore, *H. akashiwo* may sometimes have a considerable grazing impact on populations of co-occurring *Synechococcus* spp. in Masan and Shiwha Bays.

The results of the present study are ecologically important to planktonic communities for the following reasons: (1) in the marine planktonic food webs, some raphidophytes are able to feed on one of the most abundant photosynthetic microorganisms in the world's oceans (Ferris & Palenik 1998, Li 1998). Also, *Heterosigma akashiwo* may sometimes have a considerable grazing impact on populations of co-occurring *Synechococcus* sp.; thus, we should take raphidophytes into consideration as important predators on *Synechococcus* sp. (2) *Chattonella ovata*, *C. subsalsa*, and *H. akashiwo* feed well on *Synechococcus* sp., but *Fibrocapsa japonica* rarely does. Thus, the roles of *C. ovata*, *C. subsalsa*, and *H. akashiwo* may be different from *F. japonica* in planktonic food webs and bloom dynamics.

Fig. 8. Ingestion rate (*IR*) of *Heterosigma akashiwo* on *Synechococcus* sp. as a function of initial prey concentration (*pc*). Data are mean ± SE. The curve was fitted by a linear equation using all treatments in the experiment: $IR = 1.18 \times 10^{-6} \times pc$, $r^2 = 0.508$
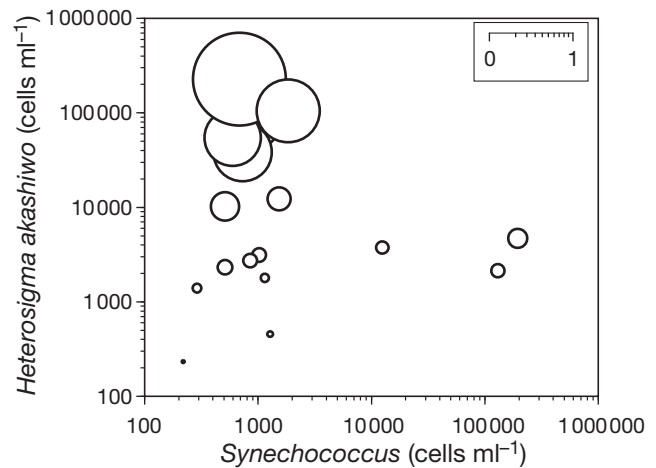
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