Effects of *Heterosigma akashiwo* (Raphidophyceae) on protist grazers: laboratory experiments with ciliates and heterotrophic dinoflagellates

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ABSTRACT: *Heterosigma akashiwo* is a fish-killing raphidophyte capable of forming dense blooms. Since microzooplankton consume a large fraction of coastal phytoplankton production and readily ingest cells in the *H. akashiwo* size range, the formation of such blooms indicates that grazing has been inhibited. Growth rates of 7 cultured protist grazer species were measured in the presence of 2 *H. akashiwo* strains. Both strains were toxic to the 3 largest ciliates tested; all experienced higher mortality in the presence of *H. akashiwo* than when starved. Addition of *Rhodomonas* sp., a high-quality prey species for many protists, sometimes ameliorated *H. akashiwo* toxicity but in other instances did not. Two smaller ciliate species and 2 heterotrophic dinoflagellates were either unaffected by *H. akashiwo* or were able to survive and grow on it. For grazers susceptible to the toxicity, higher *H. akashiwo* cell concentrations and older *H. akashiwo* cultures were both associated with higher mortality. No toxicity was associated with filtrate from *H. akashiwo* culture or from the presence of *H. akashiwo* cells that were not ingested, so the toxic effect of this raphidophyte to large ciliates seems to depend on ingestion of the cells. Mortality of large ciliates, often a major component of coastal microzooplankton communities, is likely an important element of *H. akashiwo*'s capability to form persistent blooms.

KEY WORDS: Microzooplankton · Growth · Ingestion · Toxicity · Raphidophyte

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INTRODUCTION

Heterosigma akashiwo is a bloom-forming raphidophyte species, typically rare in the plankton but capable of forming dense, persistent aggregations. *H. akashiwo* is distributed globally in euryhaline, coastal environments, and high cell concentrations are often associated with low-salinity sea surface layers (Honjo 1993, Taylor & Haigh 1993, Hershberger et al. 1997). Blooms of *H. akashiwo* (formerly referred to as *Olisthodiscus luteus* and *Heterosigma carterae*; Hara & Chihara 1987, Throndsen 1996) have caused massive deaths of penned fish in numerous Pacific Rim countries, including the United States, Chile, Japan, Korea and New Zealand (Honjo 1993, Horner et al. 1997, Khan et al. 1997, and references therein). The mechanism of ichthyotoxicity is not well understood (see below), although toxicity level has been related to environmental conditions (salinity, irradiance, temperature) and growth stage (Ono et al. 2000, Haque & Onoue 2002).

Blooms of *Heterosigma akashiwo*, or indeed of any phytoplankton species, indicate that removal of phytoplankton by grazers is reduced relative to growth and accumulation rates of phytoplankton cells (Strom 2002). This is an unusual situation in the marine plankton. Rates of zooplankton grazing are often a substantial fraction of phytoplankton growth rates, even in coastal waters, with most of this grazing attributable to microzooplankton (Calbet & Landry 2004). *H. akashiwo*, in particular, has the potential to be a good food for protists and other microzooplankton because phytoplankton similar in size and morphology to *H. akashiwo* are eaten readily by numer-

ous microzooplankton grazers. Reduced population growth rates or mortality of protist grazers in the presence of *H. akashiwo* could partially explain the formation and persistence of *H. akashiwo* blooms. Although there have been several previous studies of *H. akashiwo* effects on heterotrophic protists (Verity & Stoecker 1982, Nakamura 1998, Kamiyama & Arima 2001, Jeong et al. 2002, 2003), none have surveyed a range of taxa, and only one (Verity & Stoecker 1982) was designed so that *H. akashiwo* toxicity could be distinguished from nutritional insufficiency. We studied the effects of *H. akashiwo* on the growth of heterotrophic dinoflagellates and both tintinnid and aloricate ciliates. The species examined are common and seasonally abundant members of temperate coastal plankton communities.

There is no general agreement as to the mode of *Heterosigma akashiwo*'s fish toxicity. Much research has focused on production of reactive oxygen species (ROS) and their role in damaging gill tissue (Yang et al. 1995, Oda et al. 1997, Twiner & Trick 2000). A second hypothesis is that mucus, produced by either the alga or the fish, clogs the gills and leads to asphyxiation (Nakamura et al. 1998, Oda et al. 1998). Production of brevetoxin-like neurotoxins has been reported for several strains (Khan et al. 1997, Ono et al. 2000, Haque & Onoue 2002), and there is evidence that an association with bacteria is required for toxicity (Carrasquero-Verde 1999). Recently Twiner et al. (2004) investigated the effect of *H. akashiwo*-derived extracellular organics on cell metabolism, while Marshall et al. (2003) demonstrated a synergism between ROS and free fatty acids in the ichthyotoxicity of the closely related *Chattonella marina*. In addition to ichthyotoxicity, *H. akashiwo* is thought to have allelopathic effects on certain other phytoplankton species (Pratt 1966). *H. akashiwo* may well have multiple modes of toxicity that affect different types of aquatic organisms in different ways. At present, however, there is no accepted chemical measure of toxin content in this species.

Our approach to assessing *Heterosigma akashiwo* toxicity was to use the growth response of potential protist grazers as a measure of the toxic effect. The use of biological responses of marine organisms to assay for toxicity has a long history. Here the approach not only reveals any toxic effect regardless of chemical mechanism, but by employing representative members of an important group of phytoplankton consumers, does so in a way that is relevant to understanding *H. akashiwo* bloom dynamics. In designing these experiments, we wished to distinguish between toxicity and nutritional insufficiency. An algal prey species may fail to support the growth of consumers if essential dietary biochemicals are absent; this is distinct from, and has different ecological consequences than, nega-

tive effects of a deleterious compound (Jonasdottir et al. 1998, Colin & Dam 2002).

Our study addressed the question of toxicity versus nutritional insufficiency in 2 ways: (1) In treatments where *Heterosigma akashiwo* was offered alone, toxicity was defined as a growth rate significantly lower than that in starved controls. Since the energy expended by protists during prey capture is considered incidental to their total energy budget (Fenchel 1987), even a nutritionally insufficient food should not reduce the growth rate further than starvation unless toxicity plays a role. (2) We included treatments in which *H. akashiwo* was mixed with *Rhodomonas* sp., a high-quality prey species for many protist grazers. If *H. akashiwo* were nutritionally insufficient, its presence in the mixture might reduce growth relative to the *Rhodomonas*-only control: grazers would eat less of the high-quality prey species because they would also be consuming the nutritionally insufficient *H. akashiwo*. Only if *H. akashiwo* is toxic, however, would the mixed diet lead to protist growth rates lower than those in the starved control. Inclusion of the mixed diet treatment also allowed us to look for a possible amelioration of *H. akashiwo* toxicity by *Rhodomonas* sp.

We found reductions in growth rate consistent with toxicity for at least 3 of the protist grazers studied. This prompted additional investigation to explore the mechanism and consequences of *Heterosigma akashiwo* toxicity. Additional questions addressed were: (1) Is toxicity dependent upon *H. akashiwo* concentration? (2) Does *H. akashiwo* produce a water-soluble toxin that inhibits protist growth? (3) Does toxicity vary with *H. akashiwo* culture age? (4) Does the presence of *H. akashiwo* reduce the rate at which protist grazers ingest other prey?

MATERIALS AND METHODS

Cultures. Two non-axenic cultures of *Heterosigma akashiwo* were obtained from the Provasoli-Guillard National Center for the Culture of Marine Phytoplankton (CCMP, Boothbay Harbor, Maine, USA). One culture, CCMP1914 (strain synonyms: NWFSC-504, UWC-10), was isolated from Guemes Channel, Washington, USA (48.315° N, 122.390° W), the approximate location of most of our protist grazer isolates. The other culture, CCMP452 (strain synonym: Olisth), was isolated from Long Island Sound on the US Atlantic coast (approximately 41.0° N, 73.0° W) and has been used previously in similar experiments (Verity & Stoecker 1982), when it was known as *Olisthodiscus luteus*. Growth rates of both strains were similar (data not shown). A third algal culture, *Rhodomonas* sp., was chosen for use as a control and in mixed diets because

it is generally a high-quality food for protists, and because its orange phycoerythrin fluorescence allows it to be distinguished from *H. akashiwo* under epifluorescence microscopy. Algae were maintained in 30 psu f/2 medium without added silicate (Guillard & Ryther 1962), incubated at 15°C and 100 µmol photons $m^{-2} s^{-1}$ (13:11 h light:dark cycle), and transferred to fresh medium every 7 to 10 d to maintain exponential growth. We isolated all protist grazers from waters of northern Puget Sound, USA (48.5° N, 122.7° W), determined appropriate mixed-algal diets in accordance with Strom & Morello (1998), and maintained them in culture at Shannon Point Marine Center, Washington, USA. Grazers were cultured in a trace metal-enriched 30 psu seawater medium (hereafter 'ciliate medium') in accordance with Gifford (1985) at 12°C in dim light (ca. 2 µmol photons $m^{-2} s^{-1}$).

Samples of algae and pre-starved protists (see below) were preserved in acid Lugol's solution (10% final concentration) and measured using inverted microscopy. For all but 2 species, maximum lengths and widths of 20 individuals were obtained from digital images (CoolSnap CF camera) and Optimas 6.2.1 image-analysis software. *Strombidium* sp. SPMC68 and *Eutintinnus* sp. were both lost from culture before measurements were taken. Six preserved *Strombidium* sp. remaining from a prior experiment were measured as described above. For *Eutintinnus* sp., a photograph of known magnification was used to estimate its size.

Effect of *Heterosigma akashiwo* on protist growth rate. Seven species of protist grazers (Table 1) were tested for their population growth response to the 2 strains of *Heterosigma akashiwo.* Algal prey were grown in batch culture, then sampled during mid-exponential phase and counted using a haemocyto-

meter. Protist grazers were starved prior to use. Tintinnids were poured through a 10 or 20 µm plastic screen (80 µm for *Noctiluca scintillans*), then resuspended in prey-free ciliate medium. Most of the medium surrounding aloricate choreotrichs was siphoned off through a 10 µm screen (reverse concentration) and replaced with prey-free medium. Ciliates were then incubated for 24 h to allow any remaining ingested food to be digested. *Amphidinium longum* was too small to be separated physically from its prey, so it was simply not fed for 6 d, during which time most of the prey cells disappeared.

Grazer density was estimated by counting live cells in replicate droplets of known volume under a dissecting microscope; grazers were then added to triplicate polycarbonate bottles (incubation volumes 50 or 100 ml, depending on grazer species). Six diet treatments were used: (1) starved (= ciliate medium only); (2) *Rhodomonas* sp.; (3) *Heterosigma akashiwo* CCMP452; (4) *H. akashiwo* CCMP1914; (5) *Rhodomonas* sp. + *H. akashiwo* CCMP452, or (6) *Rhodomonas* sp. + *H. akashiwo* CCMP1914. *H. akashiwo* was always supplied at 2×10^3 cells ml^{-1}, and *Rhodomonas* sp. at 1×10^4 cells ml^{-1} (Table 2). The former species is larger than the latter (*H. akashiwo* CCMP452: 16.1 × 11.6 µm; *H. akashiwo* CCMP1914: 14.8 × 9.7 µm; *Rhodomonas* sp.: 10.1 × 5.5 µm), so the 1:5 *H. akashiwo*:*Rhodomonas* sp. ratio by cell number gave approximately equal concentrations by prey volume of each prey type. A concentration of 1×10^4 cells ml^{-1} *Rhodomonas* sp. was previously shown to be well above the concentration at which protist growth rates saturate (Zirbel 2001). Further, 2×10^3 cells ml^{-1} *H. akashiwo* was chosen as a reasonable simulation of the concentration grazers might experience in a bloom, given field observations of 10^3 to 10^4 cells ml^{-1} at bloom peaks (Taylor & Haigh 1993, Kamiyama et al. 2000). In this and all subsequent experiments, the bottles were incubated at 12°C in the dark, unmixed for 2 to 3 d, with 10 ml samples taken daily and fixed with acid Lugol's solution (10% final concentration). Each preserved sample was transferred to a 10 ml settling chamber and the protist grazers enumerated using inverted microscopy (transmitted light illumination). Growth rates (µ, doublings d^{-1}) were then calculated using the formula:

$$\mu = (\ln T_t - \ln T_0)/[t \times \ln(2)]$$

where T_t = protist abundance at time t, and T_0 = protist abundance at $t = 0$.

Table 1. Cell dimensions (µm) of protist grazers (±1 SD) used in experiments with *Heterosigma akashiwo*. Lorica dimensions are given in parentheses for tintinnids. n = 20 for all size determinations except *Eutintinnus* sp. and *Strombidium* sp. SPMC68 (see text above)

Taxon	Genus and species	Strain ID	Length	Width
Tintinnid	*Coxliella* sp.	SPMC82	44 ± 8 (61 ± 12)	27 ± 3 (40 ± 4)
	Metacylis sp.	SPMC84	34 ± 7 (42 ± 12)	20 ± 2 (28 ± 2)
	Eutintinnus sp.	SPMC80	38 (125)	15 (20)
Aloricate choreotrich	*Strombidium* sp. (chloroplast-retaining)	SPMC68	57 ± 7	41 ± 5
	Strombidium sp.	SPMC92	40 ± 2	20 ± 3
Dinoflagellate	*Amphidinium longum*	SPMC98	18 ± 2	9 ± 1
	Noctiluca scintillans	SPMC74	296 ± 54	271 ± 48

Table 2. Summary of experimental treatments and initial algal concentrations (cells ml^{-1}). R.sp. = *Rhodomonas* sp.; H.a. = *Heterosigma akashiwo*; n = number of replicates per treatment; nd = not determined. Both *H. akashiwo* strains were from the Provasoli Guillard National Center for the Collection of Marine Phytoplankton (CCMP). Initial protist grazer concentrations (cells ml^{-1}) shown in parentheses after grazer species names

Expt	Grazer species	Treatment	Initial algal concentration		n
			R. sp	H. a	
Growth	*Coxliella* sp. (8.6), *Metacylis* sp. (16.2), *Strombidium* sp. SPMC68 (5.8), *Eutintinnus* sp. (47.0), *Amphidinium longum* (196), *Strombidium* sp. SPMC92 (10.8), *Noctiluca scintillans* (0.8)	(i) Starved (ii) R.sp. (iii) H.a. CCMP452 (iv) H.a. CCMP1914 (v) R.sp. + H.a. CCMP452 (vi) R.sp. + H.a. CCMP1914	0 1×10^4 0 0 1×10^4 1×10^4	0 0 2×10^3 2×10^3 2×10^3 2×10^3	3
H.a. concentration effect	*Coxliella* sp. (8.0), *Metacylis* sp. (9.0)	(i) Starved (ii) R.sp. (iii) R.sp. + H.a. CCMP452 (iv) R.sp. + H.a. CCMP452 (v) R.sp. + H.a. CCMP452	0 1×10^4 1×10^4 1×10^4 1×10^4	0 0 2×10^3 4×10^3 6×10^3	2 or 3
H.a. filtrate effect	*Metacylis* sp. (8.8)	(i) Starved (ii) R.sp. (iii) R.sp. filtrate (iv) R.sp. filtrate + R.sp. (v) H.a. CCMP452 filtrate (vi) H.a. CCMP452 filtrate + R.sp.	0 1×10^4 0 1×10^4 0 1×10^4	0 0 0 0 0 0	3
'Age of culture' effect	*Coxliella* sp. (10.0)	(i) Starved (ii) H.a. CCMP452 cultures of 7 different ages: H.a. only (iii) H.a. CCMP452 cultures of 7 different ages: H.a. + R.sp.	0 0 1×10^4	0 2×10^3 2×10^3	2
H.a. effect on ingestion of R.sp.	*Metacylis* sp. (18.0), *Amphidinium longum* (nd)	(i) R.sp. (ii) H.a. CCMP452 (iii) R.sp. + H.a. CCMP452	1×10^4 0 1×10^4	0 2×10^3 2×10^3	3

We did not measure feeding rates during growth experiments, but did examine grazers to see whether they had ingested *Heterosigma akashiwo*. To this end, additional 10 ml samples were taken before the incubation period began and again 0.5 to 24 h later, depending on the presumed grazing rate of the grazer being tested. These samples were preserved with 10% glutaraldehyde (0.5% final concentration) and stained with 4,6-diamidino-2-phenylindole (DAPI). The samples were refrigerated in the dark for 24 h, then filtered in their entirety onto 25 mm diameter polycarbonate membranes with pore size 0.8 to 8.0 μm, depending on grazer size. The filters were mounted on slides with immersion oil and frozen at –20°C until they could be observed with epifluorescent microscopy. Under blue light excitation, *H. akashiwo* cells fluoresce red, while *Rhodomonas* sp. cells fluoresce orange. A minimum of 100 cells was examined per slide or, if fewer than 100 cells were present, all cells were examined. A grazer population was considered to have ingested *H. akashiwo* if at least 5 grazers were observed to contain at least 1 *H. akashiwo* cell each on at least 1 slide. Because *Amphidinium longum* feeds by myzocytosis, intact cells are not ingested. For this grazer species we

looked for red-fluorescing food vacuoles. The large *Strombidium* sp. retained chloroplasts from ingested prey, masking the fluorescence of ingested prey cells in food vacuoles. For this species, we confirmed feeding on *H. akashiwo* by making behavioral observations under a dissecting microscope.

The experiment with *Noctiluca scintillans* required modification because *N. scintillans* grows more slowly than the other grazers, is more resistant to starvation, and achieves lower densities in culture. For this experiment, the pre-experiment starvation period was 7 d and the subsequent growth experiment lasted 22 d. Algal prey were counted using a Palmer-Maloney chamber on Days 3, 7, and 15, and algae were added as needed to restore initial concentrations (Table 2). Total incubation volume was 300 ml; samples of 50 ml each were taken on Days 0, 3, 7, 10, 15 and 22. Each sample was sieved through a 40 μm screen and back-rinsed into approximately 10 ml seawater, then preserved with acid Lugol's solution (10% final concentration). Inverted microscope counts and growth rate calculations were performed as described above.

***Heterosigma akashiwo* concentration gradient effect on tintinnid abundance.** Two experiments were

conducted to determine whether varying the concentration of *Heterosigma akashiwo* in mixed-algal treatments affected ciliate abundance. The tintinnids *Coxliella* sp. and *Metacylis* sp. were tested because, in growth rate experiments, both showed a marked decrease in growth rate in the presence of *H. akashiwo*. Preparations for the experiments (growth of stock cultures, pre-starvation of tintinnids) were as described above. In all treatments, the concentration of *Rhodomonas* sp. was 1×10^4 cells ml^{-1} and *H. akashiwo* strain CCMP452 was used. In the *Coxliella* sp. experiment, approximately 8 grazers ml^{-1} (final concentration) were added to duplicate bottles containing the following treatments: (1) starved (= ciliate medium only); (2) *Rhodomonas* sp.; (3) *Rhodomonas* sp. + 2×10^3 cells ml^{-1} *H. akashiwo*, (4) *Rhodomonas* sp. + 4×10^3 cells ml^{-1} *H. akashiwo*, and (5) *Rhodomonas* sp. + 6×10^3 cells ml^{-1} *H. akashiwo*. Incubation volume in the experimental bottles was 30 ml. For the *Metacylis* sp. experiment, the procedure was the same except that bottles were run in triplicate, initial grazer concentration was 9 grazers ml^{-1}, incubation volume was 100 ml, and the *H. akashiwo* addition levels were 1×10^2, 5×10^2, 1×10^3 and 2×10^3 cells ml^{-1} (Table 2). Feeding was not examined in these experiments.

Effect of *Heterosigma akashiwo* filtrate on *Metacylis* sp. abundance. An experiment was performed to determine whether *Heterosigma akashiwo* toxicity was due to dissolved substances exuded by the algae. Cell-free filtrate from *Rhodomonas* sp. and *H. akashiwo* CCMP452 was prepared by filtering approximately 100 ml of dense algal culture (initial concentrations of 8.23×10^5 cells ml^{-1} *Rhodomonas* sp., 1.84×10^5 cells ml^{-1} *H. akashiwo*) through a 5 μm pore-size polycarbonate filter overlain on a 0.7 μm effective pore-size glass fiber filter using gentle (<125 mm Hg) vacuum pressure. The filtrates were then used to set up triplicate bottles of the following treatments: (1) starved (= ciliate medium only); (2) *Rhodomonas* sp.; (3) 10% (final volume) *Rhodomonas* sp. filtrate (equivalent to 8.23×10^4 cells ml^{-1}); (4) 10% *Rhodomonas* sp. filtrate + *Rhodomonas* sp; (5) 10% *H. akashiwo* filtrate (equivalent to 1.84×10^4 cells ml^{-1}); and (6) 10% *H. akashiwo* filtrate + *Rhodomonas* sp. Initial *Rhodomonas* sp. concentrations were always 1×10^4 cells ml^{-1}, while initial *Metacylis* sp. concentration was 9 cells ml^{-1} in an incubation volume of 100 ml (Table 2).

'Age of culture' effect on *Coxliella* sp. growth rate. Observations made during our study indicated that the age of the *Heterosigma akashiwo* culture used to supply prey cells might affect ciliate growth rate. To test this hypothesis, we generated a series of duplicate *H. akashiwo* CCMP452 cultures of varying ages (hereafter 'experimental cultures') to use in a *Coxliella* sp. growth experiment. Two different 'parent cultures'

were started 6 d before the first experimental cultures were initiated. These parent cultures were used alternately to inoculate the experimental series. The parent cultures were diluted to approximately 50% after each inoculation to maintain them in exponential phase with a density of approximately 1×10^5 cells ml^{-1} at the time of inoculation. Experimental cultures were initiated at densities of approximately 2×10^4 cells ml^{-1}, representing early exponential phase. In total, 2 cultures were started on each of 7 dates between November 27, 2000, and December 18, 2000. These duplicate cultures were the basis for replication in the *Coxliella* sp. growth experiment. The growth experiment was initiated 1 d after the last inoculation. First, a FlowCAM (Fluid Imaging Technologies) was used to determine cell density in each *H. akashiwo* culture (range: 2.99×10^4 to 2.61×10^5 cells ml^{-1}). Second, 2 experimental bottles containing *Coxliella* sp. were established for each of the 14 *H. akashiwo* cultures in the age series, as well as 2 control bottles that would not have *H. akashiwo* added, for a total of 30 bottles. Half of these bottles also contained 1×10^4 cells ml^{-1} *Rhodomonas* sp. *H. akashiwo* cells were added to the experimental bottles at a density of 2×10^3 cells ml^{-1} (Table 2). Incubation volume was 75 ml.

Effect of *Heterosigma akashiwo* on ingestion of *Rhodomonas* sp. Experiments were conducted with *Metacylis* sp. and *Amphidinium longum* to determine whether the presence of *Heterosigma akashiwo* altered their ingestion of *Rhodomonas* sp. *Metacylis* sp. and *A. longum* were chosen as experimental subjects because they differed in their growth response to *H. akashiwo* and because they have different prey ingestion strategies (phagocytosis vs. myzocytosis, respectively). For each experiment, triplicate bottles were set up containing the treatments: (1) *Rhodomonas* sp.; (2) *H. akashiwo* CCMP452; and (3) *Rhodomonas* sp.+ *H. akashiwo* (Table 2). Initial *Rhodomonas* sp. and *H. akashiwo* concentrations were 1×10^4 and 2×10^3 cells ml^{-1}, respectively, and incubation volume was 60 ml. Because our previous observations indicated that *A. longum* does not ingest *H. akashiwo*, the *H. akashiwo*-only treatment was omitted for that experiment. For *Metacylis* sp., 10 ml samples were taken at the start of the experiment and 1.5 and 4.5 h after the grazers were added. The *A. longum* experiment was sampled 0, 10, 20, and 30 min after grazer addition. Grazers were fixed in glutaraldehyde and examined using epifluorescent microscopy. For *Metacylis* sp., the number of ingested *Rhodomonas* sp. and/or *H. akashiwo* cells were counted for approximately 100 ciliates on each slide. For *A. longum*, ingestion of individual prey cells could not be quantified. Instead, approximately 200 dinoflagellates per slide were scored as to whether or not they contained any ingested *Rhodomonas* sp.

For both experiments, the percentage of grazers with ingested food was calculated for each bottle and time point. For the *Metacylis* sp. experiment, numbers of ingested prey were converted to pg C using measured C content values of 27.7 pg cell^{-1} for *Rhodomonas* sp. and 218.3 pg cell^{-1} for *Heterosigma akashiwo* CCMP452 (M. J. Zirbel & S. Strom unpubl. data).

Statistical analyses. *Effect of Heterosigma akashiwo on protist growth rate:* Grazer abundance data were analyzed using ANOVAR (α = 0.05) (SPSS 10.0). Where the assumption of sphericity was violated, the Huynh-Feldt epsilon was used to adjust the degrees of freedom. A sequential Bonferroni correction (Peres-Nato 1999) was applied to the table of ANOVAR p-values, with the resulting corrected alpha = 0.05. When significant differences between treatments were indicated, *a priori* contrasts were used to make specific comparisons. The contrasts were: (1) starved control vs. *Heterosigma akashiwo* treatments; (2) *Rhodomonas* sp. vs. *Rhodomonas* sp. + *H. akashiwo* treatments; (3) *H. akashiwo* CCMP452 vs. *H. akashiwo* CCMP1914; (4) *Rhodomonas* sp. + *H. akashiwo* CCMP452 vs. *Rhodomonas* sp. + *H. akashiwo* CCMP1914; and (5) *H. akashiwo* treatments vs. *Rhodomonas* sp. + *H. akashiwo* treatments.

Heterosigma akashiwo concentration gradient effect on tintinnid abundance: Abundance data were analyzed as described above, except that significant ANOVAR results were followed by Helmert contrasts to compare each concentration of *H. akashiwo* to the higher concentrations.

Effect of Heterosigma akashiwo filtrate on Metacylis sp. abundance: Abundance data for *Rhodomonas* sp.-fed and unfed treatments were grouped and analyzed separately using ANOVAR (α = 0.05).

'Age of culture' effect on Coxliella growth rate: Growth rate data were examined using ANOVAR.

Effect of Heterosigma akashiwo on ingestion of Rhodomonas sp.: The percentage of grazers containing ingested food was calculated for each day, and the results were compared using ANOVAR (α = 0.05). For the experiment with *Metacylis* sp., Scheffé's post-hoc test was used to determine homogeneous subsets. Also for the *Metacylis* sp. experiment, pg of ingested *Rhodomonas* sp. carbon for each treatment were compared at the 1.5 and 4.5 h time points using ANOVA (α = 0.05).

RESULTS

Effect of *Heterosigma akashiwo* on protist growth rate

All protist grazers except the heterotrophic dinoflagellate *Amphidinium longum* ingested *Heterosigma akashiwo*. However, the 7 grazer species exhibited a range of growth responses to the raphidophyte, which proved toxic to some, nutritionally insufficient to others, and able to support survival or growth in 2 species. *H. akashiwo* was toxic to the 3 largest ciliates: *Coxliella* sp., *Metacylis* sp., and the chloroplast-retaining *Strombidium* sp. SPMC68. Ciliate growth rates were lower on both strains of *H. akashiwo* than when starved (Fig. 1, Table 3). This toxic effect was not ameliorated by the addition of *Rhodomonas* sp. (Fig. 1,

Table 3. Protist grazer growth rates (doublings d^{-1} ± 1 SD) in experiments testing the effect of *Heterosigma akashiwo* alone and in mixtures with *Rhodomonas* sp. (see Fig. 1). Rates were calculated based on initial and final abundances, except where otherwise noted

Grazer	Starved	*Rhodomonas* sp.	*H. akashiwo* CCMP452	*H. akashiwo* CCMP1914	*Rhodomonas* sp. + *H. akashiwo* CCMP452	*Rhodomonas* sp. + *H. akashiwo* CCMP1914
Coxliella sp.	−1.88 ± 0.24[a]	−0.62 ± 0.11	−2.95 ± 0.32[a]	−4.04 ± 0.34[a]	−2.33 ± 0.35	−2.52 ± 0.17
Metacylis sp.	−0.78 ± 0.09	−0.47 ± 0.06	−1.77 ± 0.05	−1.76 ± 0.16	−2.18 ± 0.15	−1.77 ± 0.10
Strombidium sp. SPMC68	−0.94 ± 0.23[a]	−0.41 ± 0.30[a]	−2.79 ± 0.70[a]	−4.99 ± 1.49[a]	−2.84 ± 1.27[a]	−4.13 ± 0.80[a]
Eutintinnus sp.	−0.18 ± 0.05	0.19 ± 0.03	−0.12 ± 0.07	0.03 ± 0.03	−0.01 ± 0.06	0.16 ± 0.05
Strombidium sp. SPMC92	0.09 ± 0.18	0.62 ± 0.09	0.26 ± 0.08	0.44 ± 0.14	0.65 ± 0.13	0.65 ± 0.07
Amphidinium longum	−0.18 ± 0.01	0.16 ± 0.01	−0.18 ± 0.04	−0.17 ± 0.01	0.25 ± 0.05	0.24 ± 0.12
Noctiluca scintillans	−0.22 ± 0.03	0.03 ± 0.01	−0.01 ± 0.02	−0.02 ± 0.02	0.01 ± 0.01	0.03 ± 0.02

[a]Growth rates calculated using data from Day 1 because ciliate abundance in some treatments was zero on Day 2
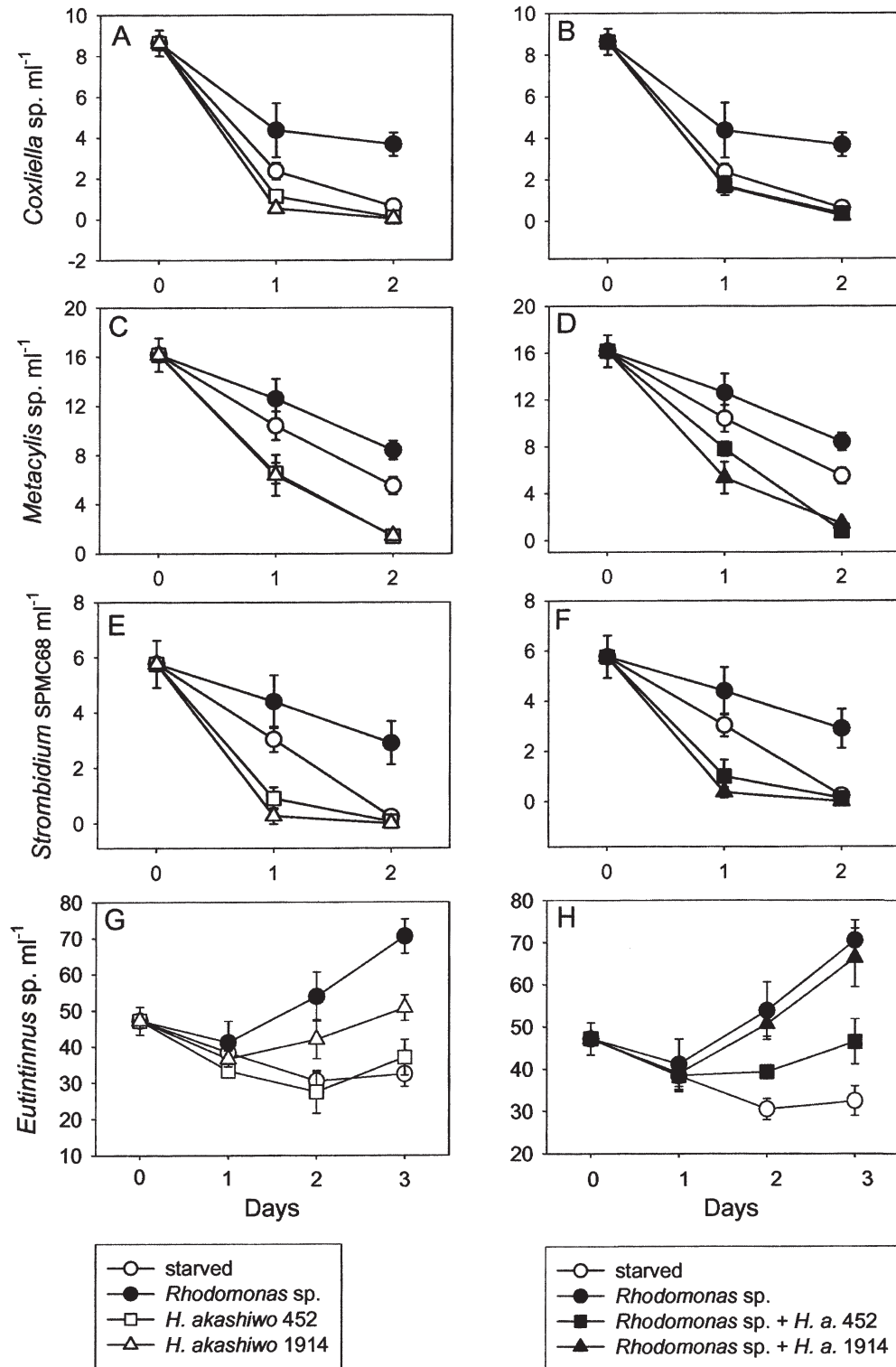
Fig. 1. (Above and following page.) Results of protist growth rate experiments, showing effect of 2 *Heterosigma akashiwo* (*H. a.*) strains (CCMP452, CCMP1914) on the abundance of (A,B) *Coxliella* sp., (C,D) *Metacylis* sp., (E,F) *Strombidium* sp. SPMC68, (G,H) *Eutintinnus* sp., (I,J) *Amphidinium longum*, (K,L) *Strombidium* sp. SPMC92, and (M,N) *Noctiluca scintillans*. Results of each growth experiment have been separated into 2 graphs for clarity: left-hand panels for each grazer species include data for *H. akashiwo* treatments, while right-hand panels include data for mixed-diet treatments. Both panels include data from starved and *Rhodomonas* sp.-fed controls. See Table 2 for experiment conditions. Error bars: ±1 SD (n = 3)

Fig. 1 (continued)

Table 3); we observed no difference between the growth rates on *H. akashiwo* alone and those on *H. akashiwo* combined with *Rhodomonas* sp. (statistical results for all growth rate experiments [Fig. 1] are reported in Table 4).

Eutintinnus sp. was the only grazer that grew at different rates on the 2 strains of *Heterosigma akashiwo* (Fig. 1, Table 3). Strain CCMP1914 supported higher growth rates than strain CCMP452. The former supported some tintinnid growth between Days 1 and 3, though less than the high-quality *Rhodomonas* sp. diet. Growth on the latter strain was indistinguishable from growth in the starved control, indicating complete nutritional insufficiency but not toxicity. This interpretation is supported by the mixed diet results. Addition of CCMP452 to *Rhodomonas* sp. reduced growth rates

relative to a diet of *Rhodomonas* sp. alone, whereas addition of CCMP1914 did not (Fig. 1).

Amphidinium longum and *Strombidium* sp. SPMC92 were unaffected by either strain of *Heterosigma akashiwo*, whether in single prey or mixed-diet treatments. *Noctiluca scintillans*, on the other hand, clearly received some modest nutritional benefit from *H. akashiwo*, which supported survival of the dinoflagellate over the 22 d course of the growth experiment (Fig. 1). This level of growth was better than that in the starved controls, in which *N. scintillans* abundance slowly declined, but worse than that in the *Rhodomonas* sp. treatment. Growth in the *H. akashiwo* + *Rhodomonas* sp. mixtures was indistinguishable from growth on *Rhodomonas* alone (Fig. 1, Table 3).

Table 4. Results of statistical analyses (p-values) for experiments testing the effect of *Heterosigma akashiwo* on protist growth. All experiments showed significant effects of treatment (ANOVAR, $\alpha = 0.05$). The 5 *a priori* contrasts specifically compared: (1) Starved vs. *H. akashiwo* treatments; (2) *Rhodomonas* sp. vs. *H. akashiwo* + *Rhodomonas* sp. treatments; (3) *H. akashiwo* CCMP452 vs. *H. akashiwo* CCMP1914; (4) *H. akashiwo* CCMP452 + *Rhodomonas* sp. vs. *H. akashiwo* CCMP1914 + *Rhodomonas* sp.; (5) *H. akashiwo* vs. *H. akashiwo* + *Rhodomonas* sp. treatments. *Significant p-values ($\alpha = 0.05$)

| Grazer | Contrasts | | | | |
	1	2	3	4	5
Coxliella sp.	0.012*	< 0.001*	0.429	0.873	0.080
Metacylis sp.	<0.001*	<0.001*	0.927	0.216	0.847
Strombidium sp. SPMC68	0.022*	<0.001*	0.559	0.523	0.874
Eutintinnus sp.	0.090	0.003*	0.002*	0.001*	<0.001*
Strombidium sp. SPMC92	0.250	0.992	0.309	0.905	0.009*
Amphidinium longum	0.698	0.232	0.796	0.950	<0.001*
Noctiluca scintillans	0.025*	0.283	0.306	0.977	0.005*

Heterosigma akashiwo concentration gradient effect on tintinnid abundance

Ciliate abundance decreased with increasing *Heterosigma akashiwo* concentration for both *Coxliella* sp. and *Metacylis* sp. (Fig. 2). In the *Coxliella* sp. experiment, all treatments containing *H. akashiwo*-yielded significantly lower ciliate abundance than the *Rhodomonas* sp. only control, and ciliate abundance decreased with increasing *H. akashiwo* concentration (ANOVAR, $\alpha = 0.05$, $p < 0.001$; all contrasts, $p < 0.006$). We observed the same effects when *Metacylis* sp. was the grazer species (ANOVAR, $\alpha = 0.05$, $p < 0.001$; all contrasts, $p < 0.001$). On Day 2 of both experiments, ciliate abundances at the lowest *H. akashiwo* concentration were higher than abundances in the starved controls (compare open and filled squares in Fig. 2).

Effect of *Heterosigma akashiwo* filtrate on *Metacylis* sp. abundance

Neither the *Rhodomonas* sp. filtrate nor the *Heterosigma akashiwo* filtrate had any effect on the abundance of *Metacylis* sp. (Fig. 3), either in unfed (ANOVAR, $p = 0.143$) or fed (ANOVAR, $p = 0.071$) treatments.
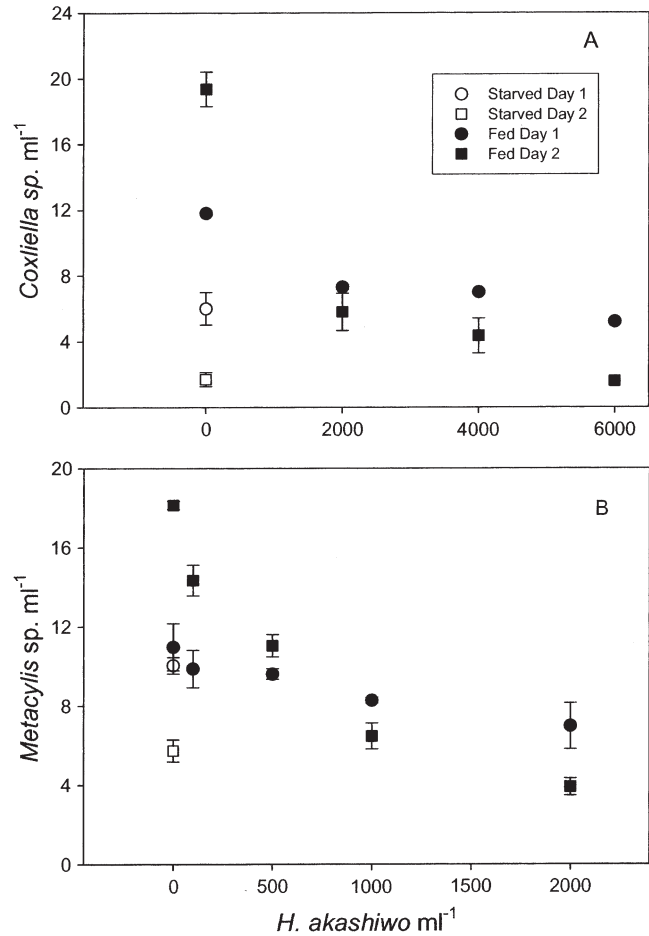

Fig. 2. Effect of varying concentrations of *Heterosigma akashiwo* CCMP452 on the abundance of (A) *Coxliella* sp. and (B) *Metacylis* sp. in a mixed-algal diet. See Table 2 for experiment conditions. Error bars: ±1 SD (n = 2)
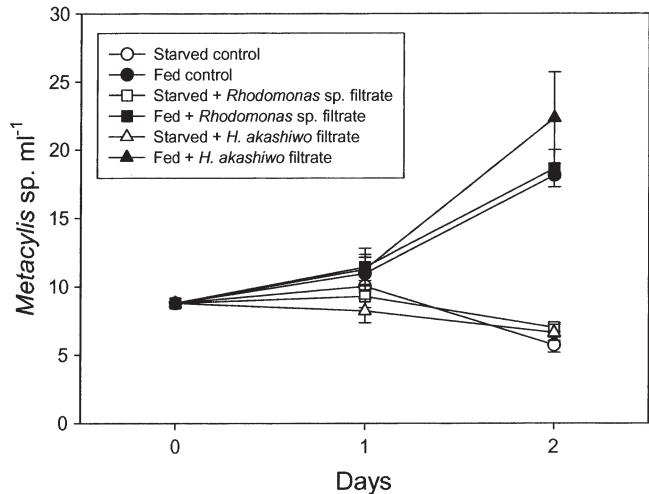

Fig. 3. Effect of *Rhodomonas* sp. and *Heterosigma akashiwo* culture filtrates on the abundance of *Metacylis* sp. Filtrates comprised 10% of total experiment volume. See Table 2 for experiment conditions. Error bars: ±1 SD (n = 3)

'Age of culture' effect on *Coxliella* sp. growth rate

The age of the *Heterosigma akashiwo* culture clearly influenced the growth rate of *Coxliella* sp. (ANOVAR, p = 0.015), with prey from younger cultures leading to a higher ciliate growth rate (lower mortality rate) than prey from the oldest cultures (Fig. 4). We observed a non-linear response of mortality to culture age, with cultures from 1 to 12 d old becoming increasingly toxic with age, and cultures older than 12 d all approximately equivalent in toxicity. All treatments with *H. akashiwo* alone led to greater ciliate mortality than the starved controls (open vs. filled circles in Fig. 4), confirming the toxicity seen during the *Coxliella* sp. growth experiment (Fig. 1). In contrast to the *Coxliella* sp. growth experiment (Fig. 1, Table 3), addition of *Rhodomonas* sp. to the *H. akashiwo* diet did reduce mortality (increase growth) in the 'age of culture' experiment. We saw this increase regardless of *H. akashiwo* culture age (ANOVAR, p < 0.001), but mixed diets overall supported substantially lower growth than the *Rhodomonas* sp.-only controls. There was no interaction between diet (*H. akashiwo* alone vs. mixed) and culture age effects on growth (ANOVAR, p = 0.762)

Effect of *Heterosigma akashiwo* on ingestion of *Rhodomonas* sp.

The presence of *Heterosigma akashiwo* had no effect on *Amphidinium longum*'s feeding on *Rhodomonas* sp. (ANOVAR, p = 0.158). Approximately 25% of the dinoflagellates had fed on *Rhodomonas* sp. after 30 min in treatments with and without *H. akashiwo*. As in the growth experiment, *A. longum* did not eat *H. akashiwo* at all. Similarly, the percentage of *Metacylis* sp. with ingested food was the same for the *Rhodomonas* sp.-only and *Rhodomonas* sp. + *H. akashiwo* treatments, with a lower percentage eating
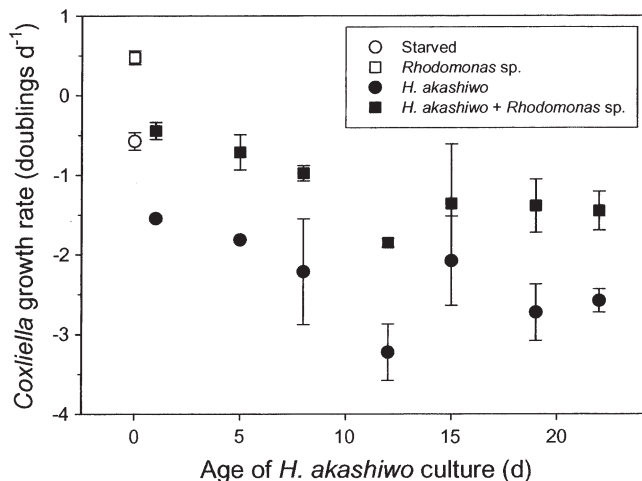

Fig. 4. Average growth rates of *Coxliella* sp. when fed *Heterosigma akashiwo* CCMP452 from cultures of varying ages, with or without added *Rhodomonas* sp. See Table 2 for experiment conditions. Error bars: ±1 SD (n = 2)

H. akashiwo alone (ANOVAR, p < 0.001) (Table 5). The amount of algal C ingested per ciliate was, however, higher on the *H. akashiwo* than on the *Rhodomonas* sp. diet, perhaps due to the greater prey availability in the *H. akashiwo* treatment or to the lower C density of the *Rhodomonas* sp. cells (0.17 vs. 0.19 pgC μm^{-3}). Ciliates contained approximately the same total amount of ingested C when fed the mixed diet as when fed *H. akashiwo* alone (1.5 h: 32.4 vs. 34.4 pgC ciliate^{-1}; 4.5 h: 46.1 vs. 43.0 pgC ciliate^{-1}). In addition, the ratio of *H. akashiwo* C to *Rhodomonas* sp. C in the food vacuoles was similar at the 2 time points (ca. 2:1). Assuming that both *H. akashiwo* and mixed-diet prey levels were above levels at which *Metacylis* sp. feeding rates saturate, the addition of *H. akashiwo* to the *Rhodomonas* sp. diet had no effect on either the percentage of ciliates in the population that were feeding, or on the overall amount of prey C ingested.

Table 5. Ingestion of *Rhodomonas* sp. and *Heterosigma akashiwo* by *Metacylis* sp. after 1.5 and 4.5 h of feeding (±1 SD, n = 3). % feeding: percentage of ciliates with ingested prey. Available prey: $\mu gC\ l^{-1}$. Ingested *Rhodomonas* sp. or *H. akashiwo*: pgC ciliate^{-1}. na: prey species not available in the treatment listed

Diet	Available prey	1.5 h			4.5 h		
		% feeding	Ingested *Rhodomonas*	Ingested *H. akashiwo*	% Feeding	Ingested *Rhodomonas*	Ingested *H. akashiwo*
Rhodomonas sp.	277	29.8 ± 2.5	10.4 ± 0.5	na	46.2 ± 3.3	19.5 ± 0.9	na
H. akashiwo	437	14.1 ± 3.3	na	34.4 ± 3.2	17.8 ± 0.1	na	43.0 ± 1.5
Rhodomonas sp. + *H. akashiwo*	714	35.5 ± 1.6	10.5 ± 0.2	21.9 ± 5.7	43.9 ± 0.8	13.7 ± 1.0	32.4 ± 3.2

DISCUSSION

Both tested strains of *Heterosigma akashiwo* were toxic to 3 species of choreotrich ciliates; *Coxliella* sp., *Metacylis* sp. and the larger *Strombidium* sp. all experienced higher mortality when fed the raphidophyte than when starved. Although another large tintinnid, *Favella taraikaensis*, was seen to reject *H. akashiwo* cells in high-speed video work (Taniguchi & Takeda 1988), these 3 ciliate species were not capable of avoiding the toxic cells. We observed ingestion by all 3 species during the growth experiments, and substantial feeding by *Metacylis* in the ingestion experiment (Table 5). Toxicity of *H. akashiwo* (then considered *Olisthodiscus luteus*) to 2 tintinnid species was also reported by Verity & Stoecker (1982). They observed mortality greater than that in starved controls for *Tintinnopsis tubulosoides* fed strain OL-1, and for *Favella* sp. fed strain Olisth (the same as CCMP452, one of the 2 strains used in our study).

Conditions during our experiments were consistent with those eliciting greatest ichthyotoxicity in 2 Japanese *Heterosigma akashiwo* strains (Ono et al. 2000, Haque & Onoue 2002). These include: very low light levels, 30 psu salinity, an *H. akashiwo* growth temperature of 15°C, and an experiment temperature of 12°C. Further support for the existence of toxicity in our strains comes from a recent study of *H. akashiwo*-produced extracellular organics. Twiner et al. (2004) found that organics from strain NEPCC560R (the same as CCMP452, used in the present study) reduced respiratory activity of mammalian cells to less than half of control rates.

While *Heterosigma akashiwo* was toxic to 3 ciliate species in our experiments, other protist grazers experienced no toxicity. At least one of these grazers (the dinoflagellate *Amphidinium longum*) did not ingest *H. akashiwo* cells, and the presence of *H. akashiwo* had no effect on growth relative to starved or *Rhodomonas* sp. control treatments. The same result was observed for the smaller *Strombidium* sp. While this ciliate did ingest the raphidophyte, we have no information on the quantity of cells eaten or the duration of feeding; a low feeding rate or short feeding duration could have alleviated any toxic effects. *Eutintinnus* sp. and *Noctiluca scintillans* clearly derived nutritional benefit from *H. akashiwo*. When fed the raphidophyte alone they fared better than in starved controls, and their growth on *Rhodomonas* sp. was not reduced by the addition of *H. akashiwo* to their diet. The higher growth rate of *Eutintinnus* sp. on *H. akashiwo* CCMP1914 than on strain CCMP452 demonstrates a nutritional difference between the strains; whether this was due to greater ingestion of CCMP1914 or to inter-strain differences in biochemical composition is not known. Differences between strains were sufficiently subtle that they had no effect on the other 6 protist grazers.

The range of growth responses seen in the 7 grazer species we studied is consistent with literature reports for other protists. Some species ingested and grew on monospecific diets of *Heterosigma akashiwo*, including the prostomatid ciliate *Tiarina fusus* (Jeong et al. 2002) and the heterotrophic dinoflagellates *Oxyrrhis marina* and *Noctiluca scintillans* (Nakamura 1998, Jeong et al. 2003). Other species did not ingest *H. akashiwo*, including *Favella taraikaensis* (Taniguchi & Takeda 1988) and *F. ehrenbergii* (Kamiyama & Arima 2001). In contrast, the *Favella* sp. studied by Verity & Stoecker (1982) did ingest *H. akashiwo* and was killed by it. These differences among *Favella* studies could be due to the various *Favella* species used, or to differences among *H. akashiwo* strains and growth conditions. Indeed, strain differences could explain even the varying responses of different grazer species, as summarized above. Unfortunately, *H. akashiwo* strain identities were not reported for several of these studies. Other harmful algal species show marked inter-strain variability in nutritional value and toxicity to protists, including *Alexandrium tamarense* (Hansen 1989, Tillmann & John 2002), *Pfiesteria piscicida* (Stoecker et al. 2000), *Chrysochromulina polylepis* (John et al. 2002), *Emiliania huxleyi* (Strom et al. 2003), and *Prorocentrum minimum* (Rosetta & McManus 2003).

Our observations of *Heterosigma akashiwo* toxicity toward large ciliates is consistent with field observations of microzooplankton community composition during *H. akashiwo* blooms. Tintinnid abundance varied inversely with *H. akashiwo* (then identified as *Olisthodiscus luteus*) concentration during blooms in Narragansett Bay (Rhode Island, USA) during 1980–1981 (Verity & Stoecker 1982). Similarly, the abundance and diversity of microzooplankton, especially tintinnid ciliates, decreased markedly during *H. akashiwo* blooms in Hiroshima Bay, Japan (Kamiyama 1995, Kamiyama et al. 2000). A dense *H. akashiwo* bloom in Burrard Inlet (Strait of Georgia, British Columbia, Canada) resulted in high mortality rates (-1.5 to -2.0 d^{-1}) of tintinnids and large (>60 μm) aloricate choreotrichs during 24 h incubation experiments. Other protist grazers in the community, including smaller aloricate choreotrichs and heterotrophic dinoflagellates, had growth rates near zero (Erenstone 2003). Large choreotrich ciliates often exert substantial grazing pressure on coastal phytoplankton (e.g. Capriulo & Carpenter 1983, Levinsen & Nielsen 2002, Johansson et al. 2004). Toxicity of *H. akashiwo* to this component of the microzooplankton community could promote formation of *H. akashiwo* blooms.

Two specific aspects of the *Heterosigma akashiwo*–ciliate interaction could further influence bloom forma-

tion and persistence. First is the response to *H. akashiwo* cell concentration. Our results show that sufficiently low concentrations of *H. akashiwo*, in combination with nutritious prey, can be tolerated by ciliates that are otherwise susceptible to the raphidophyte's toxicity. At the lowest concentrations tested, *H. akashiwo* combined with 1×10^4 cells ml^{-1} *Rhodomonas* sp. supported higher abundances of *Coxliella* sp. and *Metacylis* sp. than did starved controls (2 d time points, Fig. 2). Increasing amounts of *H. akashiwo*, however, led to progressive decreases in ciliates ml^{-1}. Survival of protist grazers at low concentrations of otherwise toxic algae was seen in one other study of *H. akashiwo* (Verity & Stoecker 1982), as well as in studies of *Prymnesium parvum* (Rosetta & McManus 2003) and various dinoflagellates (Hansen 1989, Jeong & Latz 1994, Hansen 1995, Kamiyama 1997, Kamiyama & Arima 1997). Decreasing abundance of potential consumers with increasing algal density constitutes a negative feedback, which would promote further accumulation of harmful algal cells. Such feedbacks would be enhanced by the 'age of culture' effect that we observed. *H. akashiwo* cells from older cultures were more toxic to *Coxliella* sp. than cells from younger cultures (Fig. 4). Nutrient limitation can increase toxicity in other algal species: *Alexandrium fundyense*, *A. minutum*, *Prymnesium patelliferum*, and *Pseudo-nitzschia seriata* are all known to be more toxic in stationary than in exponential growth phase (John & Flynn 2000, Graneli & Johannson 2003, Lippemeier et al. 2003, Fehling et al. 2004). To the extent that a developing bloom behaves like a batch culture, the combination of increasing *H. akashiwo* cell density and increased toxicity per cell should progressively reduce ciliate abundance and curtail grazing by this component of the microzooplankton community. Avoidance of *H. akashiwo* cells by other types of protist grazers, as seen in our study for *Amphidinium longum*, would exacerbate the accumulation of the raphidophyte, perhaps at the expense of other more palatable phytoplankton species.

The mechanism of *Heterosigma akashiwo*'s toxicity to marine organisms remains unclear. The literature offers considerable discussion and debate on the subject (see references in 'Introduction', also Twiner et al. 2001, Cembella 2003). Damage to fish gill tissue by ROS is clearly an element of the ichthyotoxicity caused by this and related raphidophytes (Yang et al. 1995, Kim et al. 1999). However, none of the proposed toxic substances (ROS, brevetoxin-like compounds, extracellular organics) has been directly tested on heterotrophic protists, the consumers with the potential to have the largest impact on *H. akashiwo* in nature. The results of our filtrate experiment show that substances toxic to protists, if dissolved, must be short-lived (i.e. requiring the continued

presence of the algal cells for effectiveness). The presence of *H. akashiwo* cells did not, however, decrease feeding on *Rhodomonas* sp. after 4.5 h (Table 5), nor did 2 d of exposure decrease survival of *Amphidinium longum* (Fig. 1), which did not eat the raphidophyte. These observations also do not support the hypothesis that *H. akashiwo* mucus prevents feeding by entangling cilia, as seen for the rotifer *Synchaeta cecilia* (Egloff 1986, *H. akashiwo* then considered *Olisthodiscus luteus*). Rather, it seems highly probable that ingestion of *H. akashiwo* by protists is required for toxicity. *H. akashiwo* toxicity to ciliates, as observed by us and by Verity & Stoecker (1982), may arise from a different mechanism than ichthyotoxicity. Tillmann & John (2002), for example, found that toxic effects of *Alexandrium* spp. on the heterotrophic dinoflagellate *Oxyrrhis marina* bore no relationship to the measured paralytic shellfish poisoning toxin content of different *Alexandrium* strains and species.

In summary, we found that 2 strains of *Heterosigma akashiwo* were toxic to several protist grazers. Consistent with research on other toxic marine organisms (McClintock & Baker 2001), different protist grazers showed a range of responses to *H. akashiwo*, with some able to avoid feeding on the cells and others able to survive and grow on the raphidophyte. The 3 largest ciliate species tested, however, were all killed by *H. akashiwo*. No toxicity was associated with filtrate from *H. akashiwo* culture or from the presence of *H. akashiwo* cells that were not ingested, so the toxic effect of this species to large ciliates seems to depend upon ingestion of the cells. Ciliates of the types we studied can be important consumers of coastal phytoplankton. Thus, the toxic effects of *H. akashiwo* could reduce microzooplankton consumption of this species in nature. Effects such as the increase in ciliate mortality with increasing *H. akashiwo* cell concentration and population age would further promote bloom development and persistence. Toxicity of *H. akashiwo* toward ciliates is likely an important element in the ability of this raphidophyte to form persistent blooms.

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