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

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

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MASTER'S THESIS

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A Thesis
Presented to
The Faculty of
Western Washington University

In Partial Fulfillment
Of the Requirements for the Degree
Master of Science

by
Sylvia Lynne Graham
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Abstract

Heterosigma akashiwo is one of the most ichthyotoxic species of phytoplankton, severely impacting marine ecosystems and economies worldwide. Microzooplankton may play a role in regulating blooms of this alga. This study tested the effects of *H. akashiwo*, when part of a mixed-prey assemblage, on the growth and feeding of microzooplankton. A saturating prey concentration of 200 µg C l^{-1} was determined for three ciliate species: *Favella* sp., *Strombidinopsis acuminatum*, and *Metacylis* sp. This was used as the total prey concentration for dual-prey experiments in which the three ciliate species were exposed to reciprocal concentrations of *H. akashiwo* and a beneficial prey species, as well as a starved control. The beneficial prey, defined as prey producing a relatively high growth rate, were *Heterocapsa triquetra* for *Favella* sp. and *S. acuminatum* and *Isochrysis galbana* for *Metacylis* sp. Toxicity was defined as grazer growth below that of the starved control. *Favella* sp. and *Metacylis* sp. exhibited a toxic response to *H. akashiwo* when it was the sole prey species; however, the presence of beneficial prey reduced this toxicity in the mixed-prey treatments. 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 subsurface seawater from East Sound, Orcas Island in September and October, 2007, were exposed to bloom-level concentrations of *H. akashiwo*. Ingestion of *H. akashiwo* was observed by epifluorescence microscopy and abundance and biomass of the major microzooplankton types were measured. Overall structure of the natural planktonic communities was unaffected by *H. akashiwo*, although slight changes in grazer size structure did occur. Bloom-concentrations of *H. akashiwo* were harmful to the smallest

grazers and beneficial to larger *Gyrodinium/Gymnodinium* dinoflagellates that were able to ingest and grow on the alga. An aloricate ciliate and a round dinoflagellate also measurably ingested *H. akashiwo*; however, the alga was not consumed by the majority of grazers. Mixed-prey assemblages offer alternative feeding opportunities to grazers and can reduce the toxicity of *H. akashiwo* that is observed in unialgal exposures.

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Introduction

Heterosigma akashiwo is a bloom-forming planktonic flagellate in the class Raphidophyceae of the Phylum Ochrophyta (Graham and Wilcox 2000). It occurs world-wide and is one of the most ichthyotoxic species of phytoplankton, having a large impact on local marine ecosystems and economies (Honjo 1993). *H. 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 year period (Honjo 1994). Major salmon mortalities have been documented in New Zealand, Canada, Chile, and the United States (Smayda 1998). 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. Economic 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 many 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 asphyxiation (Oda et al. 1997, Twiner and Trick 2000, Yang et al. 1995). 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 and Strom 2005).

Much research has focused on the effects of *H. 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 and 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 and Sherr 1994). As the main consumers of phytoplankton, microzooplankton significantly impact phytoplankton population growth rates (Calbet and 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, Matsuyama et al. 1999, Nakamura et al. 1996, Calbet et al. 2003). Yet algal blooms, toxic or otherwise, indicate that the growth and accumulation of phytoplankton cells have increased in relation to mortality and grazer consumption of phytoplankton (Smayda 1997). Such blooms may be due to the poisoning of grazers by algal toxins, low abundances of grazers, or other factors (Turner and 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 *H. 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 and Strom (2005) showed the tintinnid ciliate *Eutintinnus* sp. and the dinoflagellate *Nocticula scintillans* derived nutritional benefit from two strains of *H. akashiwo*, CCMP1914 and CCMP452, while the ciliate *Strombidium* sp. SPMC92 and the dinoflagellate *Amphidinium longum* exhibited a neutral response to both strains. In contrast, both strains induced mortality in three species of ciliates: *Coxliella* sp., *Metacylis* sp., and *Strombidium* sp.

Few studies have investigated the effects of harmful algal species when present as part of a mixed prey assemblage, yet multi-species algal assemblages more accurately represent ecological conditions in coastal waters. Existing studies show varying impacts of mixed prey assemblages on the toxicity of harmful algal species. The presence of a beneficial prey species, *Rhodomonas* sp., did not reduce *H. akashiwo*-related mortality in the three ciliate species examined by Clough and 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 and Dam 2002).

The aim of my study was to observe the effects of a strain of *H. akashiwo* (CCMP 2809) on the growth and feeding of microzooplankton grazers when it is part of a mixed prey assemblage. This strain was recently isolated in 2006 from northern Puget Sound and little is known about its impacts on microzooplankton. My study proposed to answer two questions;

1) Does *H. akashiwo*, when mixed with known beneficial prey, affect the growth of microzooplankton grazers?

2) Do local microzooplankton communities exposed to bloom-level concentrations of *H. akashiwo* a) ingest the alga? b) change in structure?

Methods

Laboratory Cultures

A strain of *Heterosigma akashiwo* isolated from northern Puget Sound in July 2006 by the lab of Dr. Suzanne Strom and deposited with the Center for Culture of Marine Phytoplankton (CCMP) in Boothbay, ME, CCMP 2809, was used for all toxicity experiments. *Heterocapsa triquetra* and *Isochrysis galbana* were used as beneficial prey in separate dual-prey experiments. Carbon content of algal cells, measured by CHN analysis, were as follows: *H. triquetra,* 1.1 ng C cell^{-1}; *Heterosigma akashiwo,* 329.3 pg C cell^{-1}; and *Isochrysis galbana,* 9.8 pg C cell^{-1}. Algal cultures were maintained in f/2 medium at 15 °C in approximately 30 psu and 112 µmol photons m^{-2} s^{-1} in a 12:12 h light:dark cycle (Clough and Strom 2005).

Three ciliate grazer species were used in the dual-prey experiments: two tintinnid ciliates, *Favella* sp. and *Metacylis* sp., and an oligotrich ciliate *Strombidinopsis acuminatum*. Grazers were maintained at 15 ºC 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 venificum, Isochrysis galbana,* and *Heterocapsa triquetra* for *Favella* sp.; *Heterocapsa triquetra, Heterocapsa rotundata, Rhodomonas* sp., *Dunaliella tertiolecta,* and *Isochrysis galbana* for *S. acuminatum* (Clough and Strom 2005); *Isochrysis galbana, Emiliania huxleyi, Synechococcus* sp. strain CC9605, and *Micromonas pusilla* for *Metacylis* sp. Incubation conditions for all single- and dual-prey experiments were the same as those for grazer culture maintenance.

Single-prey experiments

The following experiments were conducted to determine the saturating prey concentration to be used in the dual-prey experiments. The first study established the growth rates of *Favella* sp. and *Strombidinopsis acuminatum* exposed to the following increasing concentrations of *Heterocapsa triquetra*; 0, 30, 60, 100, 200, and 400 µg C l^{-1}. Prior to experimentation, grazer cultures were divided in half and preconditioned in two separate algal concentrations in order to lessen the disparity between precondition and experimental prey concentrations. This procedure reduced the influence of substantial prey fluctuations on growth rates so that observed growth rates more accurately reflect constant growth at a given prey concentration. Grazer cultures to be used for the 0, 30, and 60 µg C l^{-1} prey treatments were preconditioned in 50 µg C l^{-1} *Heterocapsa triquetra*. Grazer cultures to be used for the 100, 200, and 400 µg C l^{-1} prey treatments were preconditioned in 250 µg C l^{-1} *HETEROCAPSA triquetra*. Following 24 hours of preconditioning, the aforementioned treatments were prepared in quadruplicate 30 ml polycarbonate bottles. Average initial *Favella* sp. concentrations were 2.2 and 1.7 cells ml^{-1} for the low and high precondition food concentrations, respectively. Average initial *S. acuminatum* concentrations were 2.3 and 2.2 cells ml^{-1} for the low and high precondition food concentrations, respectively.

An additional experiment measured the growth rate of *Metacylis* sp. with two separate beneficial prey species, each at two concentrations. Quadruplicate 30 ml polycarbonate bottles were prepared with the following five treatments: 200 µg C l^{-1} or 400 µg C l^{-1} *Isochrysis galbana*, 200 µg C l^{-1} or 400 µg C l^{-1} *Emiliania huxleyi*, and a starved

control. *Metacylis* sp. was not acclimatized prior to experimentation. Average initial ciliate concentration was 2.1 cells ml^{-1}.

For both experiments, initial samples were preserved immediately to determine actual grazer abundance at the start of the experiment. All bottles were incubated in one-layer screen bags, for 24 hours, which is sufficient time to allow a significant increase in grazer abundance without an excessive decrease in prey concentration (Verity 1985; 1991). Light level within the screen bags was approximately 3.8 µmol photons m^{-2} s^{-1}. Samples were preserved in 2% acid Lugol's solution. Grazer abundance was estimated using inverted light microscopy and growth rate (µ d^{-1}) was calculated using the following equation:

$$\mu = \frac{\ln N_{t2} - \ln N_{t1}}{t_2 - t_1} \qquad \text{Equation 1}$$

where t is time and N_{t1} and N_{t2} are the number of grazers per ml at the start and end of incubation, respectively. This equation, and those that follow, were adapted from Frost (1972).

Dual-prey experiments

Favella sp. and *Strombidinopsis acuminatum* cultures were removed from their maintenance food 24 hours after their last feeding by sieving and reverse-sieving, respectively. Afterward, both cultures were acclimatized for an additional 24 hr with 77 cells ml^{-1} of *Heterocapsa triquetra*. *Metacylis* sp. was not acclimatized prior to experimentation. Instead, *Metacylis* sp. was sieved from its maintenance food 24 hours after its last feeding, and the experiment was initiated within the following 3 hours.

Average initial grazer concentrations were as follows: *Favella* sp., 1.8; *S. acuminatum*, 2.7; and *Metacylis* sp., 2.6 cells ml^{-1}. Grazers were exposed to five prey treatments consisting of reciprocal proportions of two prey types, *Heterosigma akashiwo* and a beneficial prey, all containing a total prey concentration of 200 µg C l^{-1}. Preliminary single-prey experiments showed this total prey concentration to result in saturated growth of all three grazer species. The beneficial prey species, defined as prey producing a relatively high grazer growth rate, were *Heterocapsa triquetra* for *Favella* sp. and *S. acuminatum*, and *Isochrysis galbana* for *Metacylis* sp. The five prey treatments and a starved control were prepared in quadruplicate, according to Table 1. 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 *Heterosigma 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 placed in one-layer screen bags and incubated at 15 ºC. *Favella* sp. and *S. acuminatum* were incubated for 24 hours and *Metacylis* sp. was incubated for 8.5 hours. *Metacylis* sp. required a shorter incubation period to avoid complete mortality in all *Heterosigma 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. Grazer growth rates were calculated using Equation 1. The algal growth rate (k d^{-1}) and grazing rate (g d^{-1}) were

calculated for the 100% *Heterocapsa triquetra* and 100% *Heterosigma akashiwo* treatments with *Favella* sp. and *S. acuminatum*. Algal concentration was estimated using a Sedgwick-Rafter chamber and the growth rate was calculated using the following equation:

$$k = \frac{\ln C_2 - \ln C_1}{t_2 - t_1} \qquad \text{Equation 2}$$

where C_1 and C_2 are the concentration of algae in the algae-only bottles at the start and end of incubation, respectively. Initial algal samples were not taken, thus initial concentrations were based on target, not measured, values. Ingestion rate (ng C grazer^{-1} d^{-1}) was calculated using the following equation:

$$I = \langle C \rangle \bullet F \qquad \text{Equation 3}$$

where $\langle C \rangle$ is the average prey concentration (ng C ml^{-1}) and F is the clearance rate (ml grazer^{-1} d^{-1}). $\langle C \rangle$ was determined by the following two equations:

$$\langle C \rangle = \frac{C_1^* \left[e^{(k-g)(t_2 - t_1)} - 1 \right]}{(t_2 - t_1)(k - g)} \qquad \text{Equation 4}$$

$$g = k - \left(\frac{1}{t_2 - t_1} \right) \bullet \ln \frac{C_2^*}{C_1^*} \qquad \text{Equation 5}$$

where C_1^* and C_2^* are the concentrations of algae in grazer-containing bottles at the start and end of incubation, respectively, and g is the grazing rate (g d^{-1}). Clearance rate (F ml grazer^{-1} d^{-1}) was calculated by:

$$F = \frac{g}{\overline{N}} \qquad \text{Equation 6}$$

$$\overline{N} = \frac{N_{t_2} - N_{t_1}}{\ln N_{t_2} - \ln N_{t_1}} \qquad \text{Equation 7}$$

where $\overline{N}$ is the average grazer concentration (grazers ml^{-1}) and N_{t_1} and N_{t_2} are the concentration of grazers at the start and end of incubation, respectively.

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 bloom-level concentration of 6,000 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 were collected from East Sound, Orcas Island, northern Puget Sound. 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 five 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 two 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. Carboys were rinsed with seawater prior to being filled. Two to 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 lab, approximately two hours after water collection, at which point they were placed in a temperature-controlled room for the remaining experimental set-up.

Quadruplicate 500 ml polycarbonate bottles were prepared for the following three treatments: 1) <200 µm screened seawater with addition of f/2 medium (control), 2) <200 µm screened seawater with addition of *H. akashiwo* cells, and 3) 0.2 µm filtered seawater with addition of *H. akashiwo* cells (Table 2). The 0.2 µm filtered seawater with added *H. akashiwo* (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, f/2 medium was added to Tmt 1, at a volume equal to that of the algal culture added to Tmt 2 and 3.

H. akashiwo stock culture density was calculated immediately prior to distribution into experimental bottles. Four experiments received the target algal concentration of 6,000 cells ml^{-1}; however, the algal culture did not reach adequate density for the first sampling day, resulting in a concentration of approximately 3,000 cells ml^{-1} for that day. Algal culture and f/2 medium were distributed into experimental bottles, followed by the addition of seawater. Seawater from one carboy was siphoned into Tmt 1 and 2 bottles 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 one-layer screen bags and placed outside on a rotating plankton wheel, submerged in a flow-through seawater system to maintain ambient seawater temperature and light level. In order to identify consumers of *H. akashiwo*, 100 ml samples from Tmt 2 were preserved at 0 and 1 hr and filtered for epifluorescence microscopy (Table 3). Duplicate samples were preserved in either 1% alkaline Lugol's solution or 1% glutaraldehyde in order to best preserve the wide variety of organisms associated with field samples. The alkaline Lugol's solution fixation method was initiated with 1 ml alkaline Lugol's solution, immediately followed with 2.5 ml borate-buffered formalin, and then destained with 4 ml 3% sodium thiosulfate. 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 a UV range BP 340-380 nm wavelength excitation filter. Algal cells were observed using a blue range BP 450-490 nm wavelength excitation filter. Organisms were identified based on 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 $grazer^{-1}$ hr^{-1}) was calculated for each major consumer by dividing the number of ingested *H. akashiwo* cells by the number of that particular grazer within one 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, 125 ml samples from Tmts 1 and 2 were preserved in 10% acid Lugol's solution at 0 and 24 hrs. Inverted light microscopy was used to observe a settled volume of each sample containing at least 200

organisms of length greater than 20 µm. Dinoflagellates larger than 20 µm long and all ciliates were quantified. Microbiota software 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 and 3 were preserved in 1% acid Lugol's solution after 0, 8, and 24 hrs. *Heterosigma akashiwo* cells were quantified using a Sedgewick-Rafter chamber and algal growth (k d^{-1}) and grazing (g d^{-1}) rates were calculated using Equations 2 and 5, respectively.

Statistical Analysis

In the dual-prey experiments, growth rate of *Favella* sp. was analyzed using a one-way ANOVA and the Student-Newman-Keuls post-hoc test for multiple comparisons with SPSS 15.0 software. Growth rates of *S. acuminatum* and *Metacylis* sp. did not meet the assumption of equality of variances despite using several data transformation methods. Therefore a Kruskal-Wallis test was used to analyze the growth rates of those grazers with Statistix 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. With this method, the biomass and abundance of the major microzooplankton types found in each replicate of each treatment are configured so that samples with greater similarity are placed closer together than those with less similarity. Data were square-root transformed in order to reduce the contribution of the more abundant microzooplankton types. Ordinations were made from Bray-Curtis similarities.

Table 1. Prey proportion and concentration for the dual-prey experiment treatments. Percent *Heterosigma akashiwo* treatments are based on carbon content. *Heterocapsa triquetra* was the beneficial prey for *Favella* sp. and *S. acuminatum.* *Isochrysis galbana* was the beneficial prey for *Metacylis* sp.

Percent *H.akashiwo*	*H. akashiwo*		*H. triquetra*		*I. galbana*	
	µg C l^{-1}	cells ml^{-1}	µg C l^{-1}	cells ml^{-1}	µg C l^{-1}	cells ml^{-1}
Starved	-	-	-	-	-	-
0	0	0	200	182	200	20,471
25	50	152	150	136	150	15,353
50	100	304	100	91	100	10,235
75	150	456	50	46	50	5,118
100	200	607	0	0	0	0

Table 2. Outline of objectives, and fixation and data collection methods for experiments with natural planktonic communities. Treatments were as follows: Tmt 1, <200 µm screened seawater with addition of f/2 medium (control); Tmt 2, <200 µm screened seawater with addition of *Heterosigma akashiwo* cells; and Tmt 3, 0.2 µm filtered seawater with addition of *H. akashiwo* cells.

Objective	Treatment & Sampling Time	Measurement	Fixation	Sample Size	Microscopy
Identify potential consumers of *H. akashiwo*	Tmt 2 at 0 and 1 hr	Identify microzooplankton with *H. akashiwo* cells in food vacuoles	1% alkaline Lugol's solution or 1% glutaraldehyde	100 ml	Epifluorescence microscopy
Determine microzooplankton community changes due to *H. akashiwo*	Tmt 1 and 2 at 0 and 24 hrs	Microzooplankton identification and quantification	10% acid Lugol's solution	125 ml	Inverted light microscopy
Estimate community grazing rate on *H.akashiwo*	Tmt 2 and 3 at 0, 8, and 24 hrs	*H. akashiwo* *q*uantification	1% acid Lugol's solution	20 ml	Light microscopy (Sedgewick-Rafter chamber)

Results

Single-prey experiments

Growth rate of *Favella* sp. followed a curvilinear response with increasing concentrations of *Heterocapsa triquetra*, ranging from -0.33 to 0.21 d^{-1} (Fig. 1). Negative growth indicates mortality. The growth rate of *S. acuminatum* was highly variable and showed no clear pattern of increase, with average rates ranging from 0.15 – 0.38 d^{-1}. A prey concentration of 200 µg C l^{-1} produced growth rates of 0.09 and 0.29 d^{-1} for *Favella* sp. and *S. acuminatum*, respectively. Growth rate of *Metacylis* sp. was similar among both prey species and concentrations. Average growth rates were 0.42 and 0.38 d^{-1} for the 200 and 400 µg C l^{-1} *Isochrysis galbana* treatments, respectively, and 0.38 and 0.51 d^{-1} for the equivalent *Emiliania huxleyi* treatments (Fig. 2). For all three ciliates, 200 µg C l^{-1} was determined to be a saturating prey concentration and was thus used as the total prey concentration for the dual-prey experiments.

Dual-prey experiments

Growth rate of *Favella* sp. was significantly different among the different prey treatments (ANOVA, $p < 0.05$, Table 3). *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 response to the alga (Fig. 3 a, b). Growth rates in treatments with the beneficial prey, *Heterocapsa triquetra*, were not significantly different from the starved control, indicating that the presence of *Heterocapsa triquetra* reduced the toxic effect of *Heterosigma akashiwo*.

Growth rate of *S. acuminatum* was also significantly different among the different prey treatments (Kruskal-Wallis, $p < 0.05$, Table 4). Growth rates increased with increasing concentrations of *Heterocapsa triquetra*, with a significant difference between the starved and 100% *Heterocapsa triquetra* treatments, averaging -0.17 and 0.11 d^{-1}, respectively (Fig. 3 c, d). No toxic effect of *Heterosigma akashiwo* was observed.

Growth rate of *Metacylis* sp. was also significantly different between the different prey treatments (Kruskal-Wallis, $p < 0.05$, Table 4). 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 response to the alga (Fig. 3 e, f). The growth rates of *Metacylis* sp. in the treatments with *Heterocapsa triquetra* were not significantly different than the starved control, thus showing a response similar to *Favella* sp.

Algal growth rate in algae-only controls from the first experiment was close to zero for both prey species (Table 5). Target, not measured, initial algal concentrations were used in calculating grazing rates, resulting in negative grazing rates for three samples. In order to reflect more accurate grazing levels, negative grazing rates were entered as zero for further ingestion calculations. Ingestion of *Heterosigma akashiwo* by *Favella* sp. was near zero for the 25 and 50% *Heterosigma akashiwo* treatments and rose slightly for the 75 and 100% *Heterosigma akashiwo* treatments, with averages ranging from 4.2 to 18.4 ng C $grazer^{-1}d^{-1}$, or 12.9 to 55.9 prey cells $grazer^{-1}$ d^{-1} (Fig. 4). 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 ng C $grazer^{-1}d^{-1}$, or 12.6 to 45.1 prey cells $grazer^{-1}$ d^{-1}. Ingestion in the 100% *Heterocapsa triquetra* treatment

was higher, although not significantly so, than in the 100% *Heterosigma akashiwo* treatment ($F_{1,6} = 5.742$, $p = 0.054$). Ingestion of *Heterosigma akashiwo* by *S. acuminatum* remained low at all concentrations of the alga, with averages ranging from 2.7 to 10.3 ng C grazer^{-1}d^{-1}, or 8.1 to 31.4 prey cells grazer^{-1} d^{-1}. Conversely, ingestion of *Heterocapsa triquetra* increased with increasing concentrations of that species, with averages ranging from 17.1 to 59.4 ng C grazer^{-1}d^{-1}, or 15.5 to 54.0 prey cells grazer^{-1} d^{-1}. Ingestion in the 100% *Heterocapsa triquetra* treatment was significantly higher than in the 100% *Heterosigma akashiwo* treatment, with averages of 59.4 and 6.12 ng C grazer^{-1}d^{-1}, respectively (ANOVA, $p < 0.0001$, Table 6). Both grazer species selected against *Heterosigma akashiwo* when it was offered in combination with *Heterocapsa triquetra*, as ingestion of the raphidophyte consistently remained below its proportionate abundance as a prey source (Fig. 5 and 6). This was the case when considering both mass of carbon (ng C) and abundance (number of prey cells) of prey ingested.

Natural planktonic communities

Hydrography measurements recorded near the depth of seawater collection showed trends of decreasing temperature and increasing salinity over the September and October collection period. Seawater temperature decreased from 12.6 to 10.6 ºC (Table 7). In situ temperatures were within 1.4 °C of temperatures in the flow-through seawater system in which the experimental bottles were maintained. Salinity values exhibited an overall increase during the study period, ranging from 29.5 to 30.5 psu. Chlorophyll a concentrations were estimated from in situ fluorescence measurements. The first four

collection dates showed some variability, with chlorophyll a concentrations 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 reveal distinct pycnoclines for the first four collection dates (Fig. 7). The final date, 15 October, differed from the others by having a reduced pycnocline and very high chlorophyll a concentrations within the upper 10 meters of the water column.

Average initial concentrations of added *Heterosigma akashiwo* culture ranged between 6,120 and 6,690 cells ml^{-1} for all experiments, except for 4 September which had 2,850 cells ml^{-1} (Table 8). Average growth rate of *H. akashiwo* in algae-only controls ranged between 0.008 and 0.215 d^{-1} for all five 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 two replicates with rates of 5.8 and 6.4 d^{-1}, as compared to the two other replicate values of -0.018 and 0.077 d^{-1}.

Abundance (cells l^{-1}) and biomass (µg C l^{-1}) of major microzooplankton types for the initial, and the control and added *H. akashiwo* treatments are presented in Figures 8-12. The most abundant microzooplankton types were ciliates less than 40 µm in length and *Gyrodinium/Gymnodinium* dinoflagellates between 20-39 µm in length. These two microzooplankton types generally contributed the most to community biomass as well, along with *Protoperidinium*-like species, unidentifiable dinoflagellates, and invertebrate larvae. Partitioning of microzooplankton into two major groups of dinoflagellates and ciliates shows dinoflagellates were the more abundant type on all dates except for 15 October. Biomass of the two major groups also followed the same pattern. Overall community abundance and biomass changed throughout the sampling period. Average

overall 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} two days later on 26 September. In October, abundance decreased to below 25,000 cells l^{-1}. Average overall initial community biomass followed a similar pattern, with 56 µg C l^{-1} on 4 September, rising to 123 µg C l^{-1} on 24 September, falling to 46 µg C l^{-1} two days later and finally reaching 32 µg C l^{-1} by 15 October.

No significant treatment effect was found for community biomass or abundance for any of the experiment dates (Fig. 13 and 14). Global R values ranged from -0.20 to 0.20 and -0.17 to 0.13 for the biomass and abundance data, respectively (Table 9). Averaging abundance and biomass data for each treatment within each day revealed a distinct change in overall community structure over time that was much larger than the treatment differences (Fig. 15).

Three types of microzooplankton measurably ingested *H. 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 10). Average ingestion rates for the September experiments ranged between 0.60 and 1.10 *H. akashiwo* ingested grazer^{-1} hr^{-1}. Ingestion of *H. akashiwo* during the October experiments was negligible.

The effect of *H. 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 and 26 September, and 5 October experiments revealed two notable trends. Experiments on these three dates exhibited a decrease in the frequency of cells in

the smallest size class, 750 μm^3 $cell^{-1}$ (equivalent spherical diameter 12.4 μm), in the *H. akashiwo* treatment, with no corresponding decrease in the controls (Fig. 16, 17, and 18). This decrease was due to reduced numbers of both aloricate ciliates and G*yrodinium/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 ranges of 3,000 - 10,000 and 4,000 - 10,000 μm^3 $cell^{-1}$ (equivalent spherical diameters of 19.7 – 29.4 and 21.7 – 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.

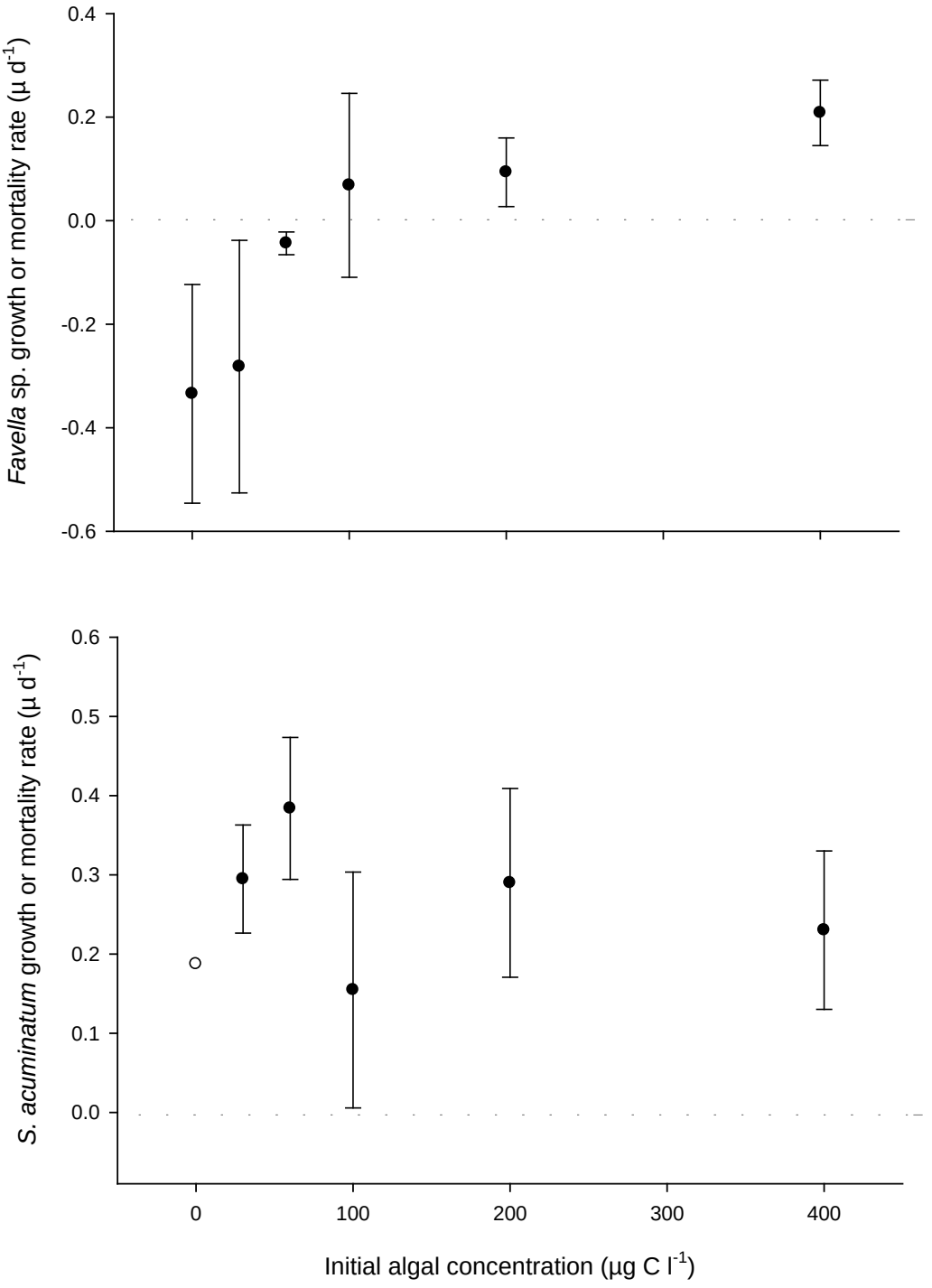

Figure 1. Growth rate (μ d^{-1}) of a) *Favella* sp. and b) *S. acuminatum* exposed to increasing concentrations of *Heterocapsa triquetra* (μg C l^{-1}) and a starved control for 24 hrs of incubation. Error bars represent standard deviation. Negative rates indicate mortality.

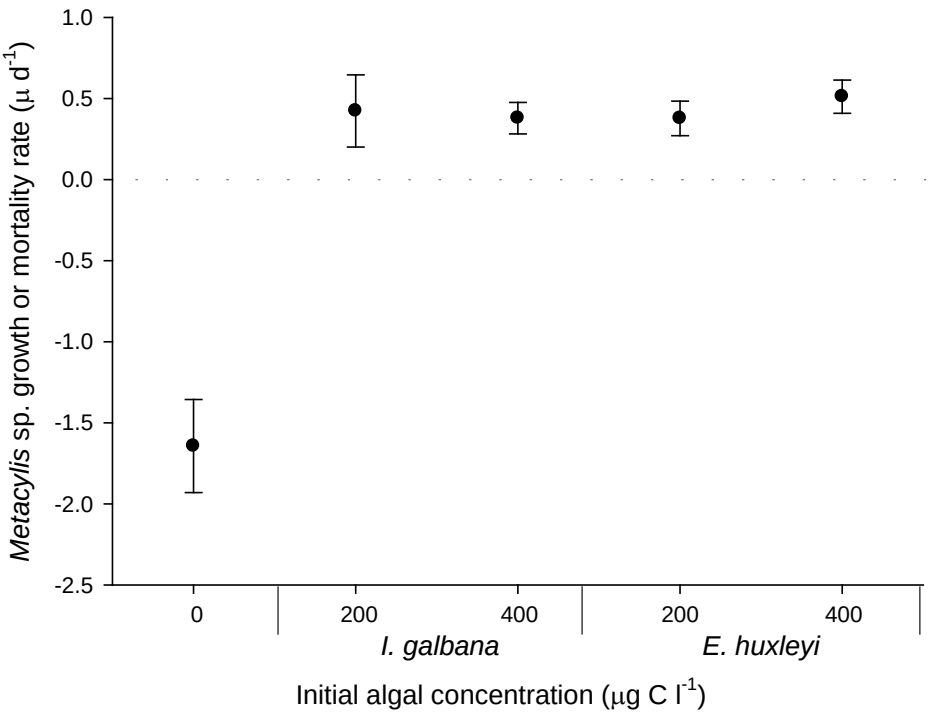

Figure 2. Growth or mortality rate (μ d^{-1}) of *Metacylis* sp. exposed to 200 and 400 μg C l^{-1} of either *Isochrysis galbana* or *Emiliania huxleyi* and a starved control for 24 hrs of incubation. Error bars represent standard deviation. Negative rates indicate mortality.

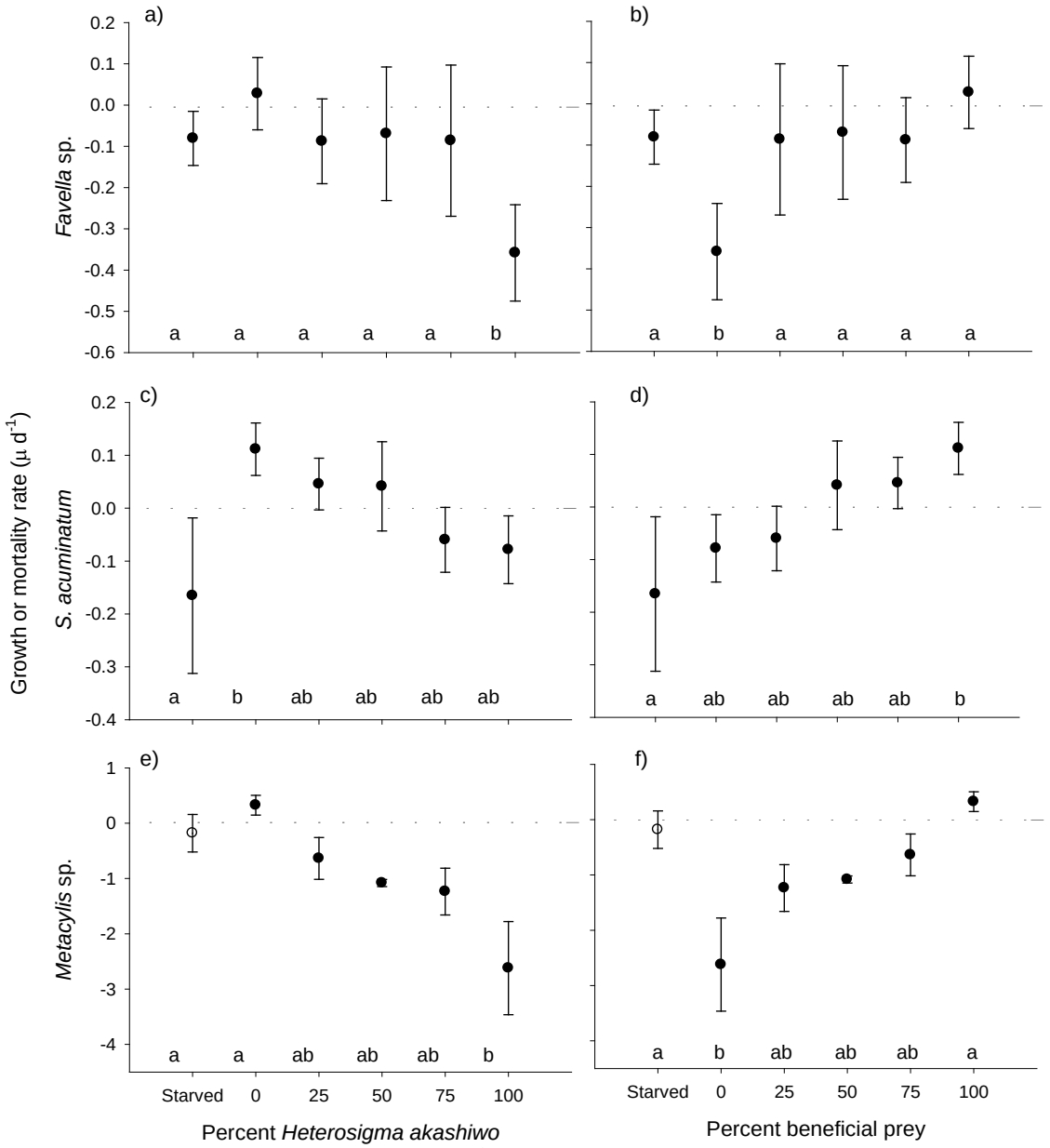

Figure 3. Growth or mortality rate (μ d^{-1}) of *Favella* sp. (a, b), *S. acuminatum* (c, d), and *Metacylis* sp. (e, f) exposed to inverse proportions of *Heterosigma akashiwo* and beneficial prey and a starved control. The beneficial prey was *Heterocapsa triquetra* for *Favella* sp. and *S. acuminatum* and *Isochrysis galbana* for *Metacylis* sp. Growth rates are shown both as a function of percent *Heterosigma akashiwo* (left column) and beneficial prey (right column). Total prey concentration was 200 μg C l^{-1} and algal carbon content was 1.1 ng, 329.3 pg, and 9.8 pg C $cell^{-1}$ for *Heterocapsa triquetra*, *Heterosigma akashiwo*, and *Isochrysis galbana*, respectively. Incubation time was 24 hours for *Favella* sp. and *S. acuminatum* and 8.5 hours for *Metacylis* sp. Treatments with similar letters are not significantly different (post-hoc Student-Newman-Keuls comparison, *Favella* sp.; comparison of mean ranks, *S. acuminatum* and *Metacylis* sp.). Error bars represent standard deviation. Negative rates indicate mortality.

Table 3. ANOVA of the growth rate (μ d^{-1}) of *Favella* sp. exposed to inverse proportions of *Heterosigma akashiwo* and *Heterocapsa triquetra*.

	SS	df	MS	F	P
Treatment	0.337	5	0.067	4.207	0.010
Error	0.288	18	0.016		
Total	0.625	23			

Table 4. Kruskal-Wallis analysis of the growth rate (μ d^{-1}) of *S. acuminatum* and *Metacylis* sp. exposed to inverse proportions of *Heterosigma akashiwo* and *Heterocapsa triquetra.*

	K-W Statistic	P
S. acuminatum	15.256	0.009
Metacylis sp.	20.235	0.001

Table 5. Mean algal growth rate (k d^{-1}) and ingestion rate (ng C $grazer^{-1}$ d^{-1} and prey cells $grazer^{-1}$ d^{-1}) for the 100% *Heterosigma akashiwo* (n=4) and 100% *Heterocapsa triquetra* (n=4) treatments of the dual-prey experiments. Total prey concentration was 200 μg C l^{-1}. Standard deviation is shown in parentheses. Algal growth rates are based on target, not measured, initial algal concentrations.

Treatment	k d^{-1}	Ingestion			
		ng C $grazer^{-1}$ d^{-1}		prey cells $grazer^{-1}$ d^{-1}	
		Favella sp.	*S. acuminatum*	*Favella* sp.	*S. acuminatum*
100% *H. triquetra*	-0.067 (0.075)	49.7 (3.46)	59.4 (3.91)	45.1 (3.15)	54.0 (3.55)
100% *H. akashiwo*	-0.075 (0.037)	18.4 (15.91)	6.12 (2.46)	55.9 (48.31)	18.6 (7.46)

Table 6. ANOVA of the ingestion rate of *S. acuminatum* in the 100% *Heterosigma akashiwo* and 100% *Heterocapsa triquetra* treatments of the dual-prey experiment.

	SS	df	MS	F	P
Algal treatment	5671.75	1	5671.75	532.04	< 0.0001
Error	63.96	6	10.66		
Total	5735.72	7			

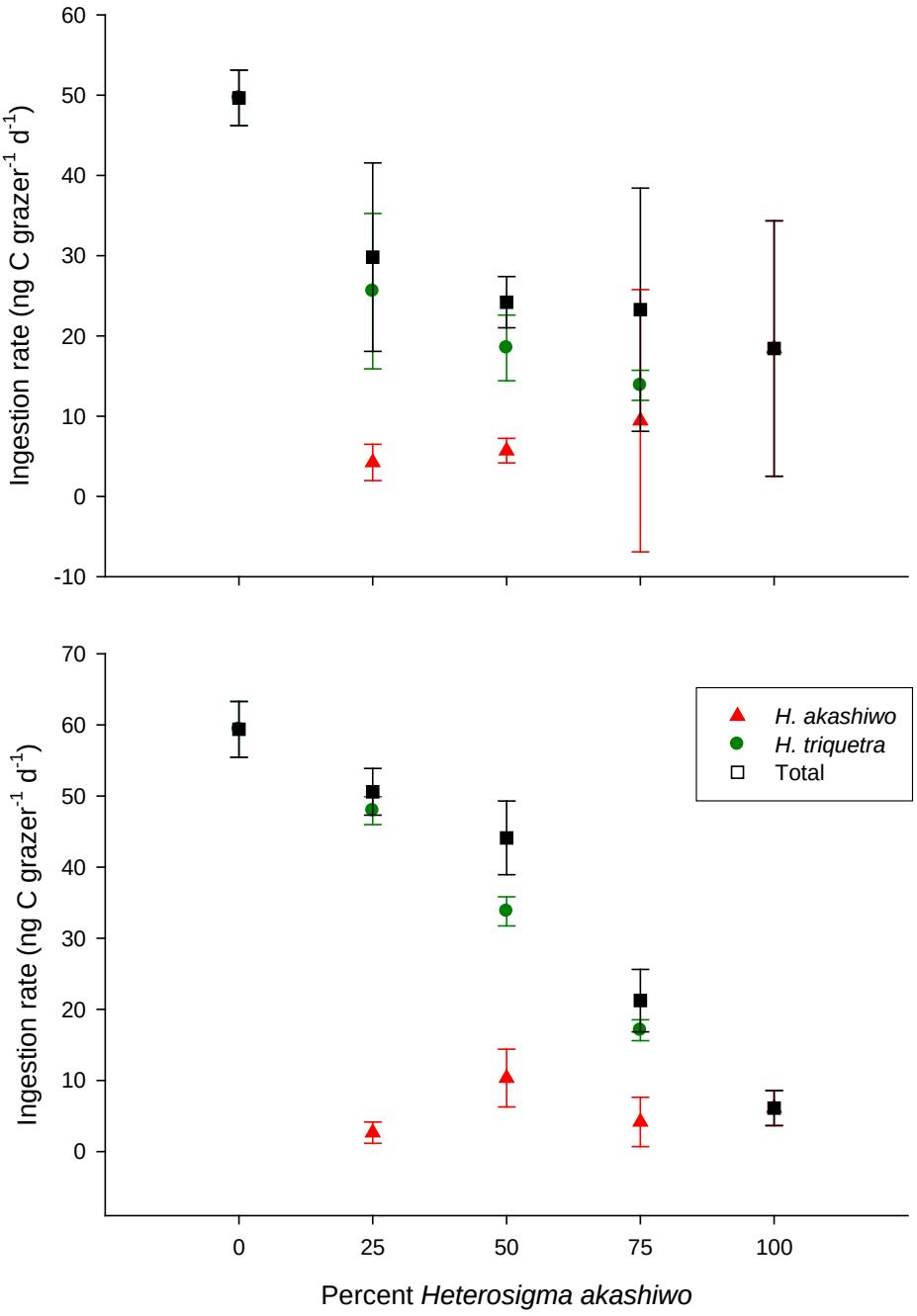

Figure 4. Average ingestion rates (ng C grazer^{-1} d^{-1}) of *Favella* sp. and *S. acuminatum* on *Heterocapsa triquetra* (●), *Heterosigma akashiwo* (▲), and total available prey (■) in the dual-prey experiments. Error bars represent standard deviation.

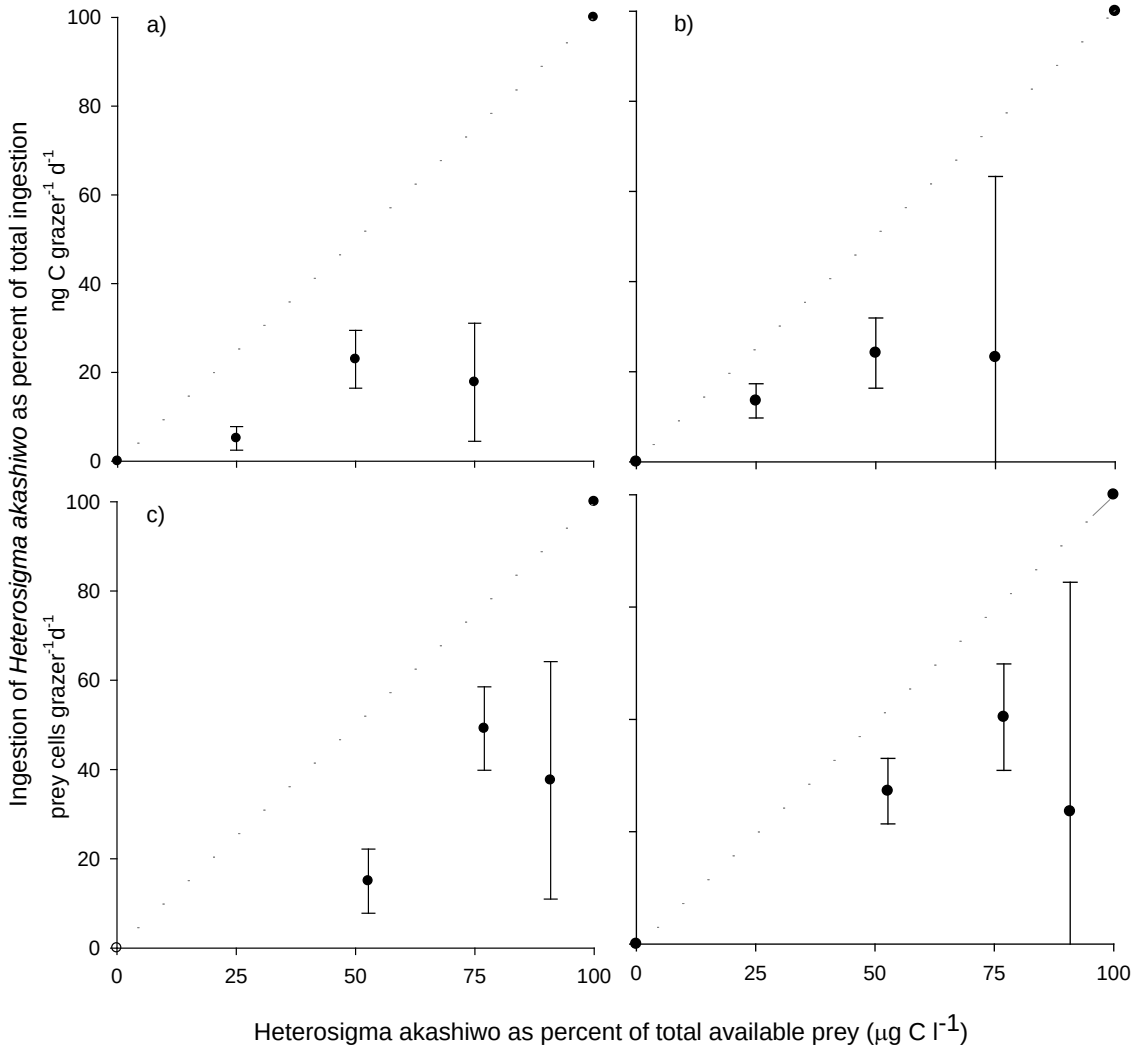

Figure 5. Ingestion of *Heterosigma akashiwo* as a percent of total ingestion versus abundance of *H. akashiwo* as a percent of total available prey by ng C grazer^{-1} d^{-1} versus µg C l^{-1} (top row) and prey cells grazer^{-1} d^{-1} versus prey cells ml^{-1} (bottom row) for *S. acuminatum* (a, c) and *Favella* sp. (b, d). Error bars represent standard deviation.

Table 7. Hydrography and chlorophyll measurements from the seawater collection location in East Sound, 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 CTD 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.

Date	Time	Salinity (psu)	Chlorophyll a ($mg\ m^{-3}$)	Temperature (°C)	Incubation Temperature (°C)
9/4	08:30	29.7	7.64	12.6	11.3
9/24	09:39	29.5	5.66	11.9	11.0
9/26	08:35	29.5	6.27	12.0	10.7
10/5	08:48	30.0	3.85	10.8	10.1
10/15	08:39	30.5	16.97	10.6	10.1

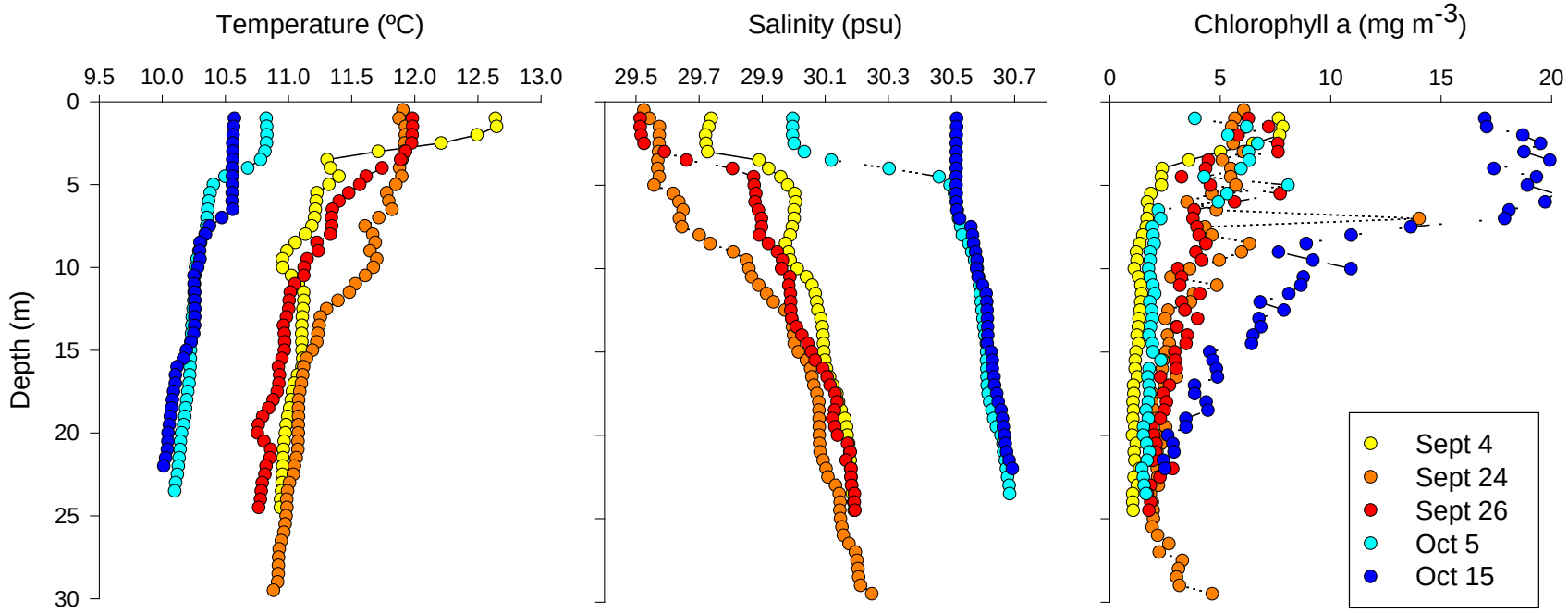

Figure 6. Temperature (°C), salinity (psu), and chlorophyll a concentration (mg m^{-3}) of the upper water column at the seawater collection location in East Sound, Orcas Island. Measurements were recorded with a CTD profiler immediately prior to seawater collection. Chlorophyll a concentration was estimated from in situ fluorescence.

Table 8. Average initial concentration (cells ml^{-1}) and growth rate (k) of added *Heterosigma akashiwo*, and grazing rate (g) on *H. akashiwo* for the experiments with natural planktonic communities (n=4). Rates are based on samples preserved at 8 and 24 hours. Eight hr samples were not taken on 4 September. Standard deviation is shown in parentheses.

Date	Average initial *H. akashiwo* concentration (cells ml^{-1})	k h^{-1}		k d^{-1}	g h^{-1}		g d^{-1}
		8 hrs	24 hrs	24 hrs	8 hrs	24 hrs	24 hrs
4 Sept	2850 (86)	-	0.009 (0.010)	0.215 (0.236)	-	0.009 (0.002)	0.204 (0.056)
24 Sept	6270 (236)	-0.001 (0.009)	0.002 (0.001)	0.058 (0.030)	-0.002 (0.006)	0.001 (0.003)	0.0315 (0.047)
26 Sept	6120 (323)	0.006 (0.019)	0.001 (0.001)	0.022 (0.029)	-0.019 (0.034)	0.003 (0.002)	0.070 (0.053)
5 Oct	6690 (191)	-0.005 (0.007)	0.003 (0.001)	0.068 (0.018)	0.129 (0.251)	0.130 (0.147)	3.108 (3.526)
15 Oct	6550 (189)	-0.007 (0.006)	0.0003 (0.002)	0.008 (0.041)	-0.005 (0.006)	-0.001 (0.002)	-0.034 (0.043)

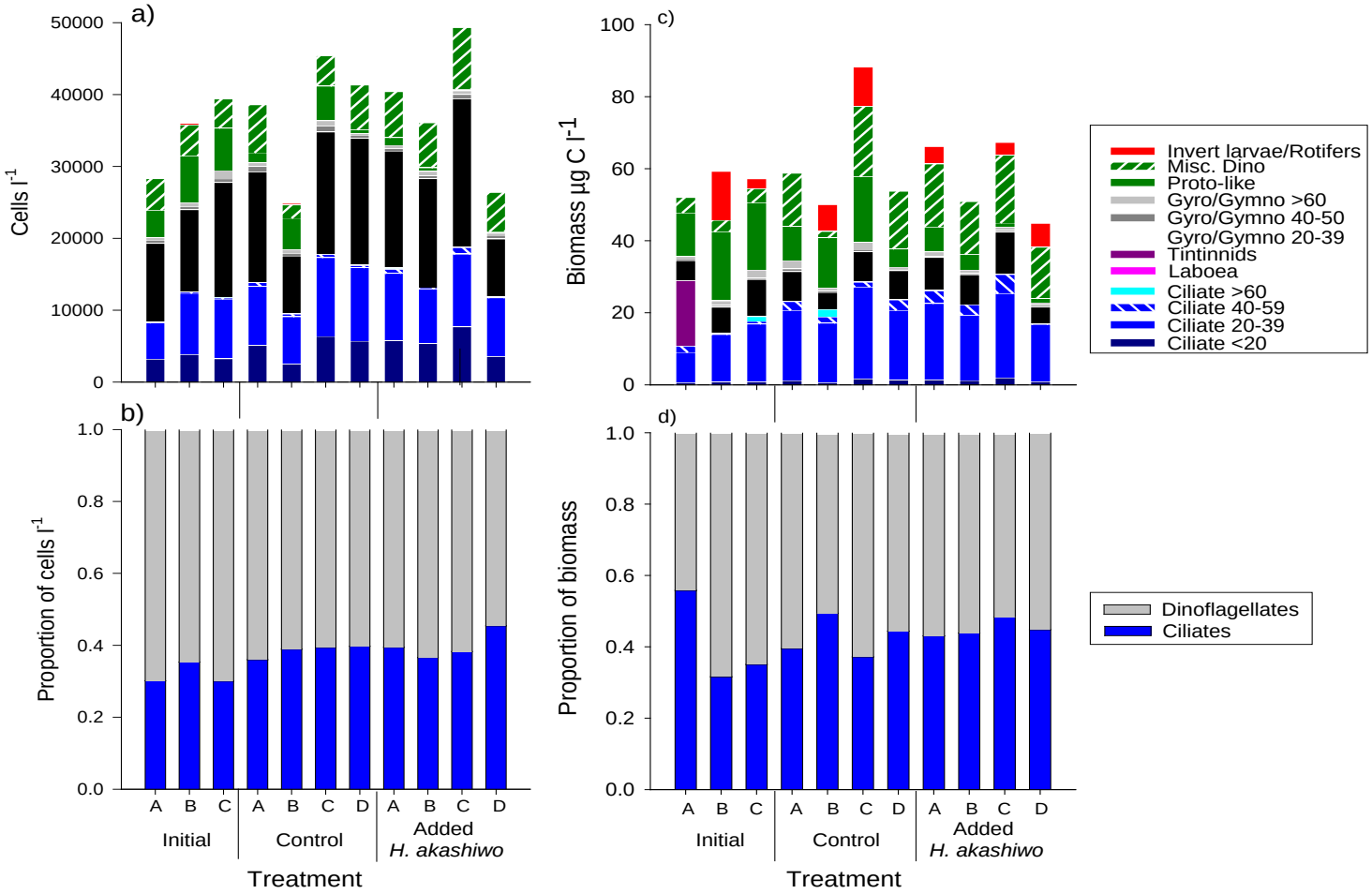

Figure 7. Microzooplankton community structure of seawater collected at 1m depth from East Sound, Orcas Island and used for the simulated *Heterosigma akashiwo* bloom experiment on 4 September, 2007. Major microzooplankton types are shown in a) cells l^{-1}, b) proportion of cells l^{-1}, c) biomass (µg C l^{-1}), and d) proportion of biomass for initial samples (n=3), and control (n=4) and added *H. akashiwo* (n=4) treatments after 24 hrs. Letters indicate replicates within each group.

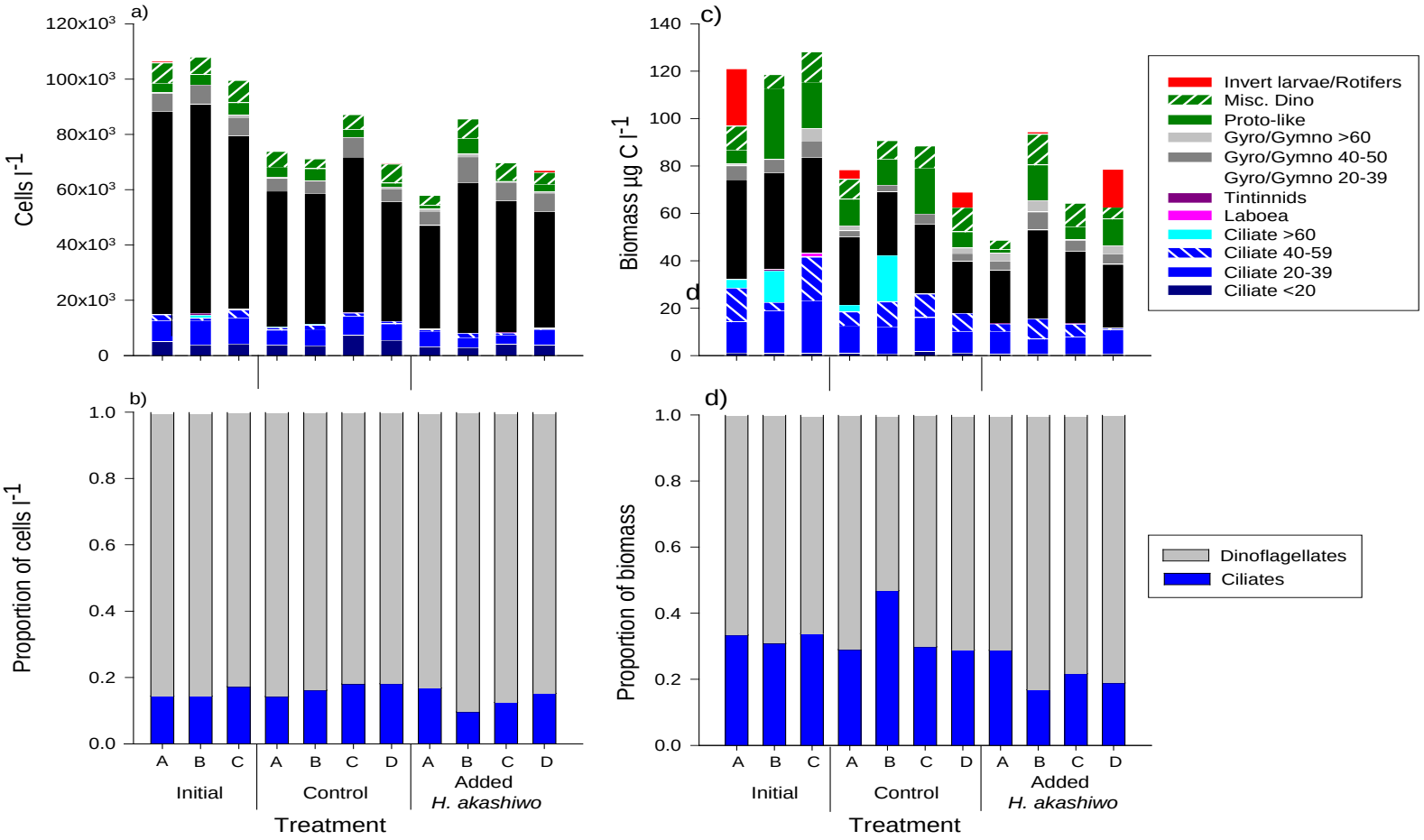

Figure 8. Microzooplankton community structure of seawater collected at 1m depth from East Sound and used for the simulated *Heterosigma akashiwo* bloom experiment on 24 September, 2007. Major microzooplankton types are shown in a) cells l^{-1}, b) proportion of cells l^{-1}, c) biomass (µg C l^{-1}), and d) proportion of biomass for initial samples (n=3), and control (n=4) and added *H. akashiwo* (n=4) treatments after 24 hrs. Letters indicate replicates within each group.

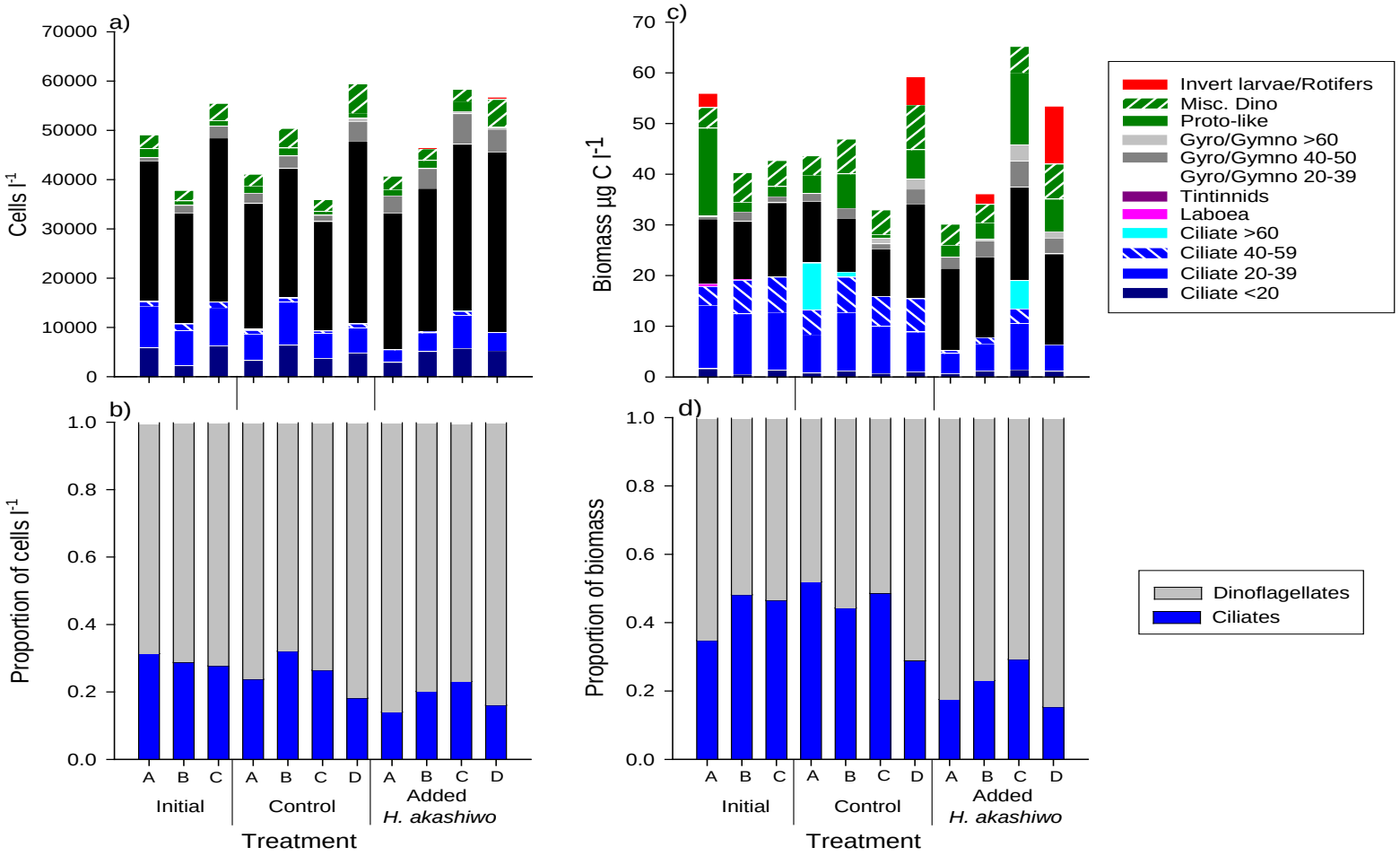

Figure 9. Microzooplankton community structure of seawater collected at 1m depth from East Sound and used for the simulated *Heterosigma akashiwo* bloom experiment on 26 September, 2007. Major microzooplankton types are shown in a) cells l^{-1}, b) proportion of cells l^{-1}, c) biomass (µg C l^{-1}), and d) proportion of biomass for initial samples (n=3), and control (n=4) and added *H. akashiwo* (n=4) treatments after 24 hrs. Letters indicate replicates within each group.

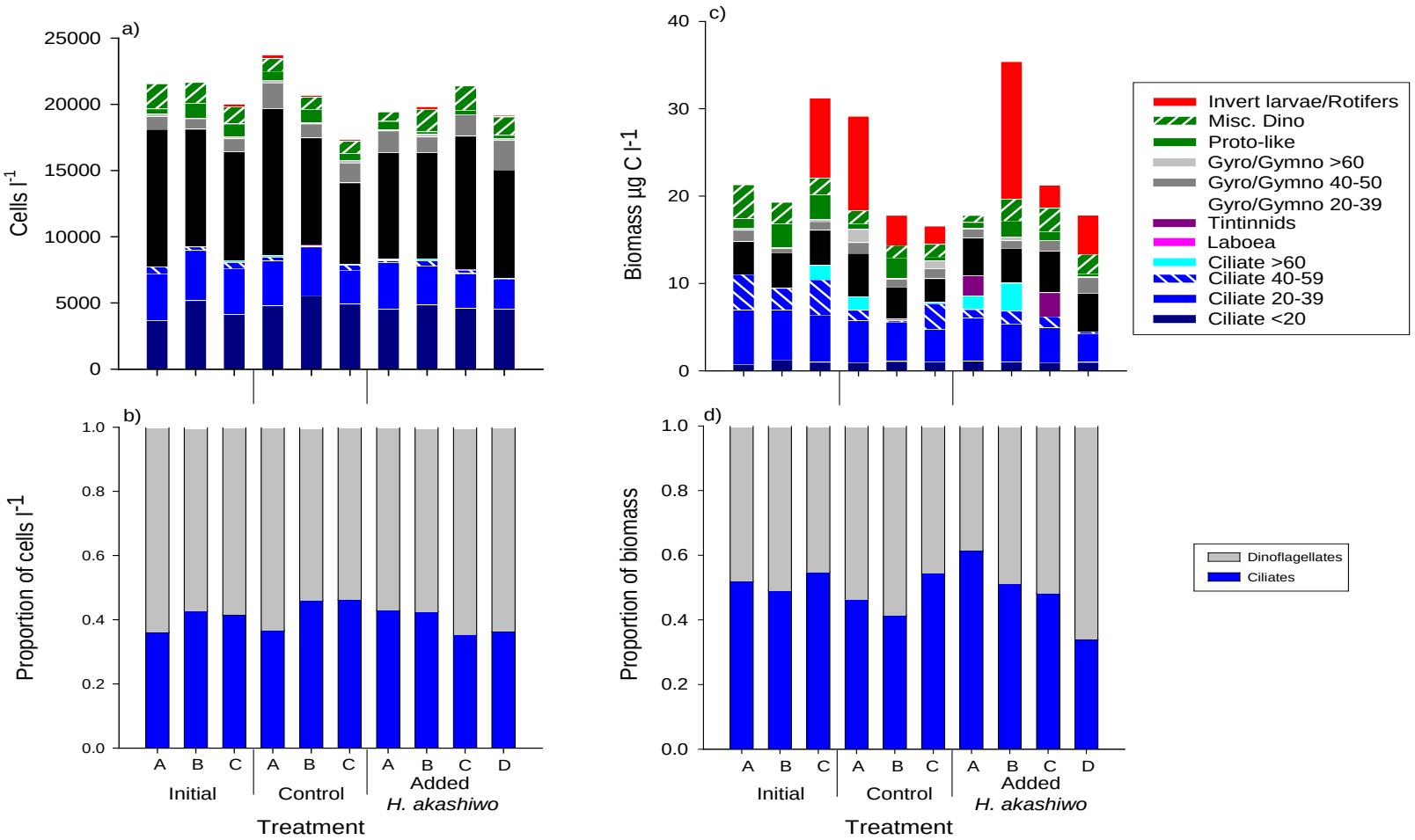

Figure 10. Microzooplankton community structure of seawater collected at 1m depth from East Sound and used for the simulated *Heterosigma akashiwo* bloom experiment on 5 October, 2007. Major microzooplankton types are shown in a) cells l^{-1}, b) proportion of cells l^{-1}, c) biomass (µg C l^{-1}), and d) proportion of biomass for initial samples (n=3), and control (n=4) and added *H. akashiwo* (n=4) treatments after 24 hrs. Letters indicate replicates within each group.

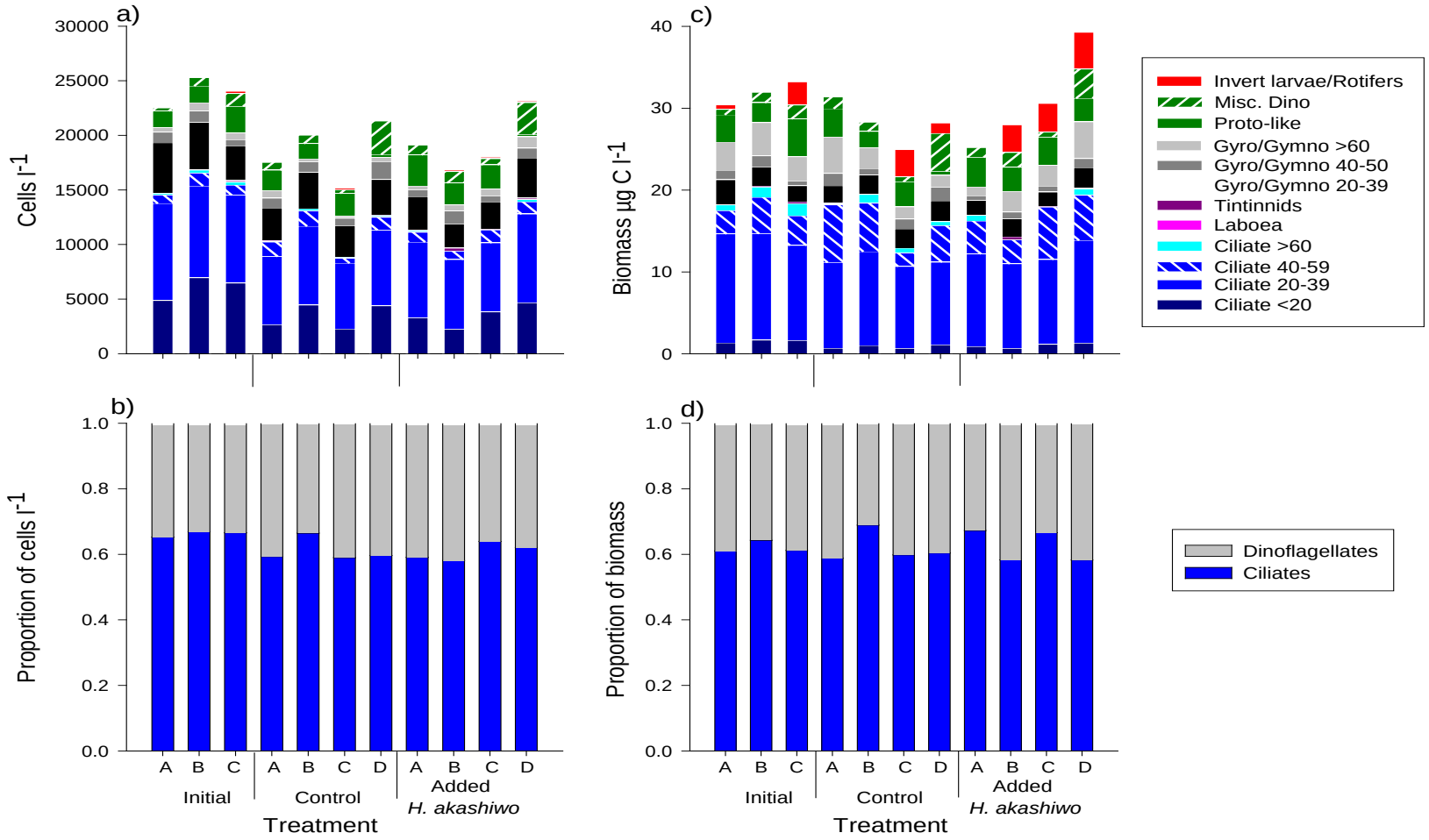

Figure 11. Microzooplankton community structure of seawater collected at 1m depth from East Sound and used for the simulated *Heterosigma akashiwo* bloom experiment on 15 October, 2007. Major microzooplankton types are shown in a) cells l^{-1}, b) proportion of cells l^{-1}, c) biomass (µg C l^{-1}), and d) proportion of biomass for initial samples (n=3), and control (n=4) and added *H. akashiwo* (n=4) treatments after 24 hrs. Letters indicate replicates within each group.

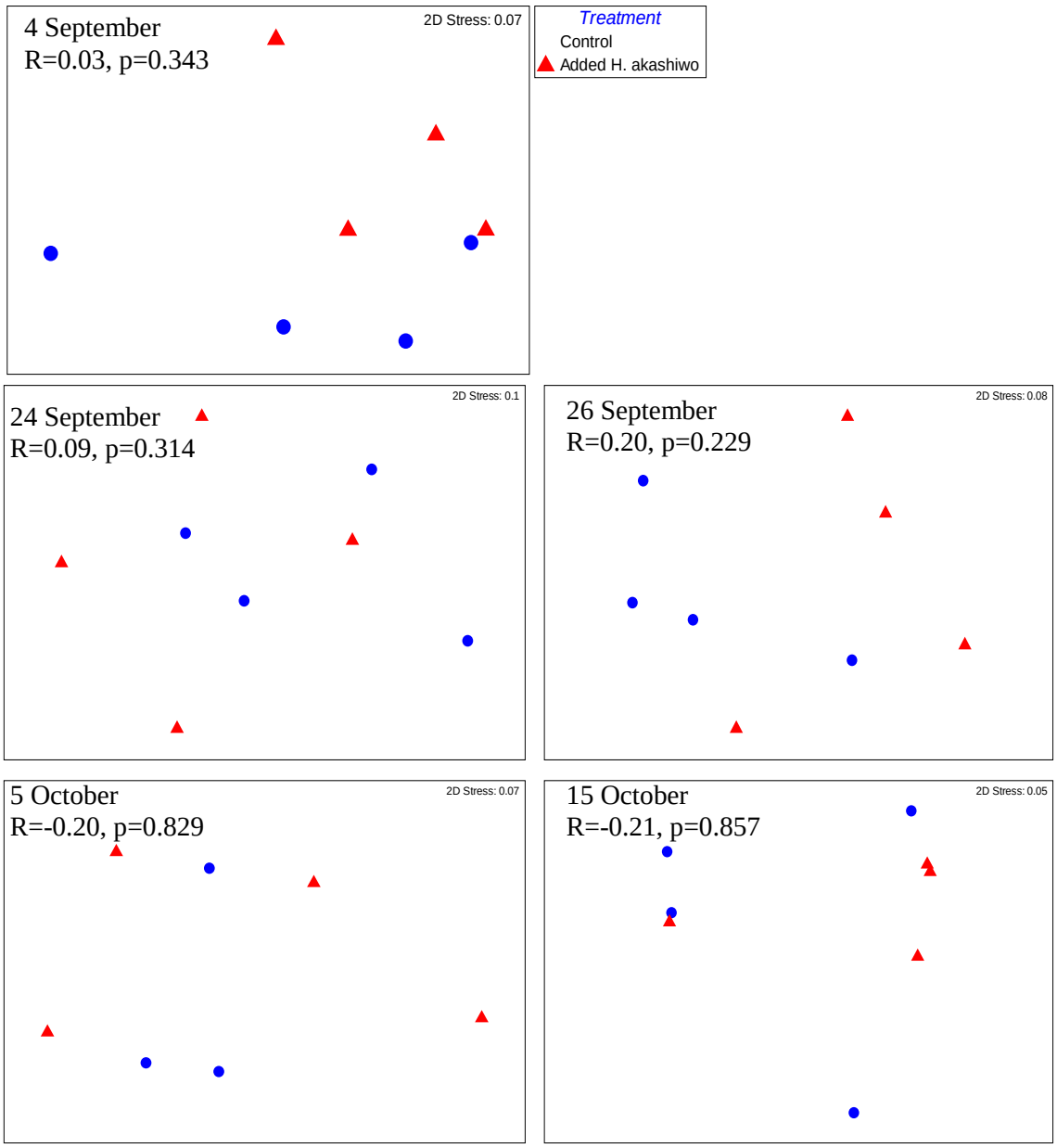

Figure 12. Multi-dimensional scaling ordination of microzooplankton biomass (µg C l^{-1}) for the control (●) and added *H. akashiwo* (▲) treatments for natural planktonic community experiments conducted on 4, 24, and 26 September and 5 and 15 October.

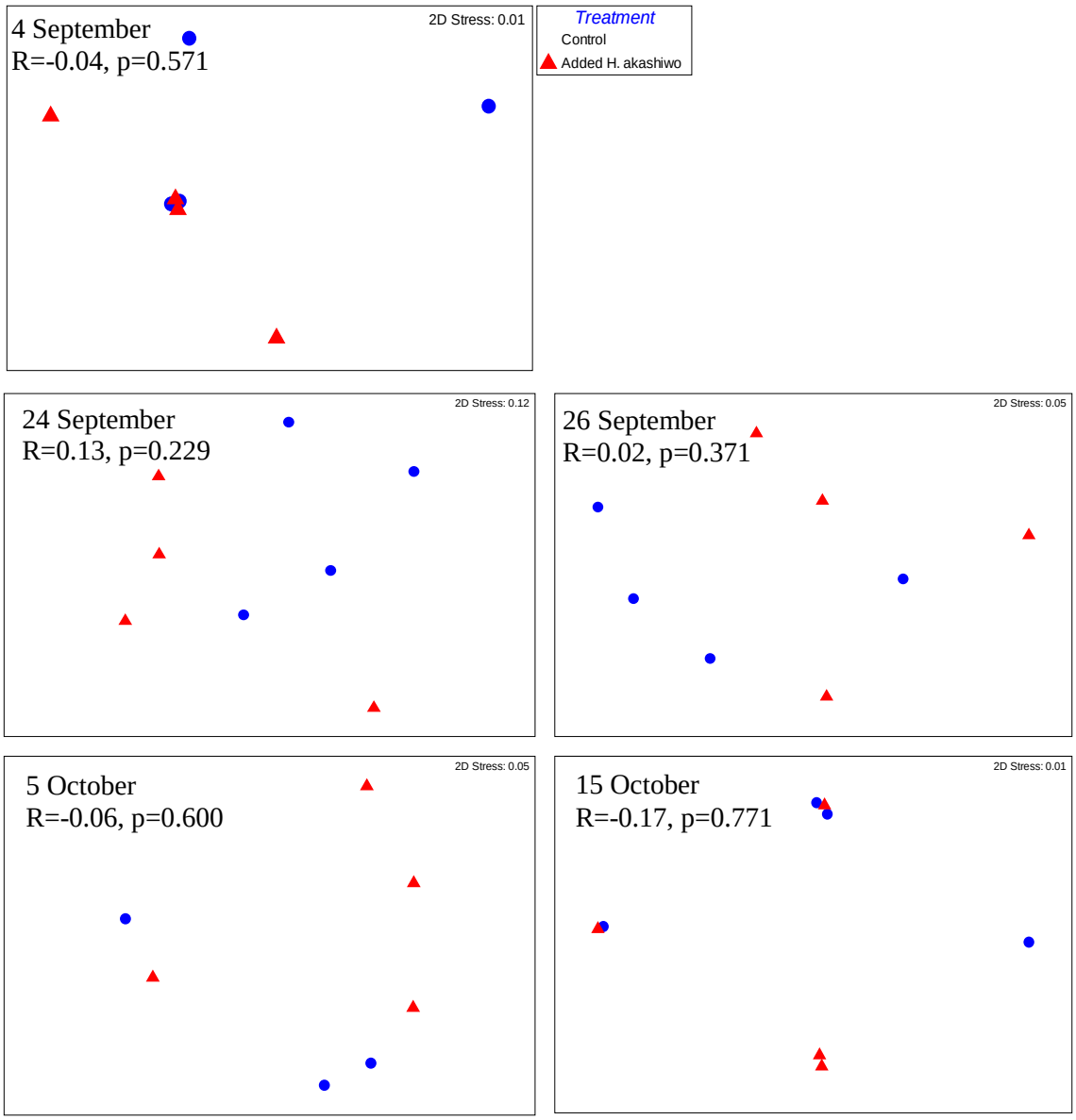

Figure 13. Multi-dimensional scaling ordination of microzooplankton abundance (cells l^{-1}) for the control (●) and added *H. akashiwo* (▲) treatments for natural planktonic community experiments conducted on 4, 24, and 26 September and 5 and 15 October.

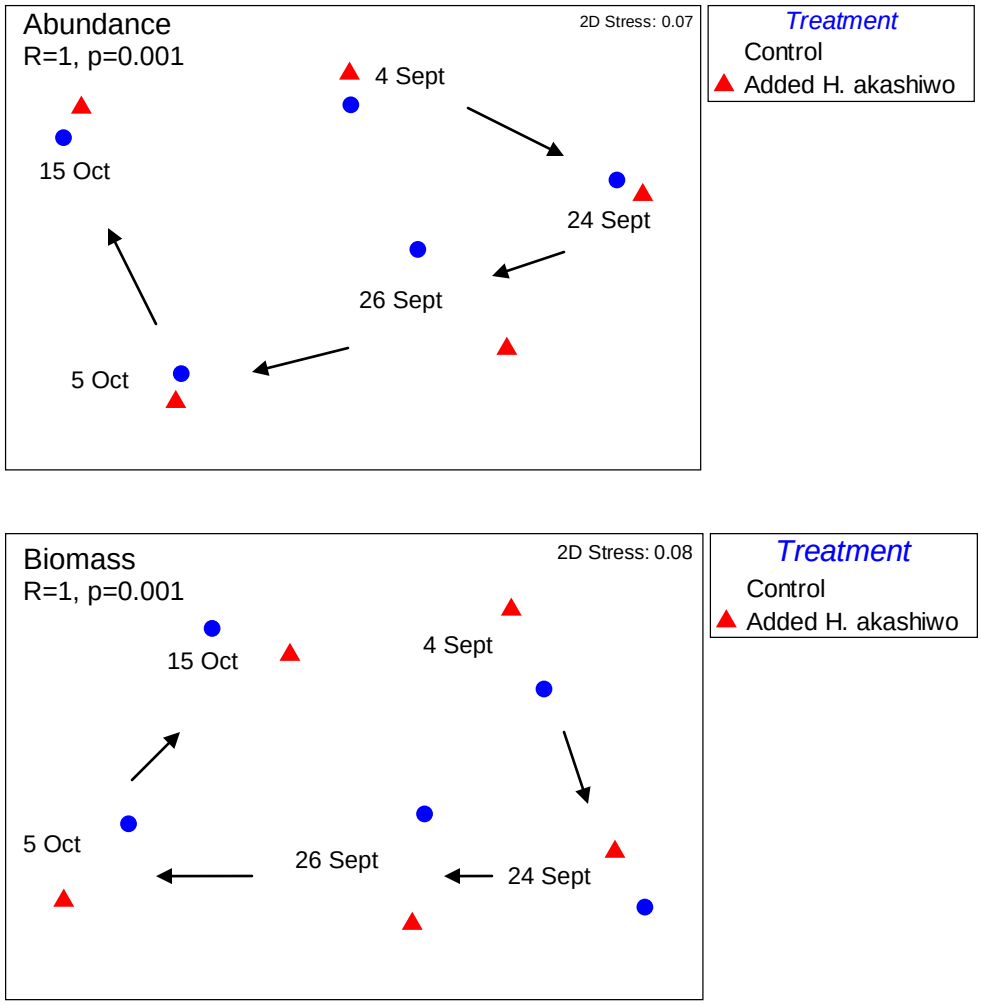

Figure 14. Multi-dimensional scaling ordination of microzooplankton abundance (cells l^{-1}) and biomass (µg C l^{-1}) averages of the control (●) and added *H. akashiwo* (▲) treatments for each experiment date.

Table 9. Average ingestion rate (*H. akashiwo* cells ingested grazer^{-1} hr^{-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 one hour. At least 100 individuals of each grazer type were quantified to calculate ingestion rate, with the exception of one replicate from 4 September (n=59). Size measurements were taken from 30 individuals of each grazer type. Standard deviation is shown in parentheses. Ingestion rates from October experiments were negligible and are not shown.

Date	Grazer type	Average length and width (µm)	Average ingestion rate (*H. akashiwo* ingested grazer^{-1} hr^{-1})
4 Sept	Aloricate ciliate	36.5 (4.9) x 28.1 (4.3)	1.10 (0.05)
24 Sept	*Gyrodinium/ Gymnodinium*	30.8 (5.8) x 15.3 (3.3)	0.60 (0.05)
26 Sept	*Gyrodinium/ Gymnodinium*	30.4 (4.9) x 17.6 (3.6)	0.61 (0.09)
26 Sept	Round dinoflagellate	31.7 (5.0) x 22.5 (3.2)	0.63 (0.07)

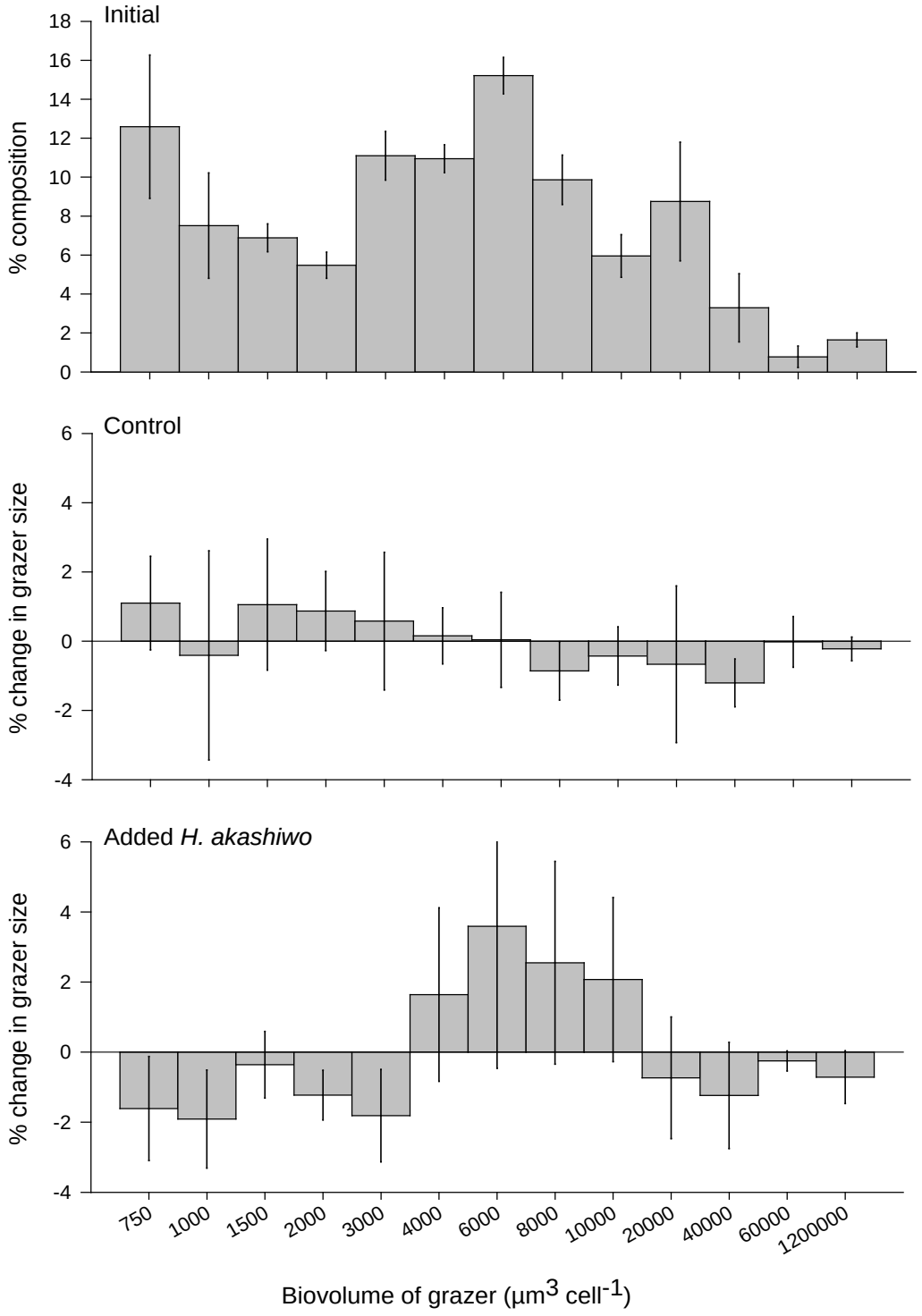

Figure 15. Percent composition of grazer sizes in the initial samples, and the percent change in grazer sizes after 24 hrs for the control and added *H. akashiwo* treatments for the experiment conducted on 24 September, 2007. Percent change in grazer sizes was calculated by subtracting the initial sample average from each of the two treatments. Error bars represent standard deviation.

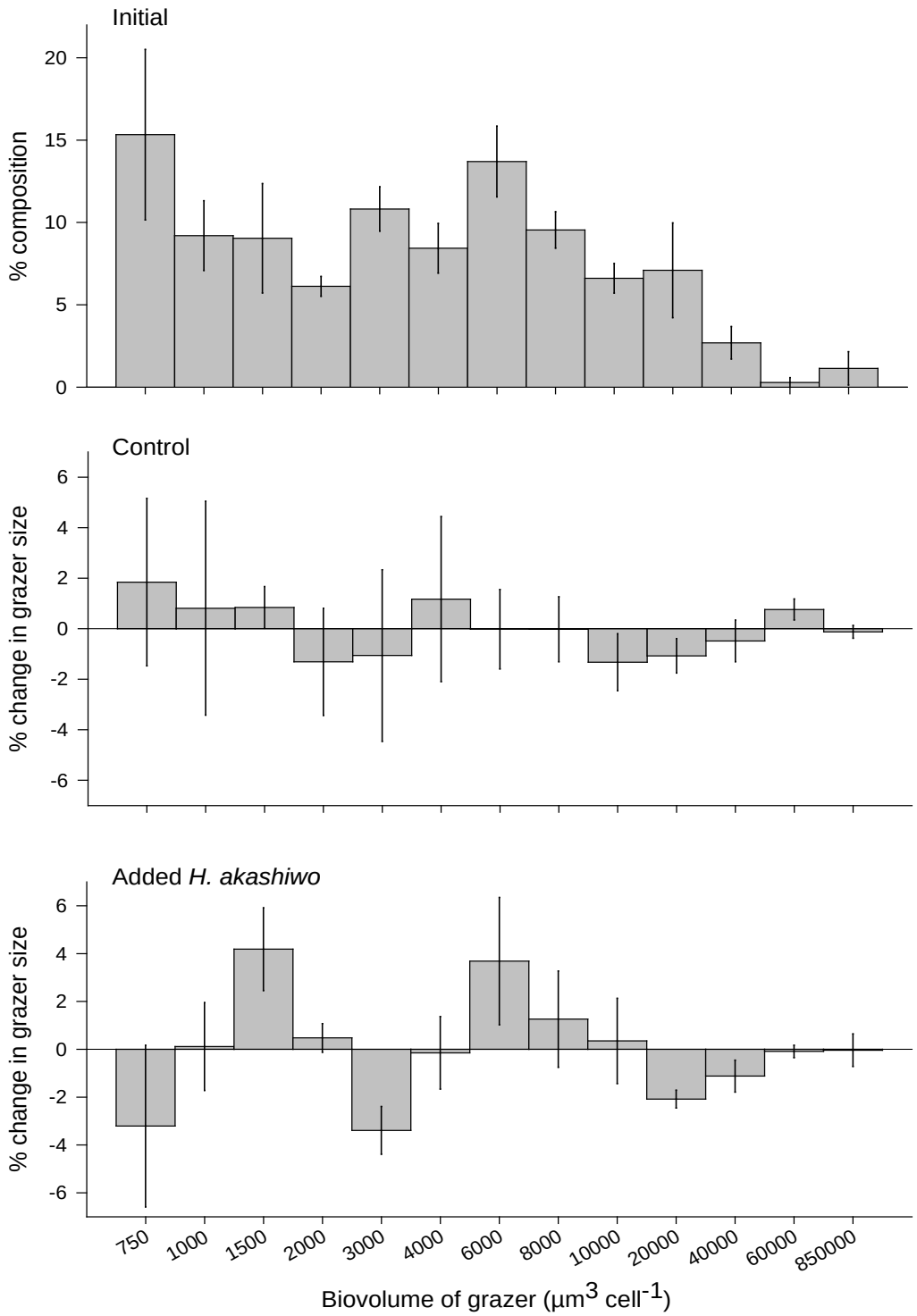

Figure 16. Percent composition of grazer sizes in the initial samples, and the percent change in grazer sizes after 24 hrs for the control and added *H. akashiwo* treatments for the experiment conducted on 26 September, 2007. Percent change in grazer sizes was calculated by subtracting the initial sample average from each of the two treatments. Error bars represent standard deviation.

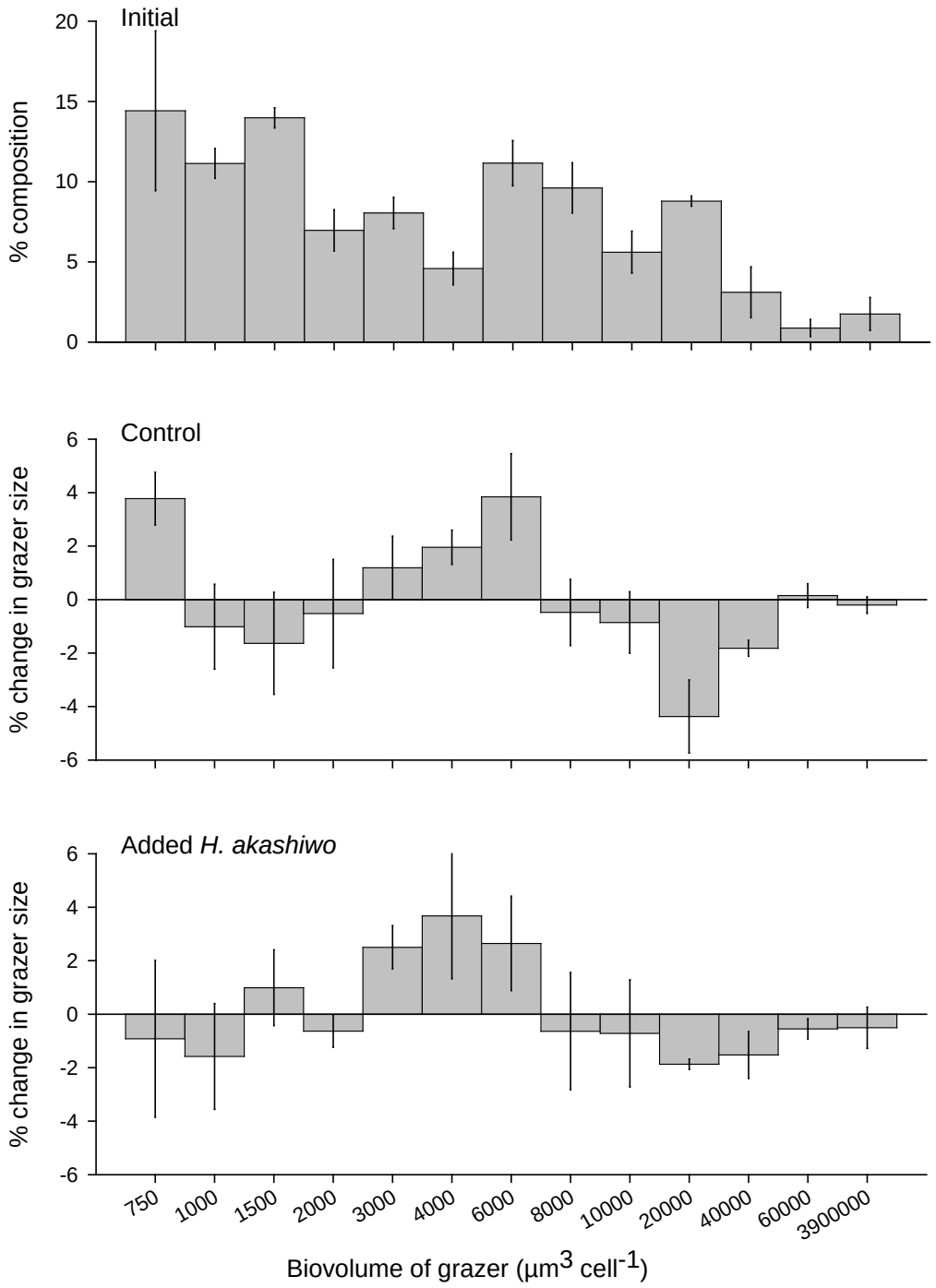

Figure 17. Percent composition of grazer sizes in the initial samples, and the percent change in grazer sizes after 24 hrs for the control and added *H. akashiwo* treatments for the experiment conducted on 5 October, 2007. Percent change in grazer sizes was calculated by subtracting the initial sample average from each of the two treatments. Error bars represent standard deviation.

Discussion

Heterosigma akashiwo was toxic to both tintinnid ciliates when it was the sole food source: *Favella* sp. and *Metacyli*s sp. both exhibited greater mortality in the 100% *Heterosigma akashiwo* treatment than in the starved control. This toxicity was not observed in treatments containing mixtures of *Heterosigma akashiwo* and beneficial prey, even at low concentrations of the beneficial alga. In mixed-prey treatments, *Favella* sp. exhibited a mortality rate similar to, but not above, that in the starved control. *Metacylis* sp. showed a trend of higher mortality in the mixed-prey treatments than in the starved control; however, the difference was not significant. In contrast to the tintinnid ciliates, *Heterosigma akashiwo* was not toxic to the oligotrich ciliate *S. acuminatum*. The growth rate of *S. acuminatum* was unaffected by the presence of *Heterosigma akashiwo*, but increased with increasing concentrations of beneficial prey, *Heterocapsa triquetra*.

Both *Favella* sp. and *S. acuminatum* ingested *Heterosigma akashiwo*; however, both ciliates selected against the alga when *Heterocapsa triquetra* was available. This suggests the ciliates were able to differentiate between the two prey species and avoided *Heterosigma akashiwo* when other prey species were available. When *Heterosigma akashiwo* was the only prey available, ingestion by *S. acuminatum* remained low, yet 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 feed more on the alga under the same conditions. It would be interesting to know if feeding rates changed during the incubation period as exposure time increased. Kamiyama and Arima (2005) also found

Favella sp. to ingest *Heterosigma akashiwo*; however, ingestion only occurred during the first 30 minutes of incubation, after which it declined to near zero. Perhaps the ingestion of *Heterosigma akashiwo* by *Favella* sp. observed in my study occurred at the beginning of incubation and declined as the grazer experienced harmful effects of the alga that led to the higher mortality rate.

The different ingestion rates between *Favella* sp. and *S. acuminatum* in the 100% *Heterosigma akashiwo* treatment may account for the difference in toxicity observed between the two ciliates. Toxicity may be partially or wholly induced through ingestion of the alga, which may explain why *Favella* sp. and not *S. acuminatum* exhibited a toxic response to the 100% *Heterosigma akashiwo* treatment. Reduced ingestion of the raphidophyte by *Favella* sp. in the mixed-prey treatments could have led to the decrease in toxicity observed in those treatments. If ingestion of *Heterosigma 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 the beneficial prey, *Heterocapsa triquetra*, in *Favella* sp. While growth rate of the grazer was not significantly inhibited in the mixed-prey treatments, ingestion of beneficial prey was hindered. The effect on feeding behavior may be a sublethal effect of *Heterosigma akashiwo*, which could have a stronger effect on grazer growth rate with a longer exposure period than that of my 24-hour experiments.

My study did not investigate the ingestion rate of *Metacylis* sp.; however, previous studies have found that it will ingest *Heterosigma akashiwo* strain CCMP452

when it is offered both alone and with a beneficial prey, *Rhodomonas* sp. (Clough and Strom 2005). In single- and mixed-prey treatments, ingestion of *Heterosigma akashiwo* was higher than that of the beneficial prey, although ingestion of *Heterosigma akashiwo* decreased when the beneficial prey was available. Strains CCMP452 and CCMP1914 were toxic to *Metacylis* sp. in both the single- and mixed-prey experiments, perhaps because of high ingestion rates. This evidence further supports the idea that, when alternative prey is present, tintinnid ciliates decrease their feeding on *Heterosigma akashiwo* and likely select against it, as observed with *Favella* sp.

Prey concentration also affects prey selection and toxicity. Colin and Dam (2002) tested the toxicity of another *Heterosigma* species, *H. carterae*, on the copepod *Acartia tonsa* in single- and mixed-prey treatments, with algal concentrations similar to those in my study. As in my study, they found reduced toxicity in the mixed-prey treatments at these low concentrations of the harmful alga. Other studies suggest the beneficial effects of alternative prey do not occur at higher concentrations of harmful algae. Clough and Strom (2005) used a *Heterosigma akashiwo* concentration of 2000 cells ml^{-1}, as compared to the concentrations in my study ranging from 152 to 607 cells ml^{-1}. This higher *Heterosigma akashiwo* concentration may explain the toxicity observed in their mixed-prey treatments, which was not seen in my study. The higher *Heterosigma akashiwo* concentration may also account for the high ingestion rates of that alga by *Metacylis* sp. Natural *Heterosigma akashiwo* blooms containing high concentrations of the alga have also caused significant decreases in tintinnid ciliate abundances, despite the presence of alternative prey species within the bloom (Kamiyama et al. 2000). Thus the

beneficial effects of alternative prey may be dependent upon low concentrations of *Heterosigma* sp.

Additional evidence shows this concentration-dependence is not universal. Hansen (1995) found the beneficial effects of alternative prey to occur in the presence of much higher concentrations of the toxic dinoflagellate *Gyrodinium aureolum*. *Favella ehrenbergii* growth remained unaffected by 1,000 to 4,000 cells ml^{-1} of *G. aureolum* when it was mixed with an equal ratio of beneficial prey. In a separate experiment in which the *G. aureolum* concentration remained at 2,000 cells ml^{-1} and the beneficial prey concentration increased from 200 to 20,000 cells ml^{-1}, growth of *F. ehrenbergii* was unaffected by the dinoflagellate until it accounted for 70% of the total prey biomass. Harmful algal species are not all equally toxic, and *G. aureolum* may be less toxic than *Heterosigma akashiwo* to *Favella* sp. Thus the effects of prey concentration on toxicity will vary depending on the algal species being used.

Considering the low *Heterosigma akashiwo* concentrations used in my study, one might conclude that the lower raphidophyte concentrations, and not the presence of beneficial 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 beneficial prey that reduced the toxicity because concentrations of the same strain of *Heterosigma akashiwo* as low as 100 cells ml^{-1} have caused toxicity in this grazer (Strom and Fredrickson, unpublished data). This algal concentration is below that of the 25% *Heterosigma akashiwo* treatment in my experiment of 152 cells ml^{-1}, thus suggesting the decrease in toxicity in the mixed-prey treatments is due to the added beneficial algae and not to a reduced abundance of *Heterosigma akashiwo*.

Tintinnid ciliates are not uniformly susceptible to toxicity by harmful algal species. Blooms of the harmful dinoflagellate *Heterocapsa circularisquama* have even led to increased growth and grazing in *Favella* sp. (Kamiyama and Matsuyama 2005). This ciliate has also grown within blooms of *Gonyaulax tamarensis*, grazing on the dinoflagellate at a rate of 0.25 d^{-1} (Watras et al. 1985).

Strombidinopsis spp. also show varying responses to other harmful algal species. *Strombidinopsis* sp. exhibited increased mortality with exposure to the alga *Prymnesium parvum* at concentrations of 5,000 to 30,000 cells ml^{-1} (Rosetta and McManus 2003). Also, concentrations of the dinoflagellate *Lucialla masanensis* similar to those used in my study were toxic to *S. jeokjo*. Conversely, *S. jeokjo* grew on similar concentrations of the dinoflagellates *Pfiesteria piscicida* and *Stoeckeria algicida* at rates of 1.61 and 1.77 d^{-1}, respectively, and readily ingested the algal cells at rates of 43 and 49 ng C $grazer^{-1}$ d^{-1}, respectively (Jeong et al. 2007). This ciliate also grew on and ingested the dinoflagellate *Cochlodinium polykrikoides* at rates of 0.85 d^{-1} and 116 ng C $grazer^{-1}$ d^{-1}, respectively (Jeong et al. 2008).

The effects of harmful algal species on microzooplankton grazers can vary with the species of both grazer and harmful algae. 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. The results of my study, along with those of previous research, support the idea that algal toxicity can be reduced or eliminated with the presence of alternative prey. Furthermore, alternative prey species can vary in the degree to which they reduce the toxic effects of harmful algae (Rosetta and McManus

2003). Therefore, the results of mixed-prey experiments will differ according to the specific grazer, beneficial prey, and harmful algal species being used.

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 and biomass of the major microzooplankton groups, which provided a variety of communities in which to test my hypothesis. The change in community structure observed among the sampling dates was likely partially due to the observed shifts in hydrography over time. The CTD depth profiles show a distinct shift in temperature and salinity between the two months; with warmer, less saline conditions in September and cooler, more saline conditions in October. This shift in hydrographic conditions is 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.

Overall community structure was not significantly affected by *H. akashiwo* on any of the sampling dates, despite the higher concentrations of 2,850 to 6,690 cells ml^{-1} *H. akashiwo* in these experiments as compared to those with grazer cultures. Nevertheless, ingestion of the alga by certain microzooplankton species and slight changes in grazer size structure were observed.

Previous examinations of natural *H. akashiwo* blooms have detected significant changes within the microzooplankton community. Kamiyama et al. (2000) found a large decrease in tintinnid ciliates during a bloom period. Tintinnid ciliates constituted a

negligible proportion of microzooplankton abundance for each of the sampling dates in my study, and thus a decrease in this population was not detectable. Alternatively, Kamiyama et al. (2000) observed 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 ng C $grazer^{-1}$ d^{-1}, respectively. The latter two 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 *H. akashiwo*; however, an aloricate ciliate, a *Gyrodinium/Gymnodinium* dinoflagellate and a round dinoflagellate were observed to measurably ingest the alga. These three 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* only ingested the alga on one 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 if 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 2001, 2002), although preliminary experiments within my study showed 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 *H. akashiwo* to be much higher than that observed in this study. Microzooplankton grazing on *H. akashiwo* during three 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 my study were within the range observed by Demir et al.; however, higher temperatures and light levels in the Delaware blooms may be 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 and Tester 1997).

Examinations of natural blooms differ from my 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, my study observed the effects of *H. akashiwo* on a naïve community within 24 hours of exposure. Perhaps this incubation time was insufficient for acclimation of the grazer community. Had the incubation time been longer, higher grazing rates and more significant changes to the community may have been observed.

The high variability of natural planktonic ecosystems made it difficult to detect clear patterns between the experimental treatments of this study. A higher treatment effect may be obtained by using a higher, yet still ecologically relevant, concentration of harmful algae.

In conclusion, bloom-level concentrations of *H. akashiwo* did not significantly change microzooplankton community structure. A slight shift in grazer sizes suggests blooms may be harmful to very small microzooplankton and benefit larger *Gyrodinium/Gymnodinium* dinoflagellates which can ingest and grow on the alga. Two other microzooplankton species also readily ingested *H. akashiwo*; however, the alga was

not consumed by the majority of grazers. The presence of additional prey species within the natural communities may have reduced the potentially toxic effects of *H. akashiwo* and offered alternative feeding opportunities, as it did in the experiments with laboratory cultures. Future research on the effects of harmful algal bloom species should include exposures to the harmful alga in prey mixtures. This provides a more complete understanding of potential microzooplankton responses to harmful algae, as negative effects to microzooplankton growth and grazing can be reduced by the presence of alternative prey. Furthermore, experiments involving mixed-prey assemblages provide a more ecologically relevant examination of the impacts of harmful algal blooms.

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