

Assessment of Microzooplankton Grazing on *Heterosigma akashiwo* Using a Species-Specific Approach Combining Quantitative Real-Time PCR (QPCR) and Dilution Methods

Elif Demir · Kathryn J. Coyne · Martina A. Doblin · Sara M. Handy · David A. Hutchins

Received: 23 June 2006 / Accepted: 17 April 2007 / Published online: 4 July 2007
© Springer Science + Business Media, LLC 2007

Abstract Delaware's Inland Bays (DIB) are subject to numerous mixed blooms of harmful raphidophytes each year, and *Heterosigma akashiwo* is one of the consistently occurring species. Often, *Chattonella subsalsa*, *C. cf. verruculosa*, and *Fibrocapsa japonica* co-occur with *H. akashiwo*, indicating a dynamic consortium of raphidophyte species. In this study, microzooplankton grazing pressure was assessed as a top-down control mechanism on *H. akashiwo* populations in mixed communities. Quantitative real-time polymerase chain reaction (QPCR) with species-specific primers and probes were used in conjunction with the dilution method to assess grazing pressure on *H. akashiwo* and other raphidophytes. As a comparison, we measured changes in chlorophyll *a* (chl *a*) to determine whole community growth and mortality caused by grazing. We detected grazing on *H. akashiwo* using QPCR in samples where chl *a* analyses indicated little or no grazing on the total phytoplankton community. Overall, specific microzooplankton grazing pressure on *H. akashiwo* ranged from 0.88 to 1.88 day⁻¹ at various sites. Experiments conducted on larger sympatric raphidophytes (*C. subsalsa*, *C. cf.*

verruculosa and *F. japonica*) demonstrated no significant microzooplankton grazing on these species. Grazing pressure on *H. akashiwo* may provide a competitive advantage to other raphidophytes such as *Chattonella* spp. that are too large to be consumed at high rates by microzooplankton and help to shape the dynamics of this harmful algal bloom consortium. Our results show that QPCR can be used in conjunction with the dilution method for evaluation of microzooplankton grazing pressure on specific phytoplankton species within a mixed community.

Introduction

Microzooplankton grazing accounts for a significant loss factor of phytoplankton and bacteria in marine systems [45]. Selective grazing by zooplankton that are <200 μm shapes phytoplankton community structure [70] and assists nutrient recycling and regeneration [26], thus establishing a link between lower and higher tropic levels [59]. Microzooplankton grazing can remove enough cells to prevent or diminish blooms [60], creating a top-down control (removal of organisms by consumers) on community dynamics that may determine the fate of harmful algal blooms. Several studies have demonstrated the direct negative effects of grazing on potentially harmful algae such as *Chattonella antiqua* [1, 69], *Micromonas* spp. [16], and *Alexandrium minutum* [7, 19]. Heterotrophic dinoflagellates have also been found to graze on laboratory cultures of the raphidophytes *Fibrocapsa japonica* [66] and *Heterosigma akashiwo* [9]. Conversely, grazing pressure on a mixed community may also indirectly favor the growth of harmful algal species, as is the case with *Aureococcus anophagefferens* [20].

Blooms of a mixed raphidophyte consortium composed of *C. subsalsa*, *Heterosigma akashiwo*, *C. cf. verruculosa*

E. Demir · K. J. Coyne · S. M. Handy · D. A. Hutchins (✉)
College of Marine and Earth Studies, University of Delaware,
Lewes, DE 19958, USA
e-mail: dahutch@usc.edu

M. A. Doblin
Institute of Water and Environmental Resource Management/
Department of Environmental Science,
University of Technology, Sydney,
Westbourne St, Gore Hill,
Sydney, NSW 2065, Australia

D. A. Hutchins
Department of Biological Sciences,
University of Southern California,
Los Angeles, CA 90089, USA

(this species is currently undergoing reclassification [6]), and *F. japonica* are common in Delaware Inland Bays (DIB) [24]. In 2000, *C. cf. verruculosa* reached a maximum density of 1×10^7 cells L^{-1} in the DIB and was linked to a major fish kill [3]. Since then, mixed blooms of raphidophytes have occurred each year from early May to late October [73]. Although no toxic events have been reported since 2000, understanding all the factors involved in bloom formation is important in assessing their potential to become harmful and their future management.

Interspecific competition may play an important role in determining the dynamics between raphidophyte species in mixed blooms. A series of laboratory experiments on local isolates of *C. subsalsa* and *H. akashiwo* indicated that *H. akashiwo* has a higher maximum growth rate (μ_{max}) and lower half saturation constant (K_s) for phosphate, nitrate, and ammonium and can grow on urea, whereas *C. subsalsa* cannot. In fact, *H. akashiwo* appears to be a better competitor for nutrients under almost all conditions and can tolerate a wider salinity, light, and temperature range than *C. subsalsa* [74]. However, in natural bloom samples, *C. subsalsa* is often more abundant than *H. akashiwo* [24], suggesting that top-down control mechanisms like grazing could offset any competitive advantage that this latter species gains from bottom-up (growth limitation of organisms as a result of environmental parameters such as nutrients) control factors.

Landry and Hassett [40] introduced the dilution method to measure microzooplankton grazing rates in natural communities, and it has been used to measure grazing in a variety of aquatic systems, from estuaries ([48] and references therein) to the open ocean [41, 63]. The design is based on a series of dilutions, with each dilution decreasing the potential of grazer/prey encounter, so that the probability of prey consumption is proportional to the dilution factor [40, 41]. As is the case with all direct environmental grazing methods, the dilution method has limitations, and there is controversy over the assumption that it is applicable to all types of marine systems. Some evidence has been presented indicating adverse effects for coastal ciliate communities, which can starve in highly diluted experimental conditions (i.e., 10% of whole seawater dilutions) and potentially lead to overestimation of grazing pressure [14]. However, this limitation has not been fully tested, and in various regions, reasonable data have been collected using the dilution method [39]. The advantage of using this method is that it requires minimal handling and few manipulations of the natural phytoplankton and microzooplankton communities. The dilution method has recently been applied in harmful algae research (e.g., [7, 8, 19, 20]) and may be the most applicable method for evaluating grazing on fragile species such as raphidophytes that require minimum disturbance.

One of the difficulties associated with the dilution method is identification and quantitative enumeration of the taxon of interest. Several methods have been developed to investigate species-specific effects of microzooplankton grazing such as use of labeled prey for establishing a qualitative relationship between specific species of predators and prey [9]. Typically, species-specific grazing experiments are performed using cultures grown in the lab. Although these are valid in determining the potential for specific microzooplankton to consume specific algae, they cannot demonstrate selective grazing (or lack thereof) in natural communities. In environmental studies, taxon-specific pigment markers have been used to differentiate selective grazing pressure on general phytoplankton groups [18, 63, 71]. The pigment analysis method requires in-depth knowledge of the entire phytoplankton community and their pigment compositions, and the sample analyses can be time consuming. Although pigment analysis is a valuable method to evaluate grazing pressure on broad taxonomic groups of phytoplankton, it is seldom able to differentiate grazing at the species level because of the lack of unique pigment signatures. In general, it is more suited for open ocean phytoplankton communities where total community structure can be better assessed using pigments compared to estuarine communities.

Another species-specific method to determine grazing pressure is by microscopic cell counts. This approach requires a high level of expertise for experiments dealing with mixed communities. Microscope cell counts are also challenging for species such as raphidophytes, which cannot be identified after preservation longer than a few days [28]. Enumeration of fixed natural samples via microscopic cell counts is challenging because raphidophytes have no cell wall and often burst after fixation [28]. Quantitative real-time polymerase chain reaction (QPCR) is a practical and efficient alternative to microscope cell counts to accurately determine changes in cell abundances in mixed natural communities [4, 5, 10, 11, 47, 52]. For QPCR, DNA is extracted from the total community, and a target gene (such as 18S rDNA) is amplified using species-specific primers and a fluorescent probe. Accumulation of the PCR product is measured as a change in fluorescence and is directly proportional to the amount of starting template [27] or number of cells of the target species (e. g., [10, 24, 52]). The product accumulation is normalized to a reference standard to correct for differences in extraction and amplification efficiencies [10]. QPCR has recently been applied to investigate vertical migration of raphidophyte species (*H. akashiwo* and *C. subsalsa*) in mesocosm experiments containing natural phytoplankton communities [24] and germination of cysts produced by these algae in DIB [53].

In this study, we used the dilution method to examine grazing pressure on *H. akashiwo* in natural blooms occur-

ring from June to October in 2004 and 2005. The grazing rates on *H. akashiwo* obtained with QPCR were compared to grazing rates for the whole community derived from chlorophyll *a* (chl *a*). We also performed a limited number of grazing experiments on natural populations of *C. subsalsa*, *C. cf. verruculosa*, and *F. japonica*. We found that *H. akashiwo*, which ranges in size from 12 to 20 μm , was subject to microzooplankton grazing pressure at various intensities, whereas the other larger raphidophyte species were not grazed. This could contribute to the dynamics of the consortium by giving other co-occurring raphidophytes, which may be too large (20–60 μm) to be consumed by microzooplankton, an advantage over *H. akashiwo*. In addition, our results demonstrate that the QPCR method can be integrated with the dilution method to evaluate microzooplankton grazing pressure on specific phytoplankton species in natural populations.

Materials and Methods

Dilution Experiments

We sampled blooms of *H. akashiwo*, other raphidophytes, and non-harmful algae in the DIB from June to November of 2004 and 2005. Water samples were collected from five different locations on 13 different dates at varying salinities (Table 1). Each location name identifies a different canal system linked to DIB. Sampling dates were within the period when *H. akashiwo* and other raphidophyte blooms are common in this area.

Samples were collected in 20-L carboys using a bilge pump and were gently gravity filtered through a 202- μm size mesh to exclude meso- (zooplankton that are 0.2 to 20 mm in size) and macrograzers (zooplankton that are 2 to 20 cm in size). After collection and pre-filtration, the communities were evaluated via microscopy to ensure that the phytoplankton and microzooplankton communities were not harmed. Part of the 202- μm pre-filtered water was filtered through 0.2- μm filter cartridges using air pressure. This filtrate was then used as a diluent for the whole water. Dilution treatments were 25, 50, 75, and 100% of whole water, in triplicates of 1-L sterilized bottles. Each bottle was enriched with NO_3^- , PO_4^{3-} , trace metals, vitamins, and FeEDTA at *f*/2 phytoplankton medium concentrations [21]. No nutrient addition treatments used in open ocean dilution experiments [41] were not applied in this study because the study sites within DIB are highly eutrophic [54, 56]. Therefore, it was assumed that phytoplankton growth was not limited by nutrients in our experiments. In the absence of this treatment, values reported in this article may be considered upper limit estimates of actual microzooplank-

ton grazing rates. Bottles were incubated at the ambient temperature of the collection site under a 12:12 light/dark cycle. Samples for extracted chl *a* and molecular analyses were collected before and after the 24-h incubation for each experiment.

Chlorophyll *a* was extracted in 90% acetone and measured fluorometrically [72]. Cell counts for raphidophytes and total community composition and density were performed on live samples using the droplet estimation method (45 μl) under light microscopy, which has a detection limit of 50,000–100,000 cells L^{-1} [2]. This method is valuable for counting total phytoplankton communities because the cells are still alive and swimming patterns can be observed, which may be essential in differentiating similarly structured algae. Microzooplankton species were identified using light microscopy from samples fixed in Lugol's solution. Mortality because of microzooplankton grazing (*g*) and apparent growth rates (*k*) of phytoplankton were obtained by plotting apparent growth rates vs dilution factor, and this rate is reported as per day (day^{-1}), according to Landry and Hassett [40]. GraphPad Prism (GraphPad Software, San Diego, CA, USA) was used for statistical comparisons of the slopes of regression analysis [22].

Molecular Methods

DNA from initial (T_0) and final (T_{24}) samples were collected by gently filtering (~ 85 kPa) the water samples onto 3- or 5- μm polycarbonate filters. The filters were submerged in cetyltrimethylammonium bromide (CTAB) buffer (100 mM Tris-HCl [pH 8], 1.4 M NaCl, 2% [w/v] CTAB, 0.4% [v/v] β -mercaptoethanol, 1% [w/v] polyvinylpyrrolidone, 20 mM EDTA [13]). The plasmid pGEM was included in the buffer at a concentration of 20 ng mL^{-1} as an internal standard to correct for differences between samples based on DNA extraction and amplification efficiencies [10]. Samples were then stored at -80°C , and DNA was extracted as described in Coyne *et al.* [11]. After spectrophotometric quantification, each DNA sample was diluted 1:200 with LoTE (3 mM Tris-HCl [pH 7.5], 0.2 mM EDTA) to obtain DNA concentrations of 1.84–62.12 $\text{ng } \mu\text{L}^{-1}$ for QPCR.

We used QPCR to quantify raphidophyte species' cell densities from initial (T_0) and final (T_{24}) samples for each experiment using an ABI Prism 7500 Real Time PCR detection system (Applied Biosystems). Microscope cell counts of raphidophytes in undiluted whole water samples were performed as described in Coyne *et al.* [10] and used as density calibrators for calculating cell densities from QPCR results [10]. *Heterosigma akashiwo* calibrators were prepared from samples that were confirmed (by PCR) to be free of a co-occurring newly described raphidophyte species [12] that is morphologically indistinguishable from

Table 1 Microzooplankton grazing results for the bloom seasons in 2004 and 2005 (numbered 1 through 13)

Expt	Date	Location	Salinity (psu)	Total number of species	Target prey species	Initial cell density (L^{-1})	Total chl a		QPCR					
							r^2	k (day^{-1}) 95% conf. int.	r^2	k (day^{-1}) 95% conf. int.	g (day^{-1}) 95% conf. int.	g (day^{-1}) 95% conf. int.		
2004														
1	June 29	Williamson Creek	12	16	<i>H. akashiwo</i>	3.08×10^7	NS	–	–	–	–	–	–	–
2	July 22	Love Creek	15	NA	<i>H. akashiwo</i>	1.20×10^7	0.78	0.85 (0.64 to 1.06)	0.82 (1.12 to -0.5)	–	–	–	–	–
3	July 31	Torquay Canal	24	10	<i>H. akashiwo</i>	1.00×10^4	0.42	1.54 (1.09 to 1.99)	0.80 (1.46 to 0.14)	–	–	–	–	–
4	Sept 29	Russell's Canal	21	8	<i>H. akashiwo</i>	3.30×10^6	0.51	0.39 (0.26 to 0.52)	0.28 (0.46 to 0.09)	0.47	1.61 (0.65 to 2.57)	1.88 (3.38 to 0.48)	–	–
5	Sept 30	Russell's Canal	21	8	<i>H. akashiwo</i>	4.09×10^7	0.35	0.21 (0.09 to 0.33)	0.19 (0.36 to 0.01)	0.65	0.18 (-0.51 to 0.88)	1.61 (2.90 to 0.92)	–	–
6	Oct 5 ^a	Derrickson Creek	13	NA	<i>Pseudopedinella</i> sp.	9.00×10^6	0.97	1.11 (1.04 to 1.18)	0.77 (0.87 to 0.66)	–	–	–	–	–
7	Oct 8	Derrickson Creek	6	10	<i>H. akashiwo</i>	8.80×10^6	NS	–	ND	–	–	–	–	–
8	Oct 11 ^a	Russell's Canal	22	7	<i>H. akashiwo</i>	1.14×10^7	NS	0.46 (0.32 to 0.60)	0.11 (0.31 to 0.08)	0.84	0.87 (0.63 to 1.10)	1.12 (1.48 to 0.75)	–	–
9	Nov 17 ^a	Indian River Bay Canal	20	NA	<i>Euglena</i> sp.	6.00×10^7	0.65	0.42 (0.22 to 0.63)	0.56 (0.85 to 0.27)	–	–	–	–	–
2005														
10	June 30	Indian River Bay Canal	22	8	<i>C. subsalsa</i>	8.78×10^5	NS	–	ND	NS	–	–	–	–
					<i>C. cf. verruculosa</i>	1.15×10^7	NS	–	–	NS	–	–	–	–
11	July 14	Holt's Landing	20	5	<i>H. akashiwo</i>	1.72×10^6	NS	–	ND	0.55	0.85 (0.47 to 1.24)	0.88 (1.44 to 0.32)	–	–
12	July 26	Russell's Canal	19	5	<i>C. cf. verruculosa</i>	4.00×10^5	0.38	0.34 (0.20 to 0.49)	0.24 (0.45 to 0.02)	NS	–	–	–	–
					<i>C. subsalsa</i>	5.00×10^4	NS	–	–	NS	–	–	–	–
13	Sept 2	Russell's Canal	24	NA	<i>C. cf. verruculosa</i>	8.50×10^5	0.73	0.33 (0.21 to 0.44)	0.39 (0.55 to 0.22)	NS	–	–	–	–
					<i>F. japonica</i>	1.38×10^5	NS	–	–	NS	–	–	–	–

Coefficients of grazing (g) and apparent growth rate (k), ($n=3$), are given using chlorophyll a and QPCR results with upper and lower 95% confidence intervals.

NA Not available, NS insignificant r^2 (used when no grazing was detected, included for comparison reasons), ND grazing not detected

^aRaphidophyte preblooms/blooms were used for all experiments except *Pseudopedinella* sp. and *Euglena* sp.

Table 2 List of primer and probe sequences used for QPCR analysis of the raphidophytes *Chattonella subsalsa*, *C. cf. verruculosa*, *Fibrocapsa japonica*, and *Heterosigma akashiwo*, and for amplification of the pGEM plasmid

DNA target	Primer/Probe	Sequence (5'–3')	References
<i>Chattonella subsalsa</i> 18S rDNA	Cs 1350F	CTAAATAGTGTGGGTAATGCTTAC	Coyne <i>et al.</i> [10]
	Cs 1705R	GGCAAGTCACAATAAAGTTCCAA	
	Raph Probe	CAACGAGTACTTTCCTTGCCGGAA	
<i>Chattonella cf. verruculosa</i> 18S rDNA	Cv 1561F	ATGCATACAGCGAGTCTAGA	Handy <i>et al.</i> [23]
	Cv 1780R	TCACTCCGAAAAGTGCAAC	
	Cv Probe	CAAGAGTACCCAGGCCTCTCGACC	
<i>Fibrocapsa japonica</i> 18S rDNA	Fc 1350F	TGCTTTAGTCATTGTGTGCAG	This study
	Fc 1705R	ACCACAACTAATGAGGAGGC	
	Fc Probe	CCCAGGCCTACCGCCAAGTTGTA	
<i>Heterosigma akashiwo</i> 18S rDNA	Hs 1350F	CTAAATAGTGTGCGTAATGCTTCT	Coyne <i>et al.</i> [10]
	Hs 1705R	GGCAAGTCACAATAAAGTTCCAT	
	Hs Probe	CAAGGAGTAACGACCTTTTGCCGGAA	
<i>Chattonella cf. verruculosa</i> 18S rDNA	M13F	CCCAGTCACGACGTTGTAAAACG	Coyne <i>et al.</i> [10]
	pGEM R	TGTGTGGAATTGTGAGCGGA	
	pGEM Probe	CACATAGAATACTCAAGCTTGCATGCCTGCA	

F Forward primer, R reverse primer

Heterosigma by light microscopy [10, 12]. Thermal cycling parameters for the QPCR reactions are described in Coyne *et al.* [10]. Briefly, we amplified DNA in triplicate 25- μ L reactions using 2.5 μ L of 1:200 diluted template, 12.5 μ L of Taqman Universal Master Mix (Applied Biosystems), 0.9 μ M of a species-specific forward primer, 0.9 μ M of a species-specific reverse primer, and 0.2 μ M of a species-specific probe. The species-specific primers and probes (listed in Table 2) were designed to amplify a unique fragment of the 18S rRNA gene. All these primer/probe sets were previously reported in other studies [10, 23], with the exception of the *F. japonica* set. The specificity of the primers and probe were evaluated as described in Coyne *et al.* [10]. Primer/probe concentrations were optimized as described in Coyne *et al.* [10] and for all species were 0.9 μ M and 0.2 μ M, respectively, except for *C. cf. verruculosa* for which concentrations of 0.3 μ M for primers and 0.25 μ M for probe were used. The threshold cycle number (Ct) was determined for each sample and calibrator. Co-extracted pGEM plasmid DNA was also amplified for the diluted DNA samples in separate reactions using pGEM-specific primers and probe. The concentrations of raphidophytes were calculated using the comparative ($\Delta\Delta$ Ct) method [10, 44] in which amplification of the target species in each sample is first normalized to the internal standard, pGEM, and then compared to the abundance of the target species in the calibrator samples. These concentrations were then used to calculate apparent phytoplankton growth and grazing mortality as described previously. Reaction efficiencies for the pGEM and *H. akashiwo* assays were 91 and 90%, respectively.

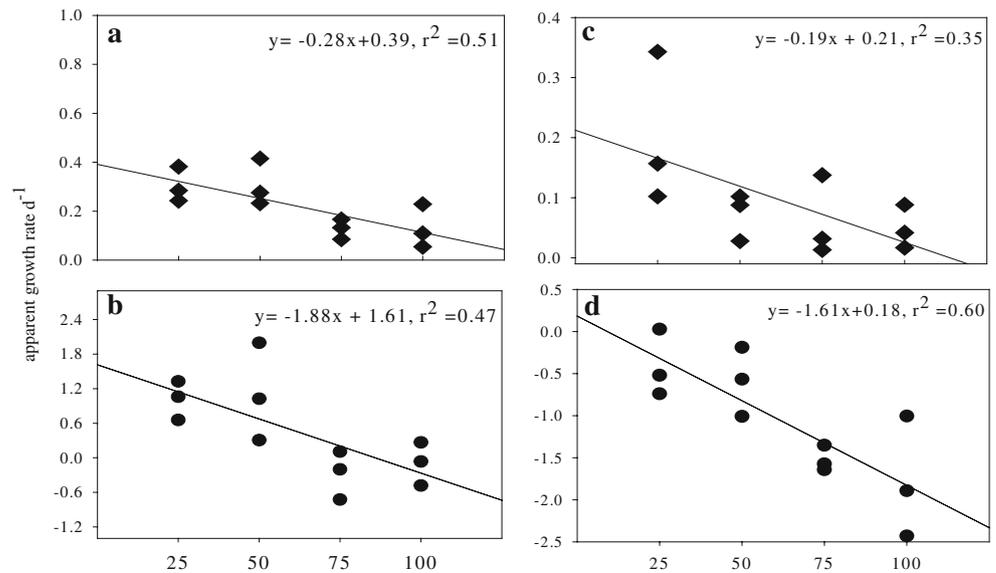
Results

Microzooplankton Grazing Pressure on *Heterosigma akashiwo*

Temperatures of the water collected ranged from 19–30°C, except for Expt 9 where the water was 6°C (Table 1). There was considerable variability in salinity (6–24 psu); however, neither of these parameters appeared to affect the grazing rates directly. *Heterosigma akashiwo* abundances were greater than 10⁶ cells L⁻¹ in all of these experiments except for Expt 3 where the density was 1 \times 10⁴ cells L⁻¹ (Table 1). The highest abundance was observed in Expt 5 (40 \times 10⁶ cells L⁻¹) followed by Expt 1 (30 \times 10⁶ cells L⁻¹). In Expt 1, 4, 7, and 8, the phytoplankton community composition was evaluated by the droplet estimation method [2]. However, these estimates included only those species that could be identified by light microscopy and likely do not include very small flagellates that are below the level of detection. For these experiments, *H. akashiwo* abundances comprised 40, 99, 92, and 96%, respectively, of the phytoplankton community [73]. In Expt 1, where microzooplankton grazing was not detected, the phytoplankton community was highly diverse with 16 species compared to an average of 8 in our other experiments. Our results indicate that total chl *a*-derived microzooplankton-grazing rates are not directly related to the chl *a* levels in the environment or to *H. akashiwo* densities (Fig. 1).

Grazing rates on *H. akashiwo* and the total community for Expt 4, 5, 8, and 11 were determined by QPCR and chl *a* analyses, respectively. Expt 4 and 5 were set up within 24 h of each other, both with water collected during a bloom

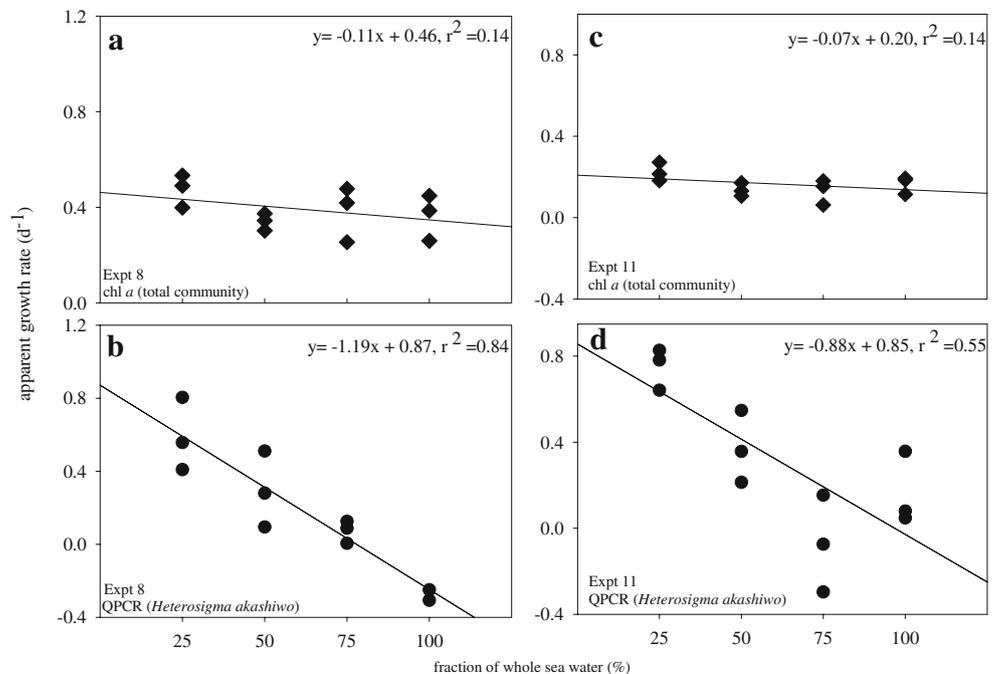
Figure 1 Apparent growth rate versus fraction of whole water in Expt 4 (*H. akashiwo*) calculated by total community chl *a* (a) and QPCR (b) and, in Expt 5, calculated by total community chl *a* (c) and QPCR (d). Lines are fit by least squares linear regression.



of *H. akashiwo* from Russell Canal, Little Assawoman Bay, DE. *Heterosigma akashiwo* density increased from 3×10^6 cells L^{-1} in Expt 4 to 40×10^6 cells L^{-1} in Expt 5 (Table 1). In Expt 4, grazing and growth of the total community (by chl *a*) were 0.28 and 0.39 day^{-1} , respectively (Fig. 1a). Grazing rates on *H. akashiwo* obtained from QPCR data (Fig. 2b) were significantly higher (1.88 day^{-1} , $p=0.019$) than grazing rates on the total community (0.28 day^{-1} , Fig. 1a). In Expt 5,

as well, the grazing and growth rates for the whole community were significantly different ($p=0.001$) than for *H. akashiwo* (Fig. 1c and d). Within the course of the consecutive experiments (Expts 4 and 5), grazing on the total community obtained by chl *a* values (Fig. 1a and c) did not change significantly and neither did species-specific grazing values on *H. akashiwo* obtained through QPCR (Fig. 1b and d). However, growth of the total community

Figure 2 Apparent growth rate versus fraction of whole water in dilution Expt 8 (*H. akashiwo*) calculated by total community chl *a* (a) and QPCR (b) and, in Expt 11, calculated by total community chl *a* (c) and QPCR (d). Lines are fit by least squares linear regression.



significantly decreased ($p=0.0011$), and a greater decrease was observed for *H. akashiwo* growth rates based on QPCR results ($p<0.0001$).

In Expt 8, growth rates for the total community versus *H. akashiwo* were 0.46 and 0.87 day⁻¹, respectively (Fig. 2a,b). Grazing rates using chl *a* concentrations (0.11 day⁻¹, Fig. 2a) suggest that grazing on the total community was minimal, whereas species-specific results obtained by QPCR analysis of *H. akashiwo* indicate significantly higher grazing pressure on *H. akashiwo* (1.19 day⁻¹, Fig. 2b) compared to the total community ($p<0.0001$). Similar results were obtained in Expt 11, where microzooplankton grazing on *H. akashiwo* yielded a significant slope (0.88 day⁻¹, Fig. 2d), but was not detected on the total community (Fig. 2c).

Microzooplankton Grazing Pressure on Co-existing Raphidophytes

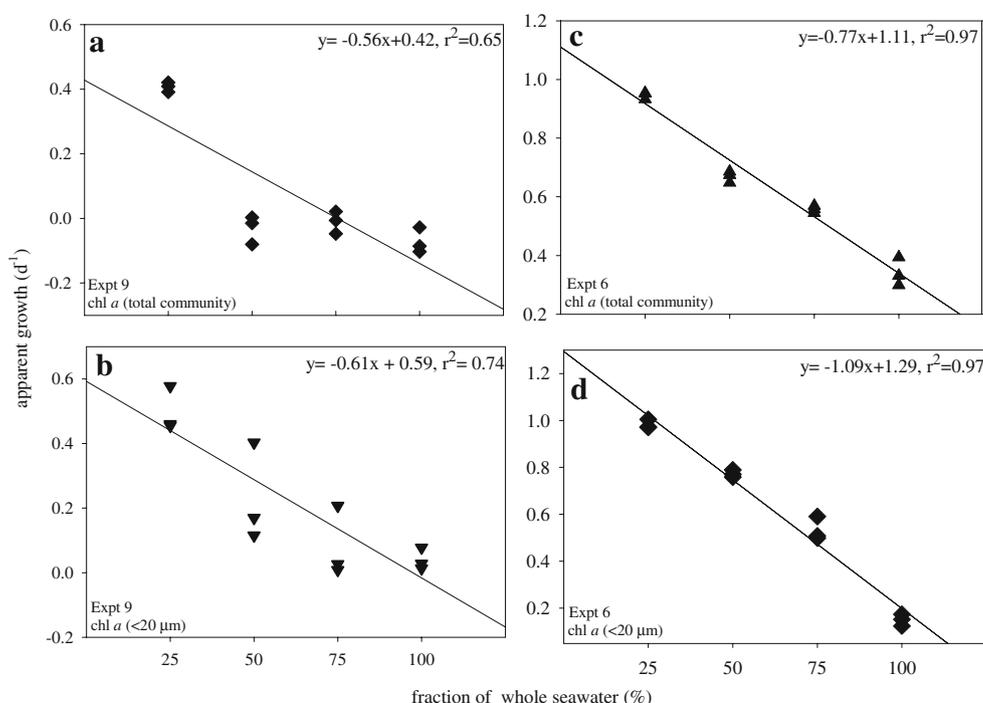
In Expt 10, 12, and 13, QPCR methods were used to investigate the microzooplankton grazing rates on other DIB raphidophytes (Table 1), including *C. subsalsa* (Expts 10 and 12), *C. cf. verruculosa* (Expts 10, 12, and 13), and *F. japonica* (Expt 13). The specificity of primers for *F. japonica* (reported in this study for the first time) in field samples from the DIB was confirmed by sequence analysis of positive PCR reactions. Application of the comparative Ct method was validated for this species with the pGEM internal standard as described in Coyne *et al.* [10]. In Expt 10, both *Chattonella* species dominated the community

with a total of 1.24×10^7 cells L⁻¹, whereas in Expts 12 and 13, total raphidophyte cell densities were ~27 and 12 times lower, respectively, than Expt 10 (Table 1). Grazing on the total community, determined by chl *a*, was either minimal (Expts 12 and 13) or not detectable (Expt 10), whereas species-specific grazing rates calculated using QPCR for *C. subsalsa*, *C. cf. verruculosa*, and *F. japonica* were consistently undetectable.

Microzooplankton Grazing Pressure on Non-harmful Algae

We also measured microzooplankton grazing during two blooms of small (<20 μm) non-harmful algae (*Pseudopedinella* sp. and *Euglena* sp.) that occurred in Dirickson Creek (Expt 6) and Indian River Bay Canal (Expt 9; tributaries of the DIB). Grazing pressure on these species was assessed for comparative purposes because both are closer in size to *H. akashiwo* than other species included in this study. In Expt 9, grazing pressure on a small (~10 μm) euglenoid species (Euglenophyceae, [65]) at a density of 60×10^6 L⁻¹ was determined by dilution experiments (Fig. 3a and 3b). In this sample, we identified two large mixotrophic dinoflagellates, *Oxyrrhis marina* and *Prorocentrum* sp. with growth rates (0.8–1.4 day⁻¹ [32, 51] and 0.21–0.33 day⁻¹ [46]) that are within the range of growth rates (0.21–1.11 day⁻¹) observed for total communities throughout this study. We evaluated microzooplankton grazing pressure by total chl *a* on unfractionated water (Fig. 3a) and for the <20-μm fraction (Fig. 3b) with no significant differences. Growth rates, however, were signif-

Figure 3 Apparent growth rate versus fraction of whole water in dilution Expt 9 (*Euglena* sp.) calculated by total community chl *a* (a) and chl *a* for the <20-μm size fraction (b) and, in Expt 6 (*Pseudopedinella* sp.), calculated by total community chl *a* (c) and chl *a* for the <20-μm size fraction (d). Lines are fit by least squares regression to the points.



icantly greater for the <20- μm size fraction compared to whole community rates ($p=0.008$).

In Expt 6, we evaluated microzooplankton grazing on the dictyophyte *Pseudopedinella* sp. (<10 μm) that occurred at a density of 9×10^6 cells L^{-1} in Dirickson Creek. Grazing pressure measured by total chl *a* (0.77 day^{-1} , Fig. 3c) was comparable to grazing on the total community (as determined by chl *a*) in Expts 2 and 3 ($0.82, 0.80 \text{ day}^{-1}$) when *H. akashiwo* was present. However, growth rates were higher for the phytoplankton community in Expt 6 (1.11 day^{-1}) compared to growth rates of the total communities that included *H. akashiwo* in Expts 2, 4, 5, and 8 (Table 1). As expected, grazing rates were significantly higher for the <20- μm size fraction (1.09 day^{-1}) of Expt 6 compared to grazing pressure on the unfractionated community (0.77 day^{-1} , $p=0.0002$, Fig. 3d), a result of the higher density of smaller phytoplankton in that particular location. Grazing pressures on the total phytoplankton communities in Expt 6 and Expt 9 were not significantly different; however, growth rates for Expt 6 were significantly higher than Expt 9 ($p<0.0001$). When comparing the <20- μm size fraction, grazing rates were also significantly higher in Expt 6 than Expt 9 ($p=0.001$).

Discussion

A consortium of at least four harmful raphidophyte species blooms annually in the DIB from mid May to the end of October. Our previous field and experimental data [74] suggested that top-down control mechanisms contribute to the dynamics of this group of algae, and we hypothesized that *H. akashiwo* (12–20 μm) can be removed by microzooplankton grazing, whereas larger raphidophyte species (*C. subsalsa*, *C. cf. verruculosa*, and *Fibrocapsa japonica*), are too large (20–50 μm) to be consumed by microzooplankton.

Although studies on laboratory cultures reveal important information on physiological characteristics and grazing rates of harmful algae [9, 55, 58, 62], they may have limited value in investigating predator/prey interactions within the natural environment. Grazing rates in natural estuarine samples, however, are much more difficult to interpret compared to laboratory cultures or offshore sites because of the complexity of environmental factors and phytoplankton communities involved [48]. For this reason, grazing pressure on natural populations of *H. akashiwo* has not been evaluated under natural environmental conditions before this study. In this article, we were able to measure variable rates of microzooplankton grazing on *H. akashiwo* in the DIB system using the dilution method in combination with species-specific molecular assays.

Grazing was measured on the total phytoplankton community by traditional chl *a* measurements. Although

chl *a* is an efficient way of evaluating total community biomass changes over time, in estuarine settings such as the DIB, the complexity of the phytoplankton community structure makes it nearly impossible to detect grazing on a specific species. Even when the species of interest is numerically dominant, total community chl *a* can often be skewed toward smaller phytoplankton or can be disproportionately contributed by a few cells of much larger but rarer species.

Quantification of phytoplankton density using microscopic cell counts is another commonly used method that provides specific information. However, some of the problems associated are: raphidophytes, as a group, cannot be reliably quantified once fixed because of their fragile nature [10, 28] and *H. akashiwo* is a relatively small alga, which is difficult to distinguish from other species, particularly, when a phylogenetically distinct species that closely resembles *H. akashiwo* in morphology is present in collected samples [8, 12]. Finally, the error in cell counts is progressively greater, as the water is diluted to measure microzooplankton grazing. Because the grazing mortality and growth rates are calculated using linear regression analysis of the dilution treatments, the propagated error in cell counts could potentially distort the results.

QPCR has been established as a sensitive and accurate method for estimating changes in cell densities of harmful algal species in environmental samples [4, 10, 17, 24, 52]. In a recent study, the application of QPCR to the analysis of *H. akashiwo* demonstrated a linear response over eight orders of magnitude with a sensitivity of four copies of the target gene [8]. In consideration of the problems associated with microscopic enumeration for this species, linear regression analysis using QPCR data to evaluate grazing pressure on *H. akashiwo* may be less subject to various errors and more accurate than cell counts. In this article, we investigated the use of QPCR in conjunction with the dilution method to differentiate between microzooplankton grazing on *H. akashiwo* and grazing on the whole community (evaluated by chl *a*) in mixed natural estuarine communities.

Potential errors and limitations of QPCR are described in [10] and include inefficiencies in filtration or errors in sample volume measurement, inaccurate pipetting of the lysis buffer, and differences in lysis efficiencies. However, these problems can be avoided by consistent treatment of all samples and the use of an internal standard (pGEM) that reduces downstream errors. Cell counts for calibrator samples need to be performed carefully. Using ambient samples as calibrators may introduce some error because target cells in mixed communities may not be as easy to count as samples from unialgal cultures. Nonetheless, the advantages of this approach outweigh the potential limitations because calibrator samples collected from the same environment contain a similar complexity of DNA and

potential PCR inhibitors as the unknown samples. Furthermore, because our objectives were to determine the relative changes in *H. akashiwo* abundance, the results of this study were not affected by errors in cell counts for calibrator samples.

The comparison of QPCR analyses with chl *a* measurements allowed us to detect species-specific and community level changes over a short time scale and at different cell densities. Our results indicate that microzooplankton grazing pressure on *H. akashiwo* can be greater than the grazing on the total phytoplankton community (Expts 4, 5, 8, and 11). These results contradict a previous study where two copepod species selected against *H. akashiwo* (in culture) and did not feed on this alga when it was provided in high concentrations [67]. However, considering their different feeding mechanisms and trophic interactions, differences between microzooplankton (e.g., ciliates) and mesozooplankton (e.g., copepods) grazing are to be expected. *Heterosigma akashiwo* is considered to be of low nutritional value [67], and toxic strains are known to deter grazers when provided as food [9]. These previous experiments were performed, however, using cultured *H. akashiwo*, and palatability to grazers will undoubtedly vary depending on strain and environmental conditions. Although the toxicity of *H. akashiwo* populations in the DIB is unknown, the relatively high grazing rates we observed and the lack of fish kills during blooms of *H. akashiwo* suggest they were not toxic during our study.

In September 2004, two experiments were conducted with water collected during the progression of a short-lived *H. akashiwo* bloom (Expts 4 and 5). Growth rates of the total phytoplankton community in both experiments were not significantly different ($p=0.438$), and grazing on the total community did not change. *Heterosigma akashiwo*-specific grazing also remained relatively constant with no significant difference (1.88 vs 1.61 day⁻¹, $p=0.934$) between the two experiments. Although the species-specific grazing rate on *H. akashiwo* did not change, the growth rate of *Heterosigma* decreased from 1.61 to 0.18 day⁻¹, suggesting that the bloom had reached stationary phase during Expt 5. We were able to verify this in field samples taken over the following 2 days when *H. akashiwo* cell densities fell to 3.75×10^5 cells L⁻¹. The cause of this decline in cell densities is unknown. Although grazing by microzooplankton has been reported as the only controlling factor in a few studies [41, 43], others have found that it is not the sole controlling factor for some algae [48]. Calbet *et al.* [7], for example, reported that as much as 12% of the decline in biomass during the recession phase of an *A. minutum* bloom was in addition to the daily loss via microzooplankton grazing. There may be other top-down controls such as viruses [42, 49, 64] or algicidal/algistatic bacteria [15, 25, 29] leading to the decrease in density that was observed.

In addition to *H. akashiwo*, we also evaluated microzooplankton grazing pressure on naturally occurring blooms of other raphidophyte species common to the DIB: *C. subsalsa* (45–50 μm, Expt 10 and 12), *C. cf. verruculosa* (30–40 μm, Expt 10, 12, and 13), and *F. japonica* (20–24 μm, Expt 13). Microzooplankton grazing specifically on *C. subsalsa* and *C. cf. verruculosa* was not detected in our experiments. Grazing pressure on the total phytoplankton communities (determined by chl *a*) in these experiments was also either minimal (Expts 12 and 13) or undetectable (Expt. 10). This may be the result of dominance by these relatively large raphidophytes. Although Tillmann and Reckermann [66] reported that several mixotrophic dinoflagellates could readily ingest *F. japonica* and had positive growth rates, in our study, microzooplankton grazing on *F. japonica* was also not detected. However, we were limited to one experiment in observing microzooplankton grazing pressure on this species. The *Chattonella* and *Fibrocapsa* species are much larger than *H. akashiwo* and were possibly not consumed by micrograzers for this reason.

As a comparison, we also evaluated grazing pressure on other small, non-harmful algal species collected from DIB (Fig. 3). When non-harmful small phytoplankton (<20 μm) were dominant, microzooplankton grazing pressure for the <20-μm size fraction was equal to or higher than grazing on the total community. Comparison of grazing rates from these experiments (Expt 6 and 9) to grazing pressure observed on *H. akashiwo* supports our hypothesis that size selection by microzooplankton community may possibly be affecting raphidophyte dynamics in the DIB.

This study focused on changes in abundance of specific algae (esp. *H. akashiwo*) within the context of a natural population with unknown species of grazers. However, we observed that large dinoflagellates (>50 μm) generally had high densities at the end of the experiments, which may be due in part to the addition of nutrients. However, several of the large dinoflagellates common to the DIB, such as *O. marina* [66] and *Gyrodinium* spp. [38, 68], are capable of mixotrophy and grazing on the smaller phytoplankton including *H. akashiwo* [33, 37]. Other mixotrophic dinoflagellates of similar size (*Noctiluca scintillans* [9, 50], *Cochlodinium polykrikoides* [34], *Gonyaulax polygramma* [36], *Prorocentrum* spp., *Heterocapsa triquetra*, *Scrippsiella trochoidea*, *Gymnodinium impudicum*, *Alexandrium tamarense*, *Akashiwo sanguinea*, *Gymnodinium catenatum*, and *Lingulodinium polyedrum* [35]) and some small heterotrophic dinoflagellates [30, 31] have also been reported to feed on *H. akashiwo*. The presence of these mixotrophic dinoflagellates could potentially affect our comparison of species-specific QPCR-based grazing rates on *H. akashiwo* with chl *a*-based total community grazing rates because these organisms are known to sometimes retain ingested chl *a* for extended periods of time [33]. Other general groups of

microzooplankton that were commonly seen in our experiments were identified as oligotrichous ciliates (15–60 μm), tintinnids (40–60 μm), *Strombidium* sp. (35–55 μm), rotifers (150–200 μm), barnacle nauplii (200 μm), and copepod nauplii (≥ 200 μm).

Our results support the hypothesis that removal of *H. akashiwo* by microzooplankton grazing may provide larger raphidophytes with a competitive advantage in the DIB. When Delaware isolates are compared, *H. akashiwo* is a better competitor for nutrients than *C. subsalsa* [74]. Our data suggest that the removal of *H. akashiwo* from the environment via microzooplankton grazing may be a key factor for the comparatively greater abundance of *C. subsalsa* in DIB, despite the latter species being competitively inferior with regard to most “bottom-up” control factors. Prey toxicity, however, can also help to determine the feeding preferences of microzooplankton and potentially decrease grazing pressure [61]. Further studies evaluating differences in interactions between toxic and non-toxic strains and higher trophic levels in the natural environment will be necessary to fully understand the bloom dynamics of this species. Other trophic interactions such as grazing on bacteria by *H. akashiwo* and *C. subsalsa* [57] can also contribute to the bloom dynamics and need to be considered when evaluating growth and grazing rates. As demonstrated in this article, integration of QPCR with the dilution method offers a valuable species-specific enumeration tool to carry out these investigations, and this approach can be used to study other interactions of harmful algal bloom species with grazers in aquatic ecosystems. In a broader scale, this species-specific method can also be utilized in investigating selected dominant phytoplankton species’ contribution to microbial loop carbon budgets during spring blooms.

Acknowledgments Authors would like to thank K. Portune and Y. Zhang for laboratory assistance. Financial support was provided by Delaware Sea Grant 235445, EPA STAR ECOHAB R83-1041, EPA ECOHAB R83-3221, NOAA-MERHAB NA04NOS4780240, and the Center for the DIB. We thank D. Kirchman, C. Hare, R. Dale, C. Gobler, and P. Gaffney for their input to the manuscript and also the Delaware Volunteer Phytoplankton Monitoring Group (especially Coordinator Dr. E. Whereat and M. Farestad) for valuable field assistance and field data.

References

- Amano K, Watanabe M, Kohata K, Harada S (1998) Conditions necessary for *Chattonella antiqua* red tide outbreaks. *Limnol Oceanogr* 43(1):117–128
- Andersen P, Thronsen J (2003) Estimating cell numbers. In: Hallegraeff GM, Anderson DM, Cembella AD (eds) Manual on harmful marine microalgae. UNESCO Publishing pp 99–131
- Bourdelaís A, Tomas C, Naar J, Kubanek J, Baden D (2002) New fish-killing alga in coastal Delaware produces neurotoxins. *Environ Health Perspect* 110(5):465–470
- Bowers HA, Tengs T, Glasgow HB, Burkholder JM, Rublee PA, Oldach DW (2000) Development of real-time PCR assays for rapid detection of *Pfiesteria piscicida* and related dinoflagellates. *Appl Environ Microbiol* 66:4641–4648
- Bowers HA, Tengs T, Glasgow HB Jr, Burkholder JM, Rublee PA, Oldach DW (2002) Development of real-time PCR assays for rapid detection of *Pfiesteria piscicida* and related dinoflagellates. *Appl Environ Microbiol* 68(6):3180
- Bowers HA, Tomas CR, Tengs T, Kempton JW, Lewitus AJ, Oldach DW (2006) Raphidophyceae (Chadefaud ex Silva) systematics and rapid identification: sequence analyses and real-time PCR assays. *J Phycol* 42(6):1333–1348
- Calbet A, Vaquer D, Felipe J, Vila M, Sala MM, Alcaraz M, Estrada M (2003) Relative grazing impact of microzooplankton and mesozooplankton on a bloom of the toxic dinoflagellate *Alexandrium minutum*. *Mar Ecol Prog Ser* 259:303–309
- Caron DA, Gobler CJ, Lonsdale DJ, Cerrato RM, Schaffner RA, Rose JM, Buck NJ, Taylor G, Boissonneault KR, Mehran R (2004) Microbial herbivory on the brown tide alga, *Aureococcus anophagefferens*: results from natural ecosystems, mesocosms and laboratory experiments. *Harmful Algae* 3(4): 439–457
- Clough J, Strom S (2005) Effects of *Heterosigma akashiwo* (Raphidophyceae) on protist grazers: laboratory experiments with ciliates and heterotrophic dinoflagellates. *Aquat Microb Ecol* 39(2):121–134
- Coyne KJ, Handy SM, Demir E, Whereat EB, Hutchins DA, Portune KJ, Doblin MA, Cary SC (2005) Improved quantitative real-time PCR assays for enumeration of harmful algal species in field samples using an exogenous DNA reference standard. *Limnol Oceanogr Methods* 3:381–391
- Coyne KJ, Hutchins DA, Hare CE, Cary SC (2001) Assessing temporal and spatial variability in *Pfiesteria piscicida* distributions using molecular probing techniques. *Aquat Microb Ecol* 24(3):275–285
- Demir E, Hutchins DA, Czymmek K, Coyne KJ (2007) Description of *Veridita minima* (gen. et sp. nov.) a new raphidophyte from Delaware’s Inland Bays. *J Phycol* (in press)
- Dempster EL, Pryor KV, Francis D, Young JE, Rogers HJ (1999) Rapid DNA extraction from ferns for PCR-based analyses. *Biotechniques* 27(1):66–68
- Dolan J, McKeon K (2005) The reliability of grazing rate estimates from dilution experiments: have we over estimated rates of organic carbon consumption by microzooplankton? *Ocean Sci* 1:1–7
- Doucette G (1995) Interactions between bacteria and harmful algae: a review. *Nat Toxins* 3:65–74
- Evans C, Archer SD, Jacquet S, Wilson WH (2003) Direct estimates of the contribution of viral lysis and microzooplankton grazing to the decline of a *Micromonas* spp. population. *Aquat Microb Ecol* 30(3):207–219
- Galluzzi L, Penna A, Bertozzini E, Vila M, Garces E, Magnani M (2004) Development of a real-time PCR assay for rapid detection and quantification of *Alexandrium minutum* (a dinoflagellate). *Appl Environ Microbiol* 70(2):1199–1206
- Gaul W, Antia AN (2001) Taxon-specific growth and selective microzooplankton grazing of phytoplankton in the Northeast Atlantic. *J Mar Syst* 30(3–4):241–261
- Gobler CJ, Deonarine S, Leigh-Bell J, Gastrich MD, Anderson OR, Wilhelm SW (2004) Ecology of phytoplankton communities dominated by *Aureococcus anophagefferens*: the role of viruses, nutrients, and microzooplankton grazing. *Harmful Algae* 3(4):471–483

20. Gobler CJ, Renaghan MJ, Buck NJ (2002) Impacts of nutrients and grazing mortality on the abundance of *Aureococcus anophagefferens* during a New York brown tide bloom. *Limnol Oceanogr* 47(1):129–141
21. Guillard R (1975) Culture of phytoplankton for feeding marine invertebrates. In: Smith WL, Chanley MH (eds) Culture of marine invertebrate animals. Plenum, New York pp 26–60
22. Hammer A, Gruttner C, Schumann R (2001) New biocompatible tracer particles: use for estimation of microzooplankton grazing, digestion, and growth rates. *Aquat Microb Ecol* 24:153–161
23. Handy S, Hutchins D, Cary S, Coyne K (2006) Simultaneous enumeration of multiple raphidophyte species by quantitative real-time PCR: capabilities and limitations. *Limnol Oceanogr Methods* 4:193–204
24. Handy SM, Coyne KJ, Portune KJ, Demir E, Doblin MA, Hare CE, Cary SC, Hutchins DA (2005) Evaluating vertical migration behavior of harmful raphidophytes in the Delaware Inland Bays utilizing quantitative real-time PCR. *Aquat Microb Ecol* 40(2):121–132
25. Hare CE, Demir E, Coyne KJ, Cary SC, Kirchman DL, Hutchins DA (2005) A bacterium that inhibits the growth of *Pfiesteria piscicida* and other dinoflagellates. *Harmful Algae* 4(2):221–234
26. Harrison W (1980) Nutrient regeneration and primary production in the sea. In: Falkowski PG (ed) Primary productivity in the sea. Plenum, New York pp 433–460
27. Heid CA, Stevens J, Livak KJ, Williams PM (1996) Real time quantitative PCR. *Genome Res* 6(10):986–994
28. Heywood P (1990) Phylum Raphidophyta. In: Margulis L, Corliss JO, Melkonian M, Chapman DJ (eds) Handbook of Protoctista. Jones & Bartlett, Boston pp 318–325
29. Imai I, Ishida Y, Sawayama S, Hata Y (1991) Isolation of a marine gliding bacterium that kills *Chattonella antiqua* (Raphidophyceae). *Nippon Suisan Gakkaishi* 57(7):1409–1409
30. Jeong HJ, Ha JH, Park JY, Kim JH, Kang NS, Kim S, Kim JS, Yoo YD, Yih WH (2006) Distribution of the heterotrophic dinoflagellate *Pfiesteria piscicida* in Korean waters and its feeding on mixotrophic dinoflagellates, raphidophytes, and fish blood cells. *Aquat Microb Ecol* 44:263–275
31. Jeong HJ, Kim JS, Kim JH, Kim ST, Seong KA, Kim TH, Song JY, Kim SK (2005) Feeding and grazing impact by the newly described heterotrophic dinoflagellate *Stoeckeria algicida* on the harmful alga *Heterosigma akashiwo*. *Mar Ecol Prog Ser* 295: 69–78
32. Jeong HJ, Kim JS, Yoo YD, Kim ST, Kim TH, Park MG, Lee CH, Seong KA, Kang NS, Shim JH (2003) Feeding by the heterotrophic dinoflagellate *Oxyrrhis marina* on the red-tide raphidophyte *Heterosigma akashiwo*: a potential biological method to control red tides using mass-cultured grazers. *J Eukaryot Microbiol* 50:274–282
33. Jeong HJ, Yoo YD, Kim JS, Kang NS, Kim TH, Kim JH (2004) Feeding by the marine planktonic ciliate *Strombidinopsis jeokjo* on common heterotrophic dinoflagellates. *Aquat Microb Ecol* 36 (2):181–187
34. Jeong HJ, Yoo YD, Kim JS, Kim TH, Kim JH, Kang NS, Yih WH (2004) Mixotrophy in the phototrophic harmful alga *Cochlodinium polykrikoides* (Dinophyceae): prey species, the effects of prey concentration and grazing impact. *J Eukaryot Microbiol* 51:563–569
35. Jeong HJ, Yoo YD, Park JY, Song JY, Kim ST, Lee SH, Kim KY, Yih WH (2005) Feeding by the phototrophic red-tide dinoflagellates: five species newly revealed and six species previously known to be mixotrophic. *Aquat Microb Ecol* 40: 133–155
36. Jeong HJ, Yoo YD, Seong KA, Kim JH, Park JY, Kim SH, Lee SH, Ha JH, Yih WH (2005) Feeding by the mixotrophic dinoflagellate *Gonyaulax polygramma*: mechanisms, prey species, the effects of prey concentration, and grazing impact. *Aquat Microb Ecol* 38:249–257
37. Jeong HJ, Yoon JY, Kim JS, Yoo YD, Seong KA (2002) Growth and grazing rates of the prostomatid ciliate *Tiarina fusus* on red-tide and toxic algae. *Aquat Microb Ecol* 28(3): 289–297
38. Kim JS, Jeong HJ (2004) Feeding by the heterotrophic dinoflagellates *Gyrodinium dominans* and *G. spirale* on the red-tide dinoflagellate *Prorocentrum minimum*. *Mar Ecol Prog Ser* 280:85–94
39. Landry M, Calbet A (2005) Reality checks on microbial food web interactions in dilution experiments: responses to the comments of Dolan and McKeon. *Ocean Science* 1:39–44
40. Landry MR, Hassett RP (1982) Estimating the grazing impact of marine micro-zooplankton. *Mar Biol* 67(3):283–288
41. Landry MR, Kirshtein J, Constantinou J (1995) A refined dilution technique for measuring the community grazing impact of microzooplankton, with experimental tests in the central equatorial pacific. *Mar Ecol Prog Ser* 120(1–3):53–63
42. Lawrence JE, Chan AM, Suttle CA (2002) Viruses causing lysis of the toxic bloom-forming alga *Heterosigma akashiwo* (Raphidophyceae) are widespread in coastal sediments of British Columbia, Canada. *Limnol Oceanogr* 47(2):545–550
43. Lehrter JC, Pennock JR, McManus GB (1999) Microzooplankton grazing and nitrogen excretion across a surface estuarine–coastal interface. *Estuaries* 22(1):113–125
44. Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the ($2^{-\Delta\Delta C}$) method. *Methods* 25(4):402–408
45. McManus GB, Fuhrman JA (1988) Clearance of bacteria-sized particles by natural-populations of nanoplankton in the Chesapeake Bay outflow plume. *Mar Ecol Prog Ser* 42(2): 199–206
46. Menden-Deuer S, Lessard EJ, Satterberg J, Grunbaum D (2005) Growth rates and starvation survival of three species of the pallium-feeding, thecate dinoflagellate genus *Protoperdinium*. *Aquat Microb Ecol* 41(2):145–152
47. Moorthi S, Countway P, Stauffer B, Caron D (2006) Use of quantitative real-time PCR to investigate the dynamics of the red tide dinoflagellate *Lingulodinium polyedrum*. *Microb Ecol* 52 (1):136–150
48. Murrell MC, Stanley RS, Lores EM, DiDonato GT, Flemer DA (2002) Linkage between microzooplankton grazing and phytoplankton growth in a Gulf of Mexico estuary. *Estuaries* 25(1): 19–29
49. Nagasaki K, Ando M, Imai I, Itakura S, Ando M, Ishida Y (1993) Viral infection in *Heterosigma akashiwo* (Raphidophyceae); a possible termination mechanism of the noxious red tide. In: Harmful marine algal blooms, Proceedings of the Sixth International Conference on Toxic Marine Phytoplankton: Intercept Ltd., London, UK
50. Nakamura Y (1998) Growth and grazing of a large heterotrophic dinoflagellate, *Noctiluca scintillans*, in laboratory cultures. *J Plankton Res* 20(9):1711–1720
51. Pedersen MF, Hansen PJ (2003) Effects of high pH on the growth and survival of six marine heterotrophic protists. *Mar Ecol Prog Ser* 260:33–41
52. Popels L, Cary S, Hutchins D, Forbes R, Pustizzi F, Gobler C, Coyne K (2003) The use of quantitative polymerase chain reaction for the detection and enumeration of the harmful alga *Aureococcus anophagefferens* in environmental samples along the United States East Coast. *Limnol Oceanogr Methods* 1: 92–102
53. Portune K, Coyne K, Hutchins D, Handy S, Cary S (2007) Germination of *Heterosigma akashiwo* and *Chattonella subsalsa*

- (Raphidophyceae) cysts in Delaware's Inland Bays, DE. *Aquat Microb Ecol* (in press)
54. Price KS (1998) A framework for a Delaware inland bays environmental classification. *Environ Monit Assess* 51(1–2): 285–298
 55. Rosetta CH, McManus GB (2003) Feeding by ciliates on two harmful algal bloom species, *Prymnesium parvum* and *Proocentrum minimum*. *Harmful Algae* 2(2):109–126
 56. Rozan TF, Taillefert M, Trouwborst RE, Glazer BT, Ma SF, Herszage J, Valdes LM, Price KS, Luther GW (2002) Iron–sulfur–phosphorus cycling in the sediments of a shallow coastal bay: implications for sediment nutrient release and benthic macroalgal blooms. *Limnol Oceanogr* 47(5):1346–1354
 57. Seong KA, Jeong HJ, Kim S, Kim GH, Kang JH (2006) Bacterivory by co-occurring red-tide algae, heterotrophic nanoflagellates, and ciliates on marine bacteria. *Mar Ecol Prog Ser* 322:85–97
 58. Setälä O, Autio R, Kuosa H (2005) Predator–prey interactions between a planktonic ciliate *Strombidium* sp. (Ciliophora, Oligotrichida) and the dinoflagellate *Pfiesteria piscicida* (Dinamoebiales, Pyrrophyta). *Harmful Algae* 4(2):235–247
 59. Sherr BF, Sherr EB, Newell SY (1984) Abundance and productivity of heterotrophic nanoplankton in Georgia coastal waters. *J Plankton Res* 6(1):195–202
 60. Sieracki ME, Gobler CJ, Cucci TL, Thier EC, Gilg IC, Keller MD (2004) Pico- and nanoplankton dynamics during bloom initiation of *Aureococcus* in a Long Island, NY bay. *Harmful Algae* 3(4):459–470
 61. Stoecker DK, Parrow MW, Burkholder JM, Glasgow HB (2002) Grazing by microzooplankton on *Pfiesteria piscicida* cultures with different histories of toxicity. *Aquat Microb Ecol* 28(1):79–85
 62. Stoecker DK, Stevens K, Gustafson DE (2000) Grazing on *Pfiesteria piscicida* by microzooplankton. *Aquat Microb Ecol* 22(3):261–270
 63. Strom SL, Welschmeyer NA (1991) Pigment-specific rates of phytoplankton growth and microzooplankton grazing in the open sub-arctic Pacific-Ocean. *Limnol Oceanogr* 36(1):50–63
 64. Tai V, Lawrence JE, Lang AS, Chan AM, Culley AI, Suttle CA (2003) Characterization of HaRNAV, a single-stranded RNA virus causing lysis of *Heterosigma akashiwo* (Raphidophyceae). *J Psychol* 39(2):343–352
 65. Throndsen J (1997) The planktonic marine flagellates. In: Tomas CR (ed) *Identifying marine phytoplankton*. Academic, San Diego pp 591–730
 66. Tillmann U, Reckermann M (2002) Dinoflagellate grazing on the raphidophyte *Fibrocapsa japonica*. *Aquat Microb Ecol* 26(3):247–257
 67. Tomas C, Deason E (1981) The influence of grazing by two *Acartia* species on *Olisthodiscus luteus* Carter. *PSZNI: Mar Ecol* 2:215–223
 68. Uchida T, Kamiyama T, Matsuyama Y (1997) Predation by a photosynthetic dinoflagellate *Gyrodinium instriatum* on loricated ciliates. *J Plankton Res* 19(5):603–608
 69. Uye S (1986) Impact of copepod grazing on the red-tide flagellate *Chattonella antiqua*. *Mar Bio* 92(1):35–43
 70. Vadstein O, Stibor H, Lippert B, Loseth K, Roederer W, Sundt-Hansen L, Olsen Y (2004) Moderate increase in the biomass of omnivorous copepods may ease grazing control of planktonic algae. *Mar Ecol Prog Ser* 270:199–207
 71. Waterhouse TY, Welschmeyer NA (1995) Taxon-specific analysis of microzooplankton grazing rates and phytoplankton growth-rates. *Limnol Oceanogr* 40(4):827–834
 72. Welschmeyer NA (1994) Fluorometric analysis of chlorophyll *a* in the presence of chlorophyll *b* and pheopigments. *Limnol Oceanogr* 39(8):1985–1992
 73. Whereat E (2005) Phytoplankton monitoring report. Volunteer Phytoplankton Monitoring Group, Delaware Inland Bays Citizen Monitor Program. University of Delaware, Sea Grant Marine Advisory Service Lewes, DE
 74. Zhang Y, Fu FX, Whereat E, Coyne KJ, Hutchins DA (2006) Bottom-up controls on a mixed-species HAB assemblage: a comparison of sympatric *Chattonella subsalsa* and *Heterosigma akashiwo* (Raphidophyceae) from the Delaware Inland Bays, USA. *Harmful Algae* 5(3):310–320