Growth and grazing of microzooplankton in response to the harmful alga *Heterosigma akashiwo* in prey mixtures

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ABSTRACT: Microzooplankton grazers may play a role in regulating blooms of the ichthyotoxic alga *Heterosigma akashiwo*. This study tested the effects of *H. akashiwo*, when part of a mixed-prey assemblage, on the growth and feeding of microzooplankton. Laboratory cultures of 3 ciliates, *Favella* sp., *Metacylis* sp., and *Strombidinopsis acuminatum*, were exposed to reciprocal concentrations of *H. akashiwo* and a non-toxic prey, at saturating prey concentrations. *Heterosigma akashiwo* was toxic to *Favella* sp. and *Metacylis* sp. when *H. akashiwo* was the sole prey species; however, this toxicity was eliminated in the mixed-prey treatments, likely because of avoidance of *H. akashiwo* and selective feeding on non-toxic prey. In contrast, the growth rate of *S. acuminatum* was unaffected by *H. akashiwo*. Both *Favella* sp. and *S. acuminatum* ingested *H. akashiwo* but selected against the alga when other prey was available. In addition, natural planktonic communities, collected from East Sound, Orcas Island, northern Puget Sound, in September and October 2007, were exposed to bloom-level concentrations of *H. akashiwo*. Abundances of the major microzooplankton types, primarily ciliates and *Gyrodinium/Gymnodinium* dinoflagellates, were unaffected by *H. akashiwo*, although slight changes in grazer size structure did occur. *Heterosigma akashiwo* was harmful to the smallest grazers, mainly aloricate ciliates and small *Gyrodinium/Gymnodinium* dinoflagellates, and beneficial to larger *Gyrodinium/Gymnodinium* dinoflagellates that were able to ingest and grow on the alga. The alga was not consumed by the majority of grazers. Preferential feeding on alternate prey reduces toxic effects of *H. akashiwo* on microzooplankton. Avoidance of *H. akashiwo* by a major group of grazers would promote bloom formation by reducing *H. akashiwo* mortality and focusing community grazing pressure on potential competitor species.

KEY WORDS: *Heterosigma akashiwo* · Microzooplankton · Harmful algal blooms · *Favella* · *Metacylis* · *Strombidinopsis*

INTRODUCTION

*Heterosigma akashiwo* is a bloom-forming planktonic flagellate in the class Raphidophyceae of the phylum Ochrophyta (Graham & Wilcox 2000). It occurs worldwide and is one of the most ichthyotoxic species of phytoplankton, having a large effect on local marine ecosystems and economies (Honjo 1993). *Heterosigma akashiwo* blooms have caused serious damage to fish culture operations in numerous Pacific Rim countries. Mass mortalities of yellowtail and red sea bream have been recorded in Japan, resulting in economic losses of over 2 billion yen during a 16 yr period (Honjo 1994). Major salmon mortalities have been documented in New Zealand, Canada, Chile, and the United States (Smayda 1998). *Heterosigma akashiwo* blooms have also been associated with a decrease in wild fish and invertebrate populations in the Gulf of Mexico (Livingston 2007). In the Pacific Northwest, *H. akashiwo*-related fish mortalities were first reported at Lummi Island, Washington, in 1976 and at Nanoose Bay, British Columbia, in 1986. Recently, extensive blooms in northern Puget Sound occurred in the early summers of 2006 and 2007 (S. L. Strom pers. obs.). Eco-
nomic losses to the regional salmon farming industry exceeded $15 million Canadian from 1986 to 1990 (Black et al. 1991).

The mechanism of ichthyotoxicity is not well understood for this species, although several hypotheses are being explored. One hypothesis is that mucus, secreted by the alga to encapsulate non-motile cell masses, sticks to gill lamellae and results in respiratory and osmoregulatory failure (Smayda 1998). Research has also focused on damage to gill structure and function by reactive oxygen species (ROS) produced by the alga, which may lead to asphyxiations (Yang et al. 1995, Oda et al. 1997, Twiner & Trick 2000). A neurotoxin, rather than physical damage to gill structure, may instead be responsible for fish mortality (Black et al. 1991). Production of brevetoxin-like neurotoxins has been reported for several strains (Khan et al. 1997). Heterosigma akashiwo may have several mechanisms of toxicity that produce different effects in different marine organisms. Recently, H. akashiwo has been shown to induce sublethal effects in the oyster Crassostrea virginica (Keppler et al. 2005) and to alter the metabolic activity of mammalian cells (Twiner et al. 2004). At this time, there is no accepted chemical measure of toxin content in this species (Clough & Strom 2005).

Much research has focused on the effects of Heterosigma akashiwo on fish species; however, negative effects of this alga on microzooplankton grazers may partially explain how blooms of this harmful species arise and persist. Microzooplankton grazers are often the major consumers of phytoplankton similar in size and morphology to H. akashiwo (Sherr & Sherr 1994). Microzooplankton play a major role in marine ecosystems as they are responsible for the majority of phytoplankton consumption and the regeneration of nutrients, and they constitute a vital food source for larger zooplankton (Sherr & Sherr 1994). As the main consumers of phytoplankton, microzooplankton significantly affect phytoplankton population growth rates (Calbet & Landry 2004). Furthermore, certain microzooplankton species graze on harmful algal species and likely play a role in regulating harmful algal bloom development (Watras et al. 1985, Nakamura et al. 1996, Matsuyama et al. 1999, Calbet et al. 2003, Jeong et al. 2003, 2007). Yet algal blooms, toxic or otherwise, are an indication that the growth and accumulation rates of phytoplankton cells have exceeded mortality and grazer consumption of phytoplankton (Smayda 1997). Harmful algal blooms may be due to the poisoning of grazers by algal toxins, low abundances of grazers, or other factors (Turner & Tester 1997). Mortality of microzooplankton in the presence of H. akashiwo could partially explain the formation and persistence of H. akashiwo blooms.

Existing research shows varying responses of microzooplankton species to Heterosigma akashiwo exposure. Jeong et al. (2002) found the prostomatid ciliate Tiarina fusus to exhibit positive growth when exposed to increasing concentrations of H. akashiwo. Clough & Strom (2005) showed that the tintinnid ciliate Eutintinmus sp. and the dinoflagellate Noctiluca scintillans derived nutritional benefit from 2 strains of H. akashiwo, while the ciliate Strombidium sp. and the dinoflagellate Amphidinium longum exhibited a neutral response to both strains. In contrast, both strains were toxic to 3 species of ciliates: Coxiella sp., Metacylis sp., and Strombidium sp.

Few studies have investigated the effects of harmful algal species when present as a part of a mixed-prey assemblage, yet multi-species algal assemblages more accurately represent ecological conditions in coastal waters, even during harmful algal blooms. Existing studies show varying effects of mixed-prey assemblages on the toxicity of harmful algal species. The presence of a beneficial prey species, Rhodomonas sp., did not reduce Heterosigma akashiwo-related mortality in the 3 ciliate species examined by Clough & Strom (2005). Conversely, negative effects of H. akashiwo on the calanoid copepod Acartia tonsa were reduced when the alga was offered with a beneficial prey species (Colin & Dam 2002).

The aim of our study was to observe the effects of Heterosigma akashiwo on the growth and feeding of microzooplankton grazers when it is part of a mixed-prey assemblage. We used a H. akashiwo strain isolated in 2006 from a fish-killing bloom in northern Puget Sound. Our study addressed 2 questions: (1) Does H. akashiwo, when mixed with non-toxic prey, affect the growth and grazing of microzooplankton grazers? (2) Do local microzooplankton communities exposed to bloom-level concentrations of H. akashiwo (a) ingest the alga and/or (b) change in structure?

**MATERIALS AND METHODS**

**Laboratory cultures.** A strain of Heterosigma akashiwo was isolated from northern Puget Sound (48°37’ N, 122°52’ W) in June 2006 and deposited with the Center for Culture of Marine Phytoplankton (CCMP) in Boothbay, ME, USA. This strain, CCMP 2809, was used for all toxicity experiments. Heterocapsa triqueta and Isochrysis galbana were used as non-toxic prey for separate dual-prey experiments because they were a component of grazer maintenance prey mixtures. Carbon content, measured by carbon, hydrogen, nitrogen (CHN) analysis (Hedges & Stern 1984), and size (length [L] and width [W]) of algal cells were as follows: H. triqueta: 1.1 ngC cell⁻¹, 20 μm L × 15 μm W; H. akashiwo: 329.3 pgC cell⁻¹, 22 μm L × 18 μm W; and I. galbana: 9.8 pgC cell⁻¹, 5 μm L × 5 μm
W. Algal cultures were maintained in f/2 medium at 15°C, salinity approximately 30 and 112 µmol photons m⁻² s⁻¹ in a 12:12 h light:dark cycle (Clough & Strom 2005).

Three ciliate grazer species were used in the dual-prey experiments: 2 tintinnid ciliates, Favella sp. and Metacylis sp., and a naked spirotrich (oligotrich) ciliate, Strombidinopsis acuminatum. Grazers were maintained at 15°C and approximately 3.8 µmol photons m⁻² s⁻¹ in a 12:12 h light:dark cycle with biweekly inoculation of the following mixed-algal diets: Mantoniella squamata, Karlodinium veneficum, Isochrysis galbana, and Heterocapsa triquetra for Favella sp.; H. triquetra, Heterocapsa rotundata, Rhodomonas sp., Dunaliella tertiolecta, and I. galbana for S. acuminatum (Clough & Strom 2005); I. galbana, Emiliania huxleyi, Synechococcus sp. strain CC9605, and Microgononas pusilla for Metacylis sp. A trace metal-enriched seawater medium at salinity 30 was used for grazer maintenance and all dual-prey experiments. Incubation conditions for dual-prey experiments were the same as those for grazer culture maintenance.

**Dual-prey experiments.** Preliminary numerical response experiments established that a prey concentration of 200 µgC l⁻¹ resulted in saturated growth for all 3 grazer species, and this was used as the total prey concentration for the dual-prey experiments. Favella sp. and Strombidinopsis acuminatum cultures were removed from their maintenance food 24 h after their last feeding by sieving and reverse-sieving, respectively. Reverse-sieving isolates grazers as a sieve is inserted into the stock culture and a pipette is used to remove culture media and algal prey that pass through the sieve. Afterward, both cultures were preconditioned for an additional 24 h with 77 cells ml⁻¹ of Heterocapsa triquetra. Metacylis sp. was not preconditioned prior to experimentation. Instead, Metacylis sp. was sieved from its maintenance food 24 h after its last feeding, and the experiment was initiated within the following 3 h.

Average initial grazer concentrations were as follows: Favella sp., 1.8 cells ml⁻¹; Strombidinopsis acuminatum, 2.7 cells ml⁻¹; and Metacylis sp., 2.6 cells ml⁻¹. Grazers were exposed to 5 prey treatments consisting of reciprocal proportions of 2 prey types, Heterosigma akashiwo and a non-toxic prey, all containing a total prey concentration of 200 µgC l⁻¹ (Table 1). The non-toxic prey species were Heterocapsa triquetra for Favella sp. and S. acuminatum, and Isochrysis galbana for Metacylis sp. The 5 prey treatments and a starved control were prepared in quadruplicate. Volume of algal culture added to the treatments ranged from 0.073 to 0.301 ml H. triquetra, from 0.229 to 0.916 ml H. akashiwo, and from 0.110 to 0.440 ml I. galbana.

The agent of toxicity to planktonic grazers is unknown for Heterosigma akashiwo; therefore, we chose to define toxicity on the basis of the biological response of co-occurring species. Toxicity was defined as growth or mortality below that of the starved control. Grazer mortality was calculated from cell loss. Ciliates disappear soon after death, making cell loss a suitable measurement of mortality. The experiments conducted with H. akashiwo and Heterocapsa triquetra also included triplicate algae-only bottles of each prey proportion, which were used to determine algal growth during the experimental period. Polycarbonate bottles were used and filled completely to hold a total of 45 ml. Initial samples were fixed immediately to estimate actual grazer concentrations at the start of the experiment. Bottles were incubated at 15°C and 20.8 µmol photons m⁻² s⁻¹. Favella sp. and Strombidinopsis acuminatum were incubated for 24 h, and Metacylis sp. was incubated for 8.5 h. Metacylis sp. required a shorter incubation period to avoid complete mortality in all H. akashiwo treatments so that a toxicity gradient could be observed. Samples were fixed in 2% acid Lugol’s solution.

Grazers were enumerated using inverted light microscopy for the entire sample volume, less the approximately 3 ml removed for algal quantification. The equations of Frost (1972) and Heinboekel (1978) were used to calculate grazer growth rates (µ, d⁻¹), algal growth rate (k, d⁻¹) and ingestion rates (ngC grazer⁻¹ d⁻¹) for experiments with Favella sp. and Strombidinopsis acuminatum. Initial algal concentrations were estimated from counts of algal stock cultures and known addition volumes. Final algal concentration was estimated by light microscopy. Out of a total of 60 grazing rate estimates, 3 were negative and were entered as zero for further ingestion calculations, in order to reflect more accurate grazing levels.

<table>
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<th>Percent H. akashiwo</th>
<th>H. triquetra</th>
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<td>100</td>
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<td>607</td>
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Table 1. Prey proportion and concentration for the dual-prey experiment treatments. Percent Heterosigma akashiwo treatment proportions are based on algal carbon content. Heterocapsa triquetra was the non-toxic prey for Favella sp. and Strombidinopsis acuminatum. Isochrysis galbana was the non-toxic prey for Metacylis sp. –: No prey given to the starved replicates (control).
Natural planktonic communities. The response of natural planktonic communities to simulated *Heterosigma akashiwo* blooms was studied by introducing bloom-density concentrations of *H. akashiwo* cells to whole seawater samples. A target concentration of 6000 cells ml$^{-1}$ was used based upon densities of a naturally occurring *H. akashiwo* bloom sampled in northern Puget Sound in June 2006. Seawater samples for experiments were collected from East Sound, Orcas Island, northern Puget Sound (48°37’N, 122°52’W). East Sound is an optimal collection location because the sheltered fjord experiences frequent mixing and stratification events, resulting in episodically elevated phytoplankton and microzooplankton abundance (Jensen 2007). Seawater samples were collected and experiments conducted on 5 separate days during September and October, 2007.

Prior to water collection, vertical profiles of salinity, temperature, and fluorescence were measured with a conductivity, temperature, and depth (CTD) profiler (Sea-Bird Electronics) in order to compare hydrography and chlorophyll data with community composition. Near-surface water (~0.5 m) was then collected with a 4 l Niskin bottle. Silicon tubing was used to transfer seawater from the Niskin bottle into 2 carboys. During transfer, seawater was screened through 200 µm mesh to remove macrozooplankton so that the response of protist grazers would not be masked by higher trophic level interactions. Three collections were necessary to obtain the required volume of water. Gloves were used to handle all tubing and mesh in order to prevent contamination. Carboys were covered in black plastic until arrival at the laboratory, approximately 2 h after water collection, at which point they were placed in a temperature-controlled room for the remaining experimental setup.

Quadreplicate 500 ml polycarbonate bottles were prepared for the following 3 treatments: (1) <200 µm screened seawater with addition of 1/2 medium (control), (2) <200 µm screened seawater with addition of *Heterosigma akashiwo* cells, and (3) 0.2 µm filtered seawater with addition of *H. akashiwo* cells. The 0.2 µm filtered seawater with added *H. akashiwo* (Treatment [Tmt] 3) was used to calculate the growth rate of the alga during the experiment. This value was used to estimate the contribution of *H. akashiwo* growth to changes in *H. akashiwo* concentration within the microzooplankton community treatment (Tmt 2). In order to maintain equivalent nutrient levels between treatments, 1/2 medium was added to Tmt 1, at a volume equal to that of the algal culture added to Tmts 2 & 3.

Four experiments received the target algal concentration of 6000 cells ml$^{-1}$; however, the algal culture did not reach adequate density for the first sampling day, resulting in a concentration of approximately 3000 cells ml$^{-1}$ for that day. Algal culture and 1/2 medium were distributed into experimental bottles, followed by the addition of seawater. Seawater from 1 carboy was siphoned into bottles for Tmts 1 and 2 in a haphazard order. Initial samples for quantifying microzooplankton abundance were also taken from this carboy and fixed immediately. In order to equally distribute planktonic organisms, water within this carboy was gently mixed with a plunger prior to and during the transfer to experimental bottles. Seawater from the second carboy was filtered through a 0.2 µm cartridge filter and distributed into Tmt 3 bottles.

Bottles were put into 1-layer screen bags and placed outside on a rotating plankton wheel (approximately 0.5 rpm), submerged in a flow-through seawater system to maintain ambient seawater temperature and light level. In order to identify consumers and determine ingestion rates of *Heterosigma akashiwo*, 100 ml samples from Tmt 2 were preserved at 0 and 1 h and filtered for epifluorescence microscopy. Samples were preserved in 1% glutaraldehyde, and slides were prepared with 20 µm pore size, 25 mm diameter polycarbonate filters. Cells were stained with 10 µg ml$^{-1}$ DAPI stain in order to observe cell nuclei for grazer identification. Nuclear characteristics were observed using an ultraviolet (UV, 340 to 380 nm bandpass) excitation filter. Algal cells were observed using a blue 450 to 490 nm bandpass excitation filter. Organisms were identified on the basis of morphology, size, nuclei, presence of cilia, and presence and pattern of chloroplasts. The number of ingested *H. akashiwo* cells was quantified for at least 100 individuals of the more abundant grazer types. Ingestion rate (*H. akashiwo* cells ingested grazer$^{-1}$ h$^{-1}$) was calculated for each major consumer by dividing the number of ingested *H. akashiwo* cells by the number of that particular grazer within 1 sample. For each grazer type found to ingest *H. akashiwo*, 30 individuals were measured to obtain length and width dimensions using Image-Pro Plus 5.0 software.

For determination of microzooplankton community changes in response to *Heterosigma akashiwo*, 125 ml samples from Tmts 1 and 2 were preserved in 10% acid Lugol’s solution at 0 and 24 h. A higher concentration of acid Lugol’s solution was used to preserve field samples owing to the range of planktonic ciliates within a sample. Inverted light microscopy was used to observe a settled volume of each sample containing at least 200 organisms longer than 20 µm. Dinoflagellates longer than 20 µm and all ciliates were quantified. Microbiota software (Roff & Hopcroft 1986) was used to measure the length and width of each individual, to calculate biovolume, and to estimate carbon content based on published carbon-to-volume ratios. In order to estimate community grazing on *H. akashiwo*, 20 ml
samples from Tmts 2 & 3 were preserved in 1% acid Lugol’s solution after 0, 8, and 24 h. *H. akashiwo* cells were quantified using a Sedgewick-Rafter chamber, and algal growth (k d−1) and grazing (g d−1) rates were calculated (Frost 1972).

**Statistical analysis.** In the dual-prey experiments, growth rate of *Favella* sp. was analyzed using a 1-way ANOVA and the Student-Newman-Keuls post-hoc test for multiple comparisons with SPSS 15.0 software. Growth rates of *Strombidinopsis acuminatum* and *Metacylis* sp. did not meet the assumption of equality of variances despite the use of several data transformation methods. Therefore a Kruskal-Wallis test was used to analyze the growth rates of those grazers with Statistix 1 software (Analytical Software).

In the natural planktonic community experiments, microzooplankton abundance and biomass were analyzed using multi-dimensional scaling (MDS) ordination and analysis of similarity (ANOSIM) with Primer 6. Data were square-root transformed to reduce the contribution of the more abundant microzooplankton types. Ordinations were made from Bray-Curtis similarities.

**RESULTS**

**Dual-prey experiments**

Growth rates of *Favella* sp. differed significantly among prey treatments (ANOVA, $F = 4.207$, p < 0.01). *Favella* sp. showed significantly increased mortality in the 100% *Heterosigma akashiwo* treatment versus the starved control, with average growth rates of −0.36 and −0.08 d−1, respectively, signifying a toxic effect of the alga (Fig. 1a,b). Growth rates in treatments with the non-toxic prey, *Heterocapsa triquetra*, were not significantly different from the starved control (Student-Newman-Keuls test, p > 0.05), indicating that the presence of *H. triquetra* eliminated the toxic effect of *H. akashiwo*.

Growth rate of *Strombidinopsis acuminatum* was also significantly different among the different prey treatments (Kruskal-Wallis statistic = 15.256, p = 0.009). Growth rates increased with increasing concentrations of *Heterocapsa triquetra*, with a significant difference between the starved and 100% *H. triquetra* treatments, averaging −0.11 and 0.17 d−1, respectively (Fig. 1c,d). No toxic effect of *Heterosigma akashiwo* was observed.

Growth rate of *Metacylis* sp. also varied with prey treatments (Kruskal-Wallis statistic = 20.235, p = 0.001). The 100% *Heterosigma akashiwo* treatment induced significantly greater mortality than the starved control, with average growth rates of −2.62 and −0.18 d−1, respectively, signifying a toxic effect of the alga (Fig. 1e,f).

The growth rates of *Metacylis* sp. in the treatments with *Heterocapsa triquetra* were not significantly different than those in the starved control, a response similar to that observed for *Favella* sp.

Growth rates in algae-only controls from the first experiment were close to zero for both prey species (Table 2). Ingestion of *Heterosigma akashiwo* by *Favella* sp. was near zero for the 25 and 50% *H. akashiwo* treatments and rose slightly for the 75 and 100% *H. akashiwo* treatments, with averages ranging from 4.2 to 18.4 ngC grazer−1 d−1, or from 12.9 to 55.9 prey cells grazer−1 d−1 (Fig. 2). Ingestion of *Heterocapsa triquetra* also increased with increasing concentrations of that species, but to a greater degree, with averages ranging from 13.8 to 49.7 ngC grazer−1 d−1, or from 12.6 to 45.1 prey cells grazer−1 d−1. Ingestion in the 100% *H. triquetra* treatment was higher, although not significantly so, than in the 100% *H. akashiwo* treatment ($F_{1,6} = 5.742$, p = 0.054). Ingestion of *H. akashiwo* by *Strombidinopsis acuminatum* remained low at all concentrations of the alga, with averages ranging from 2.7 to 10.3 ngC grazer−1 d−1, or from 8.1 to 31.4 prey cells grazer−1 d−1. Conversely, ingestion of *H. triquetra* increased with increasing concentrations of that species, with averages ranging from 17.1 to 59.4 ngC grazer−1 d−1, or from 15.5 to 54.0 prey cells grazer−1 d−1. Ingestion in the 100% *H. triquetra* treatment was significantly higher than in the 100% *H. akashiwo* treatment, with averages of 59.4 and 6.12 ngC grazer−1 d−1, respectively (ANOVA, $F = 532.04$, p < 0.0001). Both grazer species selected against *H. akashiwo* when it was offered in combination with *H. triquetra*, as proportional ingestion of the raphidophyte consistently remained below its proportionate contribution to prey availability (Fig. 3). This was the case when ingestion was calculated on the basis of either prey carbon or prey cell number.

Table 2. Mean algal growth rate k (d−1) and ingestion rate (ngC grazer−1 d−1 and prey cells grazer−1 d−1) by *Favella* sp. and *Strombidinopsis acuminatum* for the 100% *Heterosigma akashiwo* and 100% *Heterocapsa triquetra* treatments of the dual-prey experiments (n = 4). Total prey concentration was 200 µg C l−1. SD is shown in parentheses.

<table>
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<th>Treatment</th>
<th>k (d−1)</th>
<th>Ingestion</th>
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<td></td>
<td>ngC grazer−1 d−1</td>
<td>Prey cells grazer−1 d−1</td>
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<td></td>
<td><em>Favella</em> sp.</td>
<td><em>S. acuminatum</em></td>
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<tr>
<td>100%</td>
<td>−0.067</td>
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<td><em>H. triquetra</em></td>
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<tr>
<td>100%</td>
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<td>18.4</td>
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<td><em>H. akashiwo</em></td>
<td>(0.037)</td>
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Natural planktonic communities

Salinity values increased during the September to October study period, ranging from 29.5 to 30.5 (Table 3). Concurrently, seawater temperature decreased from 12.6 to 10.6°C. In situ temperatures were within 1.4°C of temperatures in the flow-through seawater system in which the experimental bottles were maintained. Chlorophyll $a$ concentrations were estimated from in situ fluorescence measurements. The first 4 collection dates showed some variability, with chlorophyll $a$ concentrations ranging between 3.85 and 7.64 mg m$^{-3}$, while on the final date concentrations rose considerably to 16.97 mg m$^{-3}$. Depth profiles revealed water
column stratification for each sampling date, with the pycnocline at an average of 5 m depth.

Average initial concentrations of added *Heterosigma akashiwo* ranged between 6120 and 6690 cells ml⁻¹ for all experiments, except for 4 September which had 2850 cells ml⁻¹ (Table 4). Average growth rate of *H. akashiwo* in algae-only controls ranged between 0.008 and 0.215 d⁻¹ for all 5 experiments. Average community grazing rate on *H. akashiwo* ranged between −0.034 and 0.204 d⁻¹, except for the 5 October experiment which had a rate of 3.11 d⁻¹. The high grazing rate on 5 October is due to 2 replicates with rates of 5.8 and 6.4 d⁻¹, as compared with the 2 other replicate values of −0.018 and 0.077 d⁻¹.

The most abundant microzooplankton types during these experiments were ciliates <40 µm in length and *Gyrodinium/Gymnodinium* dinoflagellates from 20 to 39 µm in length (Table 5). Partitioning of microzooplankton into 2 major groups of dinoflagellates and ciliates shows that dinoflagellates were the more abundant type on all dates except for 15 October. Overall microzooplankton abundance in East Sound changed throughout the sampling period. Average abundance was 35 000 cells l⁻¹ in the initial 4 September samples, rising to 105 000 cells l⁻¹ on 24 September and then falling to 48 000 cells l⁻¹ 2 d later on 26 September. In October, abundance decreased to below 25 000 cells l⁻¹.

Table 3. Hydrography and chlorophyll measurements from the seawater collection location in East Sound, Orcas Island, northern Puget Sound, and average temperature of the flow-through seawater system in which the natural planktonic community experiment bottles were maintained. *In situ* measurements were recorded at 1 m depth with a conductivity, temperature, and depth profiler immediately prior to seawater collection. Chlorophyll *a* concentration was estimated from *in situ* fluorescence. Incubation temperature was recorded every 15 min at the system intake and averaged for the time period of each experiment.

A trend of decreased abundance of oligotrich ciliates from 40 to 59 µm and >60 µm in length was observed in the added *Heterosigma akashiwo* treatment, as compared to the control, on all of the experiment dates except 4 September, with an overall average decrease of 29 and 62%, respectively. Averaging abundance data for all major microzooplankton types in each treatment within each day revealed a distinct change in overall community structure over time that was much larger than the treatment differences within a given day (Fig. 4).

Three types of microzooplankton measurably ingested *Heterosigma akashiwo* during the September
experiments, including an aloricate ciliate, a Gyrodinium/Gymnodinium dinoflagellate, and an unidentifiable round dinoflagellate. Average length of these organisms ranged from 30.4 to 36.5 µm (Table 6). Average ingestion rates for the September experiments, based on enumeration of food vacuole contents, ranged between 0.60 and 1.10 H. akashiwo grazer⁻¹ h⁻¹. Ingestion of H. akashiwo during the October experiments was negligible.

The effect of Heterosigma akashiwo on grazer size distribution was analyzed to determine whether grazer size influenced susceptibility to the alga. High variability within treatments prevented substantial differences between treatments from emerging; however, the 24 September, 26 September, and 5 October experiments revealed 2 notable trends. Experiments on these 3 dates exhibited a decrease in the frequency of cells in the smallest size class (biovolume 750 µm³ cell⁻¹) (equivalent spherical diameter 12.4 µm) in the H. akashiwo treatment, with no corresponding decrease in the controls (Fig. 5; 24 September data only). This decrease was due to reduced numbers of both aloricate ciliates and small Gyrodinium/Gymnodinium dinoflagellates in the added H. akashiwo treatment. Secondly, experiments on 24 and 26 September showed an increase in the percentage of mid-sized grazers in the H. akashiwo treatment, with no corresponding increase in the controls. This increase occurred in grazers within the biovolume ranges of 3000 to 10 000 and 4000 to 10 000 µm³ cell⁻¹ (equivalent spherical diameters of 19.7 to 29.4 and 21.7 to 29.4 µm) for 24 and 26 September, respectively. This increase in mid-sized grazers was primarily caused by an increase in Gyrodinium/Gymnodinium dinoflagellates in the added H. akashiwo treatments.
DISCUSSION

Dual-prey experiments

*Heterosigma akashiwo* was toxic to both tintinnid ciliates when it was the sole food source; *Favella* sp. and *Metacylis* sp. both exhibited greater mortality in the 100% *H. akashiwo* treatment than in the starved control. This toxicity was not observed in treatments containing mixtures of *H. akashiwo* and non-toxic prey, even at low concentrations of the non-toxic alga. In contrast to the tintinnid ciliates, *H. akashiwo* was not toxic to the oligotrich ciliate *Strombidinopsis acuminatum*. The growth rate of *S. acuminatum* was unaffected by the presence of *H. akashiwo* but increased with increasing concentrations of non-toxic prey.

*Favella* sp. and *Metacylis* sp. exhibited positive, yet low, growth rates in the 100% non-toxic prey treatment, potentially because of insufficient prey diversity. The *Favella* species has not demonstrated substantial growth on 1 prey species alone, yet it grows well on a prey mixture that includes *Heterocapsa triquetra*. Secondly, the low growth rate may be due to potentially unhealthy grazers. While poor condition may have made the grazers more susceptible to toxicity, several experiments have shown *Heterosigma akashiwo* to be harmful to tintinnids (Verity & Stoecker 1982, Kamiyama et al. 2000, Clough & Strom 2005).

Both *Favella* sp. and *Strombidinopsis acuminatum* ingested *Heterosigma akashiwo*; however, both ciliates selected against the alga when *Heterocapsa triquetra* was available. This suggests that the ciliates were able to differentiate between the 2 prey species and avoided *H. akashiwo* when other prey species were available. When *H. akashiwo* was the only prey available, ingestion by *S. acuminatum* remained low, while feeding by *Favella* sp. increased slightly above that observed in the mixed-prey treatments. This suggests that *S. acuminatum* generally avoids consuming the alga even when it is the only available prey.
whereas Favella sp. will ingest more algae under those same conditions. Ingestion of *H. akashiwo* by Favella sp. may have occurred at the beginning of incubation and declined to near zero as the grazer experienced harmful effects of the alga that led to the higher mortality rate, as observed by Kamiyama & Arima (2001). The different ingestion rates between Favella sp. and *Strombidinopsis acuminatum* in the 100% *Heterosigma akashiwo* treatment may account for the difference in toxicity observed between the 2 ciliates. Toxicity may be partially or wholly induced through ingestion of the alga, which would explain why Favella sp. and not *S. acuminatum* exhibited a toxic response to the 100% *H. akashiwo* treatment. Reduced ingestion of *H. akashiwo* by Favella sp. in the mixed-prey treatments could have led to the decrease in toxicity observed in those treatments. Verity & Stoecker (1982) suggest that *H. akashiwo* (previously *Olisthodiscus luteus*) toxicity in Favella sp. is induced by ingestion or direct contact with the alga. Tintinnid growth rate was inhibited by *H. akashiwo* exposure, yet *H. akashiwo*-conditioned media did not substantially decrease grazer growth, when also combined with alternative prey. Differential prey uptake has been observed in ciliates (Verity 1991), and Favella sp. have been shown to reject *H. akashiwo* cells (Taniguchi & Takeda 1988, Stoecker et al. 1995), signifying selective prey consumption among grazers. If ingestion of *H. akashiwo* rises only when it is the sole prey source, and ingestion plays a role in toxicity, then the presence of alternative prey would reduce this toxicity by allowing the grazer to shift its ingestion from the raphidophyte to a more beneficial prey source.

The presence of *Heterosigma akashiwo* also resulted in a decrease in feeding on non-toxic prey by Favella sp. While growth rate of the grazer was not significantly inhibited in the mixed-prey treatments, ingestion of non-toxic prey was hindered. Reduced overall feeding rates may be a sublethal effect of *H. akashiwo*, which could have a stronger effect on grazer growth rate with a longer exposure period than that of our 24 h experiments. Egloff (1986) also observed reduced feeding on non-toxic prey in rotifers exposed to *H. akashiwo*.

Selective feeding and toxicity depend on prey concentration. Colin & Dam (2002) tested the toxicity of another *Heterosigma* species, *Heterosigma carterae*, on the copepod *Acartia tonsa* in single- and mixed-prey treatments, with algal concentrations similar to those used here. As in our study, they found reduced toxicity in the mixed-prey treatments at similarly low concentrations of the harmful alga. In contrast, in at least some cases the beneficial effects of alternative prey do not occur at higher concentrations of harmful algae. Clough & Strom (2005) used a *Heterosigma akashiwo* concentration of 2000 cells ml–1, as compared to the concentrations in our study ranging from 152 to 607 cells ml–1. In addition, Clough & Strom (2005) used a different strain of the alga, which may have differed in physiology, including toxicity, from the strain used in our study (K. A. Fredrickson, R. Crim, K. Coyne, S. L. Strom unpubl.). This higher *H. akashiwo* concentration, along with interstrain differences, may explain the toxicity observed in their mixed-prey treatments, which was not seen in our study. Natural *H. akashiwo* blooms containing high concentrations of the alga have also caused significant decreases in tintinnid ciliate abundances, despite the

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Table 6. Average ingestion rate (*Heterosigma akashiwo* cells ingested grazer–1 h–1) and average length and width (µm) of microzooplankton grazers from the natural planktonic community experiments conducted in September 2007. Rates are based on samples preserved after 1 h. SD is shown in parentheses.

<table>
<thead>
<tr>
<th>Date</th>
<th>Grazer type</th>
<th>Average length and width (µm)</th>
<th>Average ingestion rate (<em>H. akashiwo</em> cells ingested grazer–1 h–1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4 Sep</td>
<td>Aloricate ciliate</td>
<td>36.5 (4.9) × 28.1 (4.3)</td>
<td>1.10 (0.05)</td>
</tr>
<tr>
<td>24 Sep</td>
<td><em>Gyrodinium/Gymnodinium</em></td>
<td>30.8 (5.8) × 15.3 (3.3)</td>
<td>0.60 (0.05)</td>
</tr>
<tr>
<td>26 Sep</td>
<td><em>Gyrodinium/Gymnodinium</em></td>
<td>30.4 (4.9) × 17.6 (3.6)</td>
<td>0.61 (0.09)</td>
</tr>
<tr>
<td>26 Sep</td>
<td>Round dinoflagellate</td>
<td>31.7 (5.0) × 22.5 (3.2)</td>
<td>0.63 (0.07)</td>
</tr>
</tbody>
</table>
up to 4000 cells ml$^{-1}$ of *G. aureolum* when it was mixed with an equal ratio of beneficial prey. Harmful algal species are not all equally toxic, and *G. aureolum* may be less toxic than *H. akashiwo* to *Favella* sp. Thus, the effects of prey concentration on toxicity will vary depending on the algal species and strain being used.

Considering the low *Heterosigma akashiwo* concentrations used in our study, one might conclude that the lower *H. akashiwo* concentrations, and not the presence of non-toxic prey, led to the reduction in toxicity in the mixed-prey treatments. In the case of *Favella* sp., it is most likely the presence of non-toxic prey that reduced the toxicity, because concentrations of the same strain of *H. akashiwo* as low as 100 cells ml$^{-1}$ are toxic to this grazer (S. L. Strom & K. A. Fredrickson unpubl. data). This algal concentration is below that of the 25% *H. akashiwo* treatment in our experiment of 152 cells ml$^{-1}$, thus indicating that the decrease in toxicity in the mixed-prey treatments is due to the added non-toxic algae and not to a reduced abundance of *H. akashiwo*.

Ciliates are not uniformly susceptible to toxicity by harmful algal species. *Favella* sp. have exhibited increased growth and grazing with exposure to blooms of the harmful dinoflagellates *Heterocapsa circularisquama* (Kamiyama & Matsuyama 2005) and *Gonyaulax tamarensis* (Watras et al. 1985). Likewise, *Strombidinopsis* spp. also show varying responses to other harmful algal species. *Strombidinopsis* sp. exhibited increased mortality with exposure to the alga *Prymnesium parvum* (Rosetta & McManus 2003) and the dinoflagellates *Lucialla masanensis* and *Pfiesteria piscicida*. Conversely, *Strombidinopsis jeokjo* readily ingested and grew on the dinoflagellates *Pfiesteria piscicida*, *Stoeckeria algicida* (Jeong et al. 2007), and *Cochlodinium polykrikoides* (Jeong et al. 2008). Different toxicity responses may be due to varying modes of toxicity among harmful algal species, as well as diverse cellular and behavioral characteristics among grazers. Furthermore, alternative, non-toxic prey species vary in the degree to which they reduce the toxic effects of harmful algae (Rosetta & McManus 2003). Therefore, the results of mixed-prey experiments will depend upon the specific grazer, non-toxic prey, and harmful algal species being used.
Natural planktonic communities

The natural planktonic community experiments tested the effects of *Heterosigma akashiwo* exposure on many types of microzooplankton. Each sampling date revealed a different community based on the abundance of major microzooplankton groups, which provided a variety of communities in which to test the effects of *H. akashiwo*. The change in community structure observed among the sampling dates is likely related in part to the observed shifts in hydrography over time. We observed a distinct shift in temperature and salinity between the 2 mo, with warmer, less saline conditions in September and cooler, more saline conditions in October. This shift in hydrographic conditions was likely caused by an interchange of seawater masses in the area through physical oceanographic processes. In addition to changing hydrography, an influx of seawater could also bring different populations of planktonic organisms to the area, resulting in the changes in community structure and possibly the variation in ingestion observed over time. Chlorophyll *a* values were relatively high for each sampling date; therefore, alternative prey species were abundant in each of the seawater samples.

The added *Heterosigma akashiwo* concentration of approximately 6000 cells ml⁻¹ reflects the average cell density observed during a large bloom in northern Puget Sound in June 2006. This bloom lasted roughly 6 d. Smaller-scale blooms in the region in 2007 and 2009 contained slightly lower cell densities of 4000 to 5000 cells ml⁻¹.

Overall microzooplankton community structure was not significantly affected by *Heterosigma akashiwo* on any of the sampling dates, despite the higher concentrations of 2850 to 6690 cells ml⁻¹ *H. akashiwo* in these experiments as compared to those with grazer cultures. Nevertheless, decreased abundance of large ciliates, slight changes in grazer size structure, and ingestion of the alga by certain microzooplankton species were observed.

*Heterosigma akashiwo* negatively affected the growth of large ciliates ≥40 µm in length. Previous observations have shown mixed responses of this grazer group to the alga. Clough & Strom (2005) found *H. akashiwo* to be toxic to laboratory cultures of 2 ciliate species within these size classes; however, a negative response to the alga was not observed in our dual-prey experiment with *Strombidinopsis acuminatum*. Additionally, a natural bloom of *H. akashiwo* in Japan was not toxic to large aralcate ciliates, yet significant changes within the microzooplankton community did occur (Kamiyama et al. 2000). Large decreases in tintinnid ciliates have been observed during *H. akashiwo* blooms in Rhode Island (Verity & Stoecker 1982) and Japan (Kamiyama et al. 2000). The tintinnid ciliates in our study were primarily *Eutintinnus* spp., which are too small to ingest *H. akashiwo* and were likely unaffected by the alga for this reason. Alternatively, Kamiyama et al. (2000) found an increase in the abundance of *Gymnodinium sanguineum* at the beginning of the bloom. Likewise, a small increase in *Gymnodinium/Gyrodinium* dinoflagellates occurred in this study on 24 and 26 September; however, the trend was too variable for changes to be significant. The increase in this grazer type coincided with the measurable ingestion of *H. akashiwo* by the same group. It appears that ingestion of the alga promoted the growth of this grazer type. Similarly, other microzooplankton species have been shown to ingest and grow on *H. akashiwo*.

Growth rates of the prostomatid ciliate *Tiarina fusus* (Jeong et al. 2002) and the dinoflagellate *Oxyrrhis marina* (Jeong et al. 2003) increased with increasing concentrations of the alga to reach maximum rates of 0.10 and 1.43 d⁻¹, respectively. Both grazers also ingested the alga at rates of 6.5 and 1.25 ngC grazer⁻¹ d⁻¹, respectively. The latter 2 studies did not report which *H. akashiwo* strain was used, and it may be that different strains are the cause of different grazer responses to the alga.

As revealed by epifluorescence microscopy, most microzooplankton avoided ingesting *Heterosigma akashiwo*; however, an aloricate ciliate, a *Gyrodinium/Gymnodinium* dinoflagellate, and a round dinoflagellate were observed to measurably ingest the alga. These 3 grazers constituted a small enough proportion of the total community that overall community grazing rates (g, d⁻¹) remained close to zero for all dates, except 5 October. The high grazing rate on that date was probably due to the presence of one or more large invertebrate species which were not excluded by the seawater screening process. Microzooplankton grazing most likely did not cause the high grazing rate, because ingestion as observed by epifluorescence microscopy was negligible on that date. Interestingly, each grazer type that ingested *H. akashiwo* ingested the alga on only 1 date, except for the *Gyrodinium/Gymnodinium* dinoflagellate which ingested the alga on both 24 and 26 September. It may be that these particular species were not present on the other dates; however, morphologically similar grazers were observed on some of the other dates. Only a general identification of grazers was performed; therefore, each grazer type identified could consist of multiple species with potentially different feeding behaviors. Consequently, it is difficult to determine whether changes in ingestion patterns are due to a change in species composition or a change in the feeding behavior of those species. A change in feeding behavior could occur with shifts in physiological condition of the grazer, such as
cellular nutrient concentrations (Smalley et al. 2003) and growth stage (Strom 2002), or environmental conditions such as temperature (Kleppel 1992) and light level (Strom 2002), although preliminary experiments within our study showed that light level did not affect ingestion rate. More work in this area is needed to clarify the relationships between environmental conditions, cellular characteristics, and ingestion rate.

Previous studies have found community grazing on *Heterosigma akashiwo* to be much higher than that observed in this study. Microzooplankton grazing on *H. akashiwo* during 3 separate natural blooms in Delaware’s Inland Bays ranged from 0.88 to 1.88 d\(^{-1}\) (Demir et al. 2008). Grazing on *H. akashiwo* was much higher than on the total phytoplankton community, which ranged from 0.11 to 0.28 d\(^{-1}\). *Heterosigma akashiwo* concentrations used in our study were within the range observed by Demir et al.; however, an acclimated community within the Delaware blooms may be a cause for the higher grazing rates. Community composition was not reported by Demir et al. (2008) and thus cannot be evaluated as another likely cause for different grazing rates between studies. Microzooplankton grazing pressure has also been shown to be strong on other harmful species (Calbet et al. 2003), yet the effects of grazing on bloom development and regulation are variable and outcomes may be situation-specific (Turner & Tester 1997).

Examinations of natural blooms differ from those in our study in that they observe a microzooplankton community which has acclimated to the increasing concentrations of harmful algae. Grazers that can ingest and grow on the harmful algal species will be favored and likely respond by increasing their own feeding rates and abundances. In contrast, our study observed the effects of *Heterosigma akashiwo* on a naive community within 24 h of exposure. Had the incubation time been longer, higher grazing rates and more significant changes to the community may have been observed. In addition, a stronger treatment effect may have occurred with a higher, yet still ecologically relevant, concentration of *H. akashiwo*.

### Implications for harmful algal blooms

Certain microzooplankton grazers exhibit a toxic response when exposed to *Heterosigma akashiwo*, which appears to be induced by ingestion of the alga. Ingestion-related toxicity likely influences the feeding preferences of affected grazers and may contribute to the observed low ingestion of the alga. *H. akashiwo* commonly occurs as only 1 member of a multi-species phytoplankton assemblage. Mixed-prey assemblages allow microzooplankton to preferentially feed on alternative prey and decrease, or avoid, ingestion of *H. akashiwo*, thereby decreasing the toxicity of the alga.

This pattern promotes bloom formation by dual means. First, grazing mortality of *Heterosigma akashiwo* is reduced, allowing growth of the species to remain stable, or increase, as environmental or physiological conditions permit. Second, community grazing pressure is focused on alternate prey, thereby decreasing the abundance of potential competitor species. Reducing interspecific competition increases resource availability to *H. akashiwo* and thus the potential growth rate of the alga. The combination of these 2 processes significantly contributes to the growth and persistence of *H. akashiwo* blooms.

### Acknowledgements
We thank B. Bingham and G. Muller-Parker for valuable advice; K. Fredrickson, K. Bright, and J. Apple for technical support; and C. Haug, G. McKeen, and N. Schwarck for assistance with field collection and laboratory equipment. Financial support was provided by NOAA ECOHAB grant NA06NOS4780248, NSF grant OCE0551436, and Western Washington University’s Department of Environmental Science.

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