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BROAD SALINITY TOLERANCE AS A REFUGE FROM PREDATION IN THE HARMFUL RAPHIDOPHYTE ALGA *HETEROSIGMA AKASHIWO* (RAPHIDOPHYCEAE)[1]

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The ability of harmful algal species to form dense, nearly monospecific blooms remains an ecological and evolutionary puzzle. We hypothesized that predation interacts with estuarine salinity gradients to promote blooms of *Heterosigma akashiwo* (Y. Hada) Y. Hada ex Y. Hara et M. Chihara, a cosmopolitan toxic raphidophyte. Specifically, *H. akashiwo*'s broad salinity tolerance appears to provide a refuge from predation that enhances the net growth of *H. akashiwo* populations through several mechanisms. (1) Contrasting salinity tolerance of predators and prey. Estuarine *H. akashiwo* isolates from the west coast of North America grew rapidly at salinities as low as six, and distributed throughout experimental salinity gradients to salinities as low as three. In contrast, survival of most protistan predator species was restricted to salinities >15. (2) *H. akashiwo* physiological and behavioral plasticity. Acclimation to low salinity enhanced *H. akashiwo*'s ability to accumulate and grow in low salinity waters. In addition, the presence of a ciliate predator altered *H. akashiwo* swimming behavior, promoting accumulation in low-salinity surface layers inhospitable to the ciliate. (3) Negative effects of low salinity on predation processes. Ciliate predation rates decreased sharply at salinities <25 and, for one species, *H. akashiwo* toxicity increased at low salinities. Taken together, these behaviors and responses imply that blooms can readily initiate in low salinity waters where *H. akashiwo* would experience decreased predation pressure while maintaining near-maximal growth rates. The salinity structure of a typical estuary would provide this HAB species a unique refuge from predation. Broad salinity tolerance in raphidophytes may have evolved in part as a response to selective pressures associated with predation.

Key index words: **Behavior; estuary; harmful algal bloom; *Heterosigma*; protist predators; refuge; salinity**

***Abbreviations*: CCMP, Center for culture of marine phytoplankton; DAPI, 4′,6-diamidino-2-phenylindole; HAB, harmful algal bloom; RFU, relative fluorescence units; SPMC, Shannon point marine center**

The photosynthetic flagellate *Heterosigma akashiwo* is a marine raphidophyte that forms ichthyotoxic blooms. The unusual and enigmatic class Raphidophyceae harbors only a few marine planktonic species: in addition to *H. akashiwo*, these comprise *Fibrocapsa japonica* and approximately six species of *Chattonella*. The species are notoriously difficult to identify and distinguish, especially in preserved samples (Bowers et al. 2006). Most marine raphidophytes are known to be toxic, and they bloom episodically and somewhat unpredictably in temperate coastal waters worldwide (Smayda 1998). Curiously, species from two or three of the raphidophyte genera sometimes – perhaps often – co-occur during blooms (Smayda 1998, Zhang et al. 2006, Lewitus et al. 2008). All of these features have posed challenges for unraveling the ecology of the class and its component members.

H. akashiwo, along with some of the other raphidophyte species, is toxic to fish. Blooms of *H. akashiwo* have been responsible for economically devastating kills of caged fish in aquaculture operations worldwide, including salmon farmed in the United States, Canada, New Zealand, Chile, and Europe, and yellowtail and sea bream in Japan's Seto Inland Sea (Honjo 1993, Horner et al. 1997, Smayda 1998). There is also evidence for wild fish kills due to *H. akashiwo* (Horner et al. 1997, Kempton et al. 2008). The mechanism(s) of *H. akashiwo* toxicity to either fish or potential planktonic

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predators are not well understood. Candidate hypotheses (not mutually exclusive) include cell or tissue damage by reactive oxygen species (Oda et al. 1997), production of brevetoxin-like neurotoxins (Haque and Onoue 2002), and physical or physiological harm from extracellular organic material (Egloff 1986, Twiner et al. 2005). Despite this persistent enigma, factors that promote *H. akashiwo* blooms and contribute to the competitive success of the species have been identified, including intraspecies genetic and physiological variability, ability to grow on a range of nutrient sources, tendency to behaviorally accumulate at density discontinuities or in surface waters, and toxicity to or avoidance by potential predators (e.g., Watanabe et al. 1982, Clough and Strom 2005, Bearon et al. 2006, Graham and Strom 2010, Fredrickson et al. 2011, Harvey and Menden-Deuer 2011).

In the Salish Sea (the estuarine system comprising Puget Sound, the Strait of Georgia, and the Strait of Juan de Fuca on the North American west coast; Fig. 1), small-scale, ephemeral blooms of *H. akashiwo* occur every year during the spring and summer (Rensel et al. 2010). Less often, there are massive blooms that can cover 100 s of km^2; for example, in June 2006 and May 2007 in northern Puget Sound, when $>10^5$ fish were killed at Cypress Island (San Juan Archipelago) and Port Angeles fish farms (Fig. 1; Rensel 2007). In a conceptual model put forth by Taylor and Haigh (1993), *H. akashiwo* blooms initiate in the low salinity waters of northern Salish Sea embayments, particularly when water temperatures at the sediment reach 15°C and trigger emergence of resting cells. When conditions are favorable, these localized blooms can seed more extensive blooms that expand into and are transported through large areas of the Salish Sea. Bloom timing often coincides with maximum runoff from the Fraser River, by far the largest supplier of freshwater to the Salish Sea estuary. Furthermore, Rensel et al. (2010) have shown that widespread *H. akashiwo* blooms tend to occur in years with earlier and higher Fraser River flow to the Salish Sea. Coupled with *H. akashiwo*'s well-known tolerance for very low salinities (e.g., Tomas 1978, Watanabe et al. 1982, Zhang et al. 2006, Fredrickson et al. 2011), these observations suggest that salinity could be a governing variable for the initiation, expansion and persistence of *H. akashiwo* blooms.

The goal of this research was to examine how environmental factors, particularly salinity, interact with the protist predation processes that can potentially control *H. akashiwo* abundance. In general, mortality due to protist predation strongly regulates the biomass and composition of coastal phytoplankton communities. Here we describe several mechanisms by which salinity, a structuring environmental factor in estuaries, can influence protist predation and hence net growth and bloom formation by *H. akashiwo* populations. These mechanisms include (1) mismatches in the salinity tolerance of predators and prey; (2) a high level of plasticity in *H. akashiwo*'s behavioral and growth responses to salinity variations; and (3) salinity-driven effects on predation processes and *H. akashiwo* toxicity. We suggest that the broad salinity tolerance of *H. akashiwo* and likely other euryhaline raphidophyte genera has evolved in part as a response to predation pressure. Broad salinity tolerance may be a general strategy by which some harmful algal species obtain a refuge from predation and thus gain the ability to form widespread blooms.

METHODS

General culturing procedures. All isolates of *H. akashiwo* and protist predators were obtained from the Salish Sea or (for *H. akashiwo* strain CCMP 3107) nearby coastal waters (Table 1; Fig. 1). All cultures were maintained and all experiments conducted at 15°C and a salinity of 30 unless otherwise indicated. This temperature has been associated with the sudden appearance of *H. akashiwo* cells in the water column in Vancouver B. C. embayments, possibly due to excystment (Taylor and Haigh 1993). *Heterosigma akashiwo* and other phytoplankton cultures were maintained in f/2 medium without added silicate at 85–160 μmol photons $\cdot$ $m^{-2} \cdot s^{-1}$ on a 12:12 (L:D) cycle. Cultures were not axenic. Heterotrophic protists (predators) were fed maintenance diets of mixed photosynthetic flagellates, and maintained in autoclaved, filtered (0.2 μm) seawater or ciliate medium (Gifford 1985) at low irradiance (1–15 μmol photons $\cdot$ $m^{-2} \cdot s^{-1}$) on a 12:12 LD cycle. Species used for experiments, with the exception of *Oxyrrhis marina*, are regularly observed in microzooplankton communities in the central Salish Sea. Unless otherwise noted, cells were fixed in 1% acid Lugol's solution and counted using a 1 mL Sedgwick-Rafter counting chamber (SPI Supplies, West Chester, PA, USA). Most heterotrophic protists were counted in 10–50 mL settled samples in an Utermöhl chamber.

Salinity tolerance for survival and growth. Growth rate was measured over a range of salinities for four *H. akashiwo* isolates (Table 1). Salinities for these and subsequent experiments were determined with a YSI model 30 hand-held salinometer, and adjusted by addition of ultra-pure (Nanopure) water. Cells were acclimated by culturing them at salinities of either 15 or 30 for 1–2 weeks before growth rate measurements commenced. Low-salinity (≤ 15) growth rate treatments were inoculated from the salinity of 15 acclimation culture, and higher salinity (>15) treatments from the salinity of 30 acclimation culture. In some cases, growth at a given salinity was tested for strains acclimated to both 15 and 30. For strain 3150, cells were grown in 30-mL volumes in quadruplicate glass tubes at each salinity with added f/2 nutrients, and the in vivo fluorescence was measured daily (Turner 10AU fluorometer; Turner Designs, Sunnyvale, CA, USA). For all other strains, cells were grown in triplicate 12-well cell plates (0.1 mL source culture added to 4 mL f/2 at target salinity for an initial concentration of 500 cells $\cdot$ mL^{-1}). Daily measurements of relative in vivo fluorescence (RFU) were made using a SpectraMax M5 well plate reader (Molecular Devices, Sunnyvale, CA, USA). For all *H. akashiwo* strains, growth rates were estimated from the rate of change in in vivo fluorescence (e.g., slope of ln (RFU) vs. time) over the exponential phase. Data were analyzed using one-way ANOVA (PASW Statistics 18, Chicago, IL, USA) to determine whether salinity significantly affected growth rate. Rates at each salinity relative to those at 30 were then evaluated using a post hoc Dunnett *t*-test.

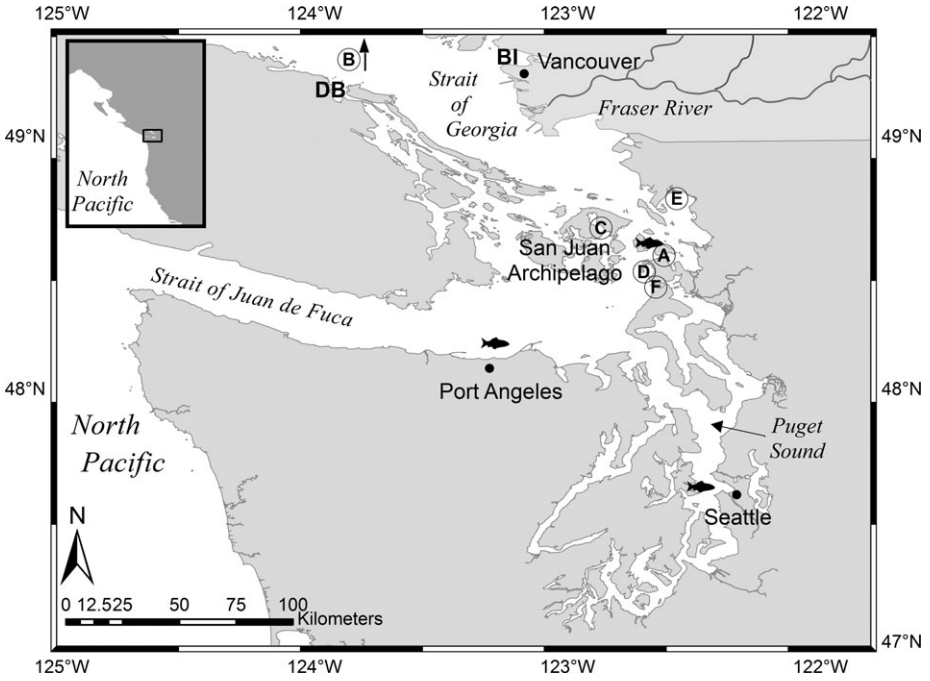

FIG. 1. Map of Salish Sea on the Pacific coast of North America, showing geographic features and sampling locations referenced in this study. Circled letters refer to isolate collection locations (see Table 1); location B (north of mapped region) is Nowish Inlet near Susan Island, British Columbia. Fish symbols show locations of salmon farms affected by *Heterosigma akashiwo* blooms. BI (Burrard Inlet) and DB (Departure Bay) refer to locations of salinity data plotted in Figure 9.

TABLE 1. Origin of *Heterosigma akashiwo* and protist predator isolates used in this study.

Species	Strain	Collection date	Code
Heterosigma akashiwo	CCMP 1914	July 1991	A
Heterosigma akashiwo	CCMP 2809	June 2006	A
Heterosigma akashiwo	CCMP 3107	Sept 2008	B
Heterosigma akashiwo	CCMP 3150	July 2007	C
Favella sp. c.f. *ehrenbergii*	SPMC 133	June 2008	D
Strombidinopsis acuminatum[a]	SPMC 114	June 2006	A
Strombidinopsis acuminatum[b]	SPMC 142	July 2010	E
Metacylis sp.	SPMC 125	Sept 2007	C
Noctiluca scintillans	SPMC 124	Sept 2007	F
Gyrodinium dominans	SPMC 103	Unknown	Denmark
Oxyrrhis marina	SPMC 107	Sept 1993	A

[a]Isolate used in toxicity experiment.
[b]Isolate used in ingestion rate experiment.
CCMP, Provasoli-Guillard National Center for Culture of Marine Phytoplankton; SPMC, Shannon Point Marine Center culture collection. Code indicates collection location as shown in Figure 1.

The salinity tolerance of heterotrophic protists was determined during *H. akashiwo* toxicity experiments, described below.

Population distributions and movement behaviors. To quantify population distributions and movement behaviors, a 30 cm tall, 5.5 cm wide 800 mL octagonal, acrylic observational chamber was used. The chamber was filled with filtered seawater using a peristaltic pump; this method allows for the creation of defined salinity structures, while also eliminating convection in the chamber (Bearon et al. 2006, Harvey and Menden-Deuer 2011). To determine the halotolerance of each species, the population distributions of protist predators and *H. akashiwo* prey were filmed in various salinity structures. The same source water was used for all culturing, growth, and behavioral experiments. *H. akashiwo* (CCMP 2809) was used in all behavioral experiments. *H. akashiwo* as well as the ciliate *Favella* sp. (c.f. *ehrenbergii*) and the heterotrophic dinoflagellate *O. marina* were filmed in a linear salinity gradient, with salinity ranging from 0 to 30. *Heterosigma akashiwo* and heterotrophic dinoflagellate *Gyrodinium dominans* were filmed in a halocline structure, in which the water column was divided evenly into a low salinity (8–10) region at the top and a high salinity (27–30) region at the bottom. In an additional experiment, *Favella* sp. and *H. akashiwo* were filmed together in a halocline structure to investigate the role of predator presence on the movement behaviors and population distributions (relative to predator-free controls) of *H. akashiwo*.

Using a syringe, organisms were introduced to the bottom of the tank through silicone tubing with an internal diameter of 1 mm. Cells were introduced slowly (10 mL $\cdot$ min^{-1}) to reduce stress to cells and disturbance of the water column. The average initial concentrations of *H. akashiwo* and heterotrophic dinoflagellates in the tank were 180 cells $\cdot$ mL^{-1} and 100 cells $\cdot$ mL^{-1}, respectively. To obtain an initial concentration of 50 *Favella* sp. $\cdot$ mL^{-1}, 2 L of ciliate culture was gently condensed to 30 mL using 20 μm Nitex mesh 15 min before introduction to the bottom of the experimental chamber.

Each experimental treatment was filmed in three independent tanks. Filming occurred at five to eight, initially random, horizons ~2–3 cm apart. Each horizon was filmed for 1 min every hour for an 11-h period. Two infrared sensitive cameras (Pixelink, Ottowa, ON, CAN) with Nikon (Melville, NY, USA) 60-mm Micro Nikkor lenses monitored ~3.2 mL of water in the center of the chamber at each horizon. The cameras were

mounted at a 45° angle with maximally overlapping fields of view to enable reconstruction of three-dimensional (3D) movement behaviors. All filming was conducted in the dark, to eliminate the potential for light-mediated behavioral responses. To view organisms, the chamber was illuminated with infrared (960 nm) light-emitting diodes. Video was captured at 30 frames · s^{-1}. To determine population distributions, each video was analyzed using the same protocol. The 2D position of each individual organism in each frame of the stereo cameras was determined, using automated ImageJ (National Institute of Health, Bethesda, MD, USA) image-processing software based on user defined settings for size and threshold to remove stationary background objects. Three-dimensional swimming paths were determined by first assembling 2D trajectories from Cartesian coordinates of each organism in each stereo frame and then joining 2D tracks based on matching space–time occurrence in the two 2D segments. Trajectories from all treatments were determined using the exact same video analysis and trajectory assembly parameters (Menden-Deuer and Grünbaum 2006). Abundances were determined by averaging the number of movement tracks per frame over the 1 min video (1800 frames). A Kolmogorov–Smirnov test was used to determine statistical significance among population distributions and movement behaviors. All statistical analyses were made using Matlab R 7.10 (Natick, MA, USA). All analyses were considered significant when $P \leq 0.05$.

Salinity tolerance for protist feeding. The photosynthetic dinoflagellate *Heterocapsa triquetra* (CCMP448), the prey species for ingestion rate measurements, was cultured as described above ("General culturing") at a salinity of 30. Preliminary tests showed that *H. triquetra* cells transferred into salinities of 15, 20, and 25 survived intact for at least 4 h; *H. triquetra* is known to be a euryhaline species, with maximum growth rates persisting at salinities of 10 or lower (Yamaguchi et al. 1997). Ciliates were cultured as described above. To prepare ciliates for ingestion experiments, *Favella* sp. (SPMC133) was separated from maintenance prey by sieving (20 μm, 6 h before experiment). Maintenance prey in the stock culture of ciliate *Strombidinopsis acuminatum* (SPMC142) was reduced by reverse concentration through a 20 μm sieve, followed by resuspension in ciliate medium. *S. acuminatum* was then allowed to graze down residual maintenance prey for 2 d before the experiment. Ingestion rates were measured by monitoring the rate of accumulation of prey cells in ciliate food vacuoles over time. Ciliates and *H. triquetra* were combined at the appropriate salinities (achieved by combining ciliate medium with ultrapure water) in triplicate and sampled after 1 h (*Favella* sp.) or in quadruplicate and sampled after 0.5 h (*S. acuminatum*). *Heterocapsa triquetra* density was 620 cells · mL^{-1}; ciliate density was 4–10 cells · mL^{-1}. *Favella* sp. samples (20 mL) were fixed in 0.5% (final concentration) glutaraldehyde; *S. acuminatum* samples (50 mL) were fixed by sequential addition of 1.5 mL alkaline Lugol's, 0.5 mL borate-buffered formalin, and destained with 6.0 mL sodium thiosulfate solution (modified method of Sherr and Sherr 1993). All samples were stained with DAPI (Sigma-Aldrich, St. Louis, MO, USA). Fixed samples were filtered onto polycarbonate filters (pore size 20 μm), oil-mounted on glass slides, and stored frozen until later examination by epifluorescence microscopy. Food vacuole contents of at least 100 ciliates per slide were enumerated based on red fluorescence of *H. triquetra* under blue light excitation. Food vacuole content estimates were corrected for background levels of prey in food vacuoles (e.g., in unfed ciliates).

Ingestion rates were analyzed as described above for growth rates. For *S. acuminatum*, data were first transformed according to log10 (ingestion rate + 1) to meet assumptions of homogeneity of variance.

Salinity effects on H. akashiwo *toxicity to heterotrophic protists.* Five species of protist predators (Table 1; all potential consumers of *H. akashiwo* and all isolated from the Salish Sea) were exposed to salinities ranging from 15 to 30 (no species except *O. marina* could survive at 10). At each salinity, treatments in quadruplicate (in triplicate for *O. marina*) consisted of an unfed control and a *H. akashiwo* CCMP 2809 treatment at 10^4 cells·mL^{-1}. The latter represents the upper range of *H. akashiwo* densities observed in the San Juan Archipelago during the widespread 2006 bloom (S. Strom, unpublished data). As previously established (Clough and Strom 2005), *H. akashiwo* was considered toxic at a given salinity if predator survival in the presence of the alga was lower than in the unfed control. A positive control treatment consisting of *Isochrysis galbana* was also used in the experiment with ciliate *Metacylis* sp. Prey algal species *H. akashiwo* and *I. galbana* were cultured at experimental salinities for 2–3 weeks before experiments were initiated. Heterotrophic protists were cultured at a salinity of 30.

To initiate experiments, predators and prey were combined in polycarbonate bottles at experimental salinities, and samples were taken from replicate, nonincubated bottles to determine initial predator concentrations (samples fixed in acid Lugol's, 2% final concentration). For the two largest species (*N. scintillans*, *Favella* sp.), bottles were sampled by sieving the entire contents (100 or 70 mL), then back rinsing the cells into a fixation jar using salinity-adjusted filtered seawater. For all other predator species, 20 mL subsamples were collected from 40 to 70 mL incubation volumes, depending on experiment. Initial cell concentrations were 1 · mL^{-1} for *N. scintillans*, 300 · mL^{-1} for *O. marina*, 12 · mL^{-1} for *Metacylis* sp., 2 · mL^{-1} for *Favella* sp., and 8 · mL^{-1} for *S. acuminatum*.

Bottles were incubated at 15°C for 24 h (ciliates) or 48 h (heterotrophic dinoflagellates). Irradiance was 5–13 μmol photons · m^{-2} · s^{-1} except in experiments with *Favella* sp. and *S. acuminatum*, when it was 90 μmol photons · m^{-2} · s^{-1}. In all cases, the light cycle was 12:12 (LD). We have previously shown that irradiance does not affect *H. akashiwo* toxicity to heterotrophic protists (S. Strom and S. Graham, unpublished data). At the end of the incubation period, all bottles were sampled as described above for initial samples. Predator abundance was determined using inverted microscopy. To assess direct effects of salinity on heterotrophic protists, % survival over time in unfed treatments was calculated as [final cells · mL^{-1}]/[initial cells · mL^{-1}] * 100. Note that a% survival value <100 denotes mortality during the incubation. To assess nutritional benefit or toxicity of *H. akashiwo* to protist predators, final predator abundance in the presence of *H. akashiwo* (Ha) was compared to average final abundance in the unfed treatment at the same salinity: [final cells · mL^{-1} with Ha]/[final cells · mL^{-1} unfed] *100. Both types of data (unfed% survival, % survival on *H. akashiwo* relative to unfed) were statistically analyzed as described for *H. akashiwo* growth rates. For *Metacylis* sp., data were first ln-transformed to meet assumptions of homogeneity of variance.

RESULTS

Tolerance of low salinity: H. akashiwo *versus protist predators.* There were major differences in salinity tolerance between *H. akashiwo* and many of its potential protistan predators. All tested Salish Sea isolates of *H. akashiwo* grew well in low salinity conditions. We observed maximal growth rates at salinities ranging from 30 to 15 (Fig. 2A) and all strains grew at high relative rates even at salinities as low as 6. However, there were strain-specific variations in

the response to low salinities. Strain 3150 showed a gradual decline in growth rate below a salinity of 15, with growth rate significantly lower at 10 and 5 than at 30 (Dunnett's *t*-test, $P < 0.05$). Maximal growth persisted to lower salinities for 1914 and 3107, with significant decreases (relative to 30) at salinities ≤ 6. Although these reductions were significant, growth at low salinity was still remarkably high for these strongly euryhaline strains (rates at 3 were 61% and 81% of the strain maxima, respectively). Strain 2809 was also tolerant of low salinities but showed a threshold-type response, with dramatic but variable growth rate decreases at three and significant reductions at 0.

Preexperiment acclimation to lower salinity further enhanced the capacity of some strains to grow at very low salinities (Fig. 2B). Both 3107 and 1914 showed significant increases in growth rate at a very low salinity of 3 when acclimated to 15 versus 30 (one-way ANOVA: 3107, $F_{1,4} = 22.039$, $P < 0.05$; 1914, $F_{1,4} = 10.449$, $P < 0.05$). For strain 3107, growth rate at 3 increased by >70% and reached near-maximal levels when cells were acclimated to the lower salinity. Only strain 2809 was not able to grow at 3, irrespective of prior acclimation.

In contrast to *H. akashiwo*, heterotrophic protists isolated from Salish Sea waters were generally not tolerant of low salinities. Only the dinoflagellate *O. marina* survived at salinities <15 (Fig. 3A). Indeed, *O. marina* proved unusually euryhaline: no tested salinity reduced survival below that observed at 30 (one-way ANOVA: $F_{4,10} = 3.019$, $P = 0.071$). In contrast, the other studied dinoflagellate, *N. scintillans*, had significantly reduced survival at all salinities <30 ($P \leq 0.017$ for all Dunnett's t comparisons), whereas the three ciliate species (Fig. 3B) all showed reduced survival at salinities <20 ($P < 0.001$ for all comparisons). These treatments were unfed, so the survival and mortality responses represent direct physiological effects of salinity on the protists.

Distributions and behaviors of H. akashiwo *and potential predators.* In the continuous salinity gradient, *H. akashiwo* cells distributed throughout the water column and actively swam at all salinities observed, indicating no avoidance of waters with salinities as low as 3. After 5 h, the largest percent of the popu-

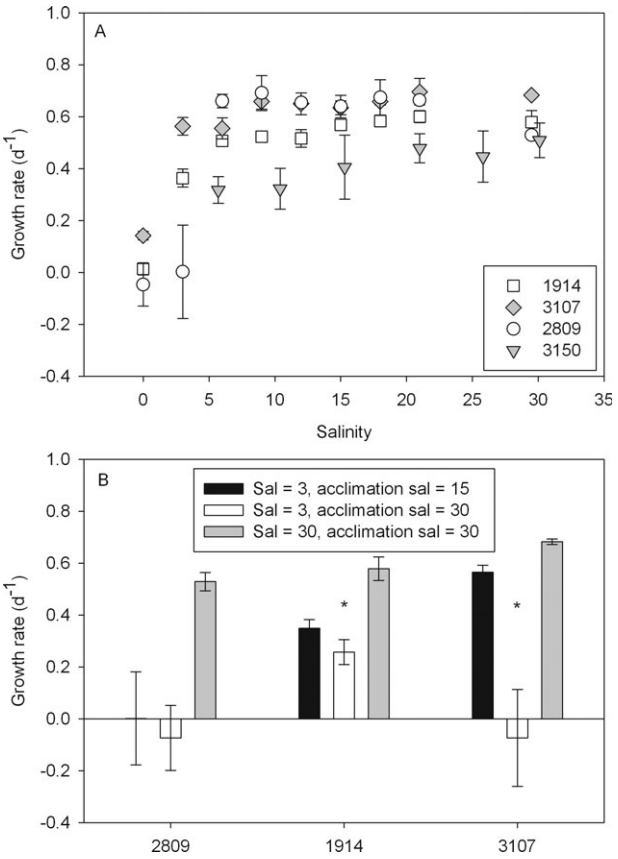

FIG. 2. Growth rates (d^{-1}) of Salish Sea *Heterosigma akashiwo* strains in f/2 (A) as a function of salinity and (B) at a salinity of 3 (vs. 30) after acclimation to either 30 or 15. Strain designations are CCMP numbers. Data are means ± 1 SD ($n = 3$ or 4); asterisks in (B) show significant differences in growth rates at a salinity of 3 between *H. akashiwo* acclimated to 15 versus 30.

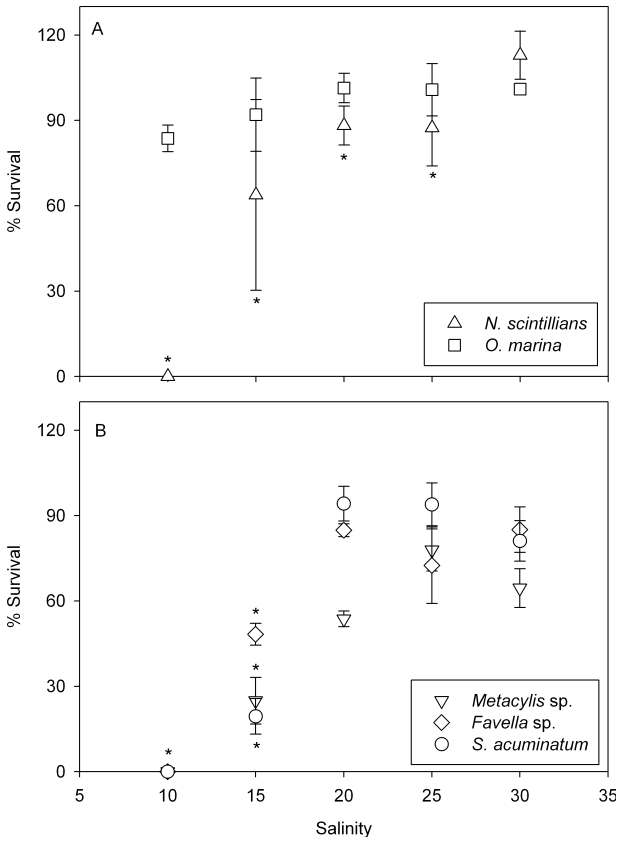

FIG. 3. Survival (final concentration as % of initial) of (A) heterotrophic dinoflagellates *Noctiluca scintillans* and *Oxyrrhis marina* and (B) ciliates *Metacylis* sp., *Favella* sp., and *Strombidinopsis acuminatum* in unfed incubations over a range of salinities. Means ± 1 SD, $n = 4$. * survival significantly lower than at 30 (Dunnett *t*-test, $P < 0.05$)

lation (18 ± 12%; mean ± SE) was found at the most saline horizon, and the smallest (7 ± 1%) at 12 (Fig. 4A). Approximately 42% of the population was found at salinities <10. This distribution did not change significantly over the 11 h of observation (K-S test, min $P = 0.09$).

Vertical distribution of *H. akashiwo* cells was affected by prior exposure to waters of varying salinity. After 5 h, maximum cell abundances were observed at salinities close to the acclimation salinity (Fig 4B). When acclimated to 15, the highest abundance of *H. akashiwo* cells (45 ± 3%) was observed at the same salinity of 15. In contrast, when cells were acclimated to 30, 39 ± 9% of the population was found at 23.5. These distributions did not change significantly over the course of the experiment (K-S test, min $P = 0.53$). Thus, although *H. akashiwo* exhibits broad halotolerance irrespective of acclimation salinity, acclimation influenced overall population distribution, with cells tending to aggregate at or near acclimation salinities.

The three heterotrophic protists tested had narrower salinity tolerances than *H. akashiwo* (Fig. 5); that is, while nearly half the *H. akashiwo* population was found at salinities ≤ 10, fewer dinoflagellates (32% and 27% for *O. marina* and *G. dominans*, respectively) and no ciliates were observed at those low salinity horizons (Fig. 5). The dinoflagellates were, however, quite tolerant of low salinities; after 5 h of observation, both species were distributed at all horizons measured. The majority of *O. marina* cells (25 ± 9%) were found at salinities of ~5 with

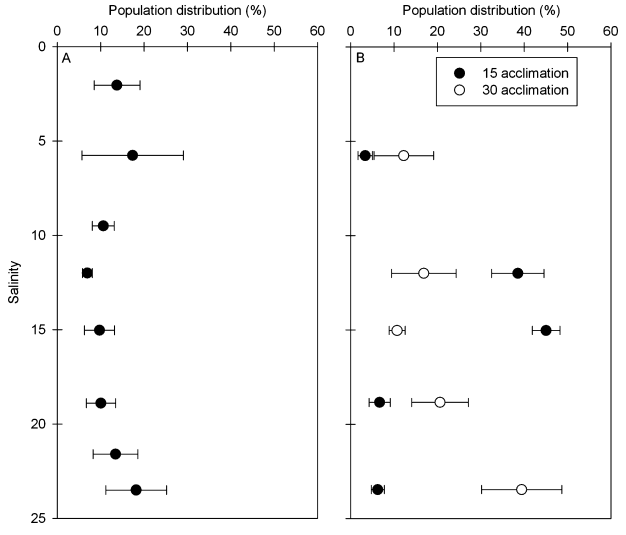

FIG. 4. Vertical distribution of *Heterosigma akashiwo* (strain CCMP2809) after 5 h in a linear salinity gradient. (A) Distribution of cells in salinity gradient ranging from 0 to 30, and (B) comparison of vertical distribution for cells grown (acclimated) in salinities of either 15 or 30 (means ± 1 SE, $n = 3$). Cells were distributed throughout the water column at every horizon observed, indicating no avoidance of low salinity waters. In (B), maximum cell abundances were observed at salinities close to the growth salinity.

<5% of the population at 2 (Fig. 5A). *G. dominans* cells aggregated preferentially to the halocline, at a salinity of about 15 (Fig. 5C); however, a substantial proportion of the population (27%) was found at 9–10, the lowest salinity horizon available in the water column given the halocline structure of this experiment. In contrast to the distributions of *H. akashiwo* and the heterotrophic dinoflagellates, the majority of the *Favella* sp. population was observed only in the most saline areas of the tank (Fig. 5B). Throughout the entire experiment, no *Favella* sp. cells were ever observed above the 15 salinity horizon (i.e., at salinities below 15). There were no significant changes in the vertical population distribution of any of the three tested heterotrophic species over the observation period (K-S test, min $P = 0.60$).

When the ciliate predator *Favella* sp. was present in the tank, *H. akashiwo* behavior was altered so that significant predator avoidance behaviors were evident both below and above the halocline. Relative to the predator-free control, *H. akashiwo* below the halocline turned 15% slower and swam 22% faster (Fig. 6A and B). Furthermore, the vertical velocity of *H. akashiwo* was in the upward direction (1.9 ± 0.1 μm · sec^{-1}) in the presence of the predator, compared to effectively zero vertical displacement in the predator-free control (Fig. 6C). Thus, the presence of the predator resulted in more linear swimming paths and faster dispersal in *H. akashiwo* below the halocline. These behaviors allowed the alga to more rapidly access predator-free areas above the halocline. Due to the low salinity, the area above the halocline was inaccessible to the ciliate predator. Nonetheless, presence of the predator in the water column induced significant differences in *H. akashiwo* motility even above the halocline. When the predator was present in the tank, *H. akashiwo* cells above the halocline exhibited more retentive movement behavior, with a 42% increase in turning rate and a 58% reduction in swimming speed (Fig. 6A and B). Vertical velocity above the halocline when the predator was present in the water column was 88% slower than when the predator was absent (Fig. 6C). Thus, the presence of the predator in the tank induced dispersive swimming behavior below the halocline and retentive swimming behavior above the halocline in *H. akashiwo*.

Salinity effects on H. akashiwo *as prey.* Feeding by protist predators was substantially impaired at low salinities (Fig. 7). Ingestion rates of ciliate *Favella* sp. on nontoxic algal prey (the dinoflagellate *H. triquetra*) were maximal at the highest tested salinity of 30, declining to 27% of this value at 20, and to essentially zero at 15. A similar decrease was seen for *S. acuminatum*. Maximum variance in the ingestion rate was seen at a salinity of 25 for both ciliates, perhaps indicating a response threshold; ingestion rates were significantly lower at 20 and 15 than at 30 (Dunnett's *t*-test, $P \leq 0.002$ in all cases).

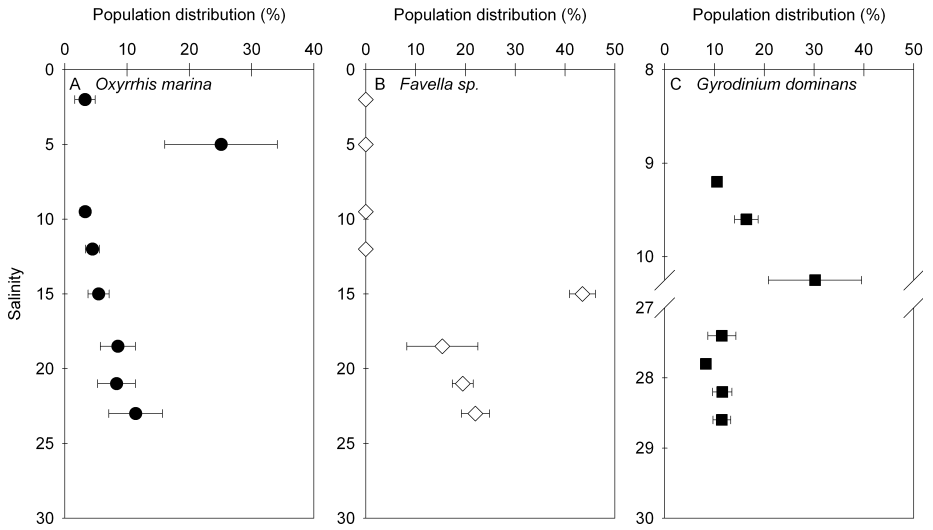

FIG. 5. Vertical distributions of (A) *Oxyrrhis marina*, (B) *Favella* sp., and (C) *Gyrodinium dominans* in salinity gradients ranging from 0 to 30 (means ± 1 SE; $n = 3$). Irrespective of salinity, both dinoflagellates (*O. marina*, *G. dominans*) distributed throughout the water column; however, ciliate *Favella* sp. was restricted to salinities >15.

Heterosigma akashiwo was not toxic to either heterotrophic dinoflagellate tested in this study (black symbols, Fig. 8). Dinoflagellate abundances during incubations increased more in the presence of *H. akashiwo* than in unfed controls (% survival >100), demonstrating that these consumers obtained nutrition from the *H. akashiwo* diet. *O. marina* populations, which showed the largest abundance response, more than doubled over 48 h; growth rates ranged from 0.40 to 0.56 d^{-1} across the tested salinity levels. *N. scintillans* growth rates were lower, ranging from −0.13 to 0.12 d^{-1}. In contrast to the heterotrophic dinoflagellates, *H. akashiwo* was toxic to the three tested ciliate species. All exhibited abundance declines during the incubation period; at most salinities, ciliate mortality was higher when fed *H. akashiwo* than in unfed treatments (% survival <100; Fig. 8).

Did salinity interact with these growth or toxicity responses? For the dinoflagellates, there was no major salinity effect on growth rates (one-way ANOVA: *O. marina*, $F_{4,10} = 0.952$, $P = 0.474$ and for *N. scintillans*, $F_{3,12} = 7.641$, $P = 0.004$; in the latter case, survival on *H. akashiwo* was slightly higher at 25 than at 30, and no other salinity-related effect was seen). These data suggest that neither the dinoflagellate's feeding behavior nor *H. akashiwo* food quality was strongly influenced by reduced salinities. This contrasts with the ciliate responses. Salinity significantly affected survival of all three species on *H. akashiwo* (one-way ANOVA: *Metacylis* sp., $F_{3,12} = 34.401$, $P < 0.004$; *Favella* sp., $F_{3,12} = 7.551$, $P < 0.004$; *S. acuminatum*, $F_{3,11} = 15.368$, $P < 0.004$). In particular, there is a strong suggestion that *H. akashiwo* became more toxic to *S. acuminatum* as salinity decreased: survival on *H. akashiwo* relative to unfed was progressively reduced as salinity declined, from 97% at 30 to 33% at 15 (Fig. 8). Percent survival reductions were significant (relative to 30) at 20 and 15 (Dunett's *t*-test, $P < 0.05$). Survival responses for the other two ciliates did not vary systematically with salinity. Note that ciliate survival at 15 was poor even in unfed treatments, leading to low cell counts and making responses to *H. akashiwo* highly variable and difficult to interpret.

DISCUSSION

Our study provides evidence that broad halotolerance in *H. akashiwo* may have evolved in part as a refuge from predation. This refuge is created through an interplay of at least three mechanisms: (1) a mismatch in the salinity tolerance of *H. akashiwo* and at least some of its protistan predators; (2) physiological and behavioral plasticity in the *H. akashiwo* response to salinity variations, including a strong ability to acclimate to low salinities; and (3) negative effects of low salinity on protist feeding rates and, in one case, on *H. akashiwo* toxicity to protist predators.

The broad salinity tolerance of raphidophytes in general, and *H. akashiwo* in particular, is well documented for isolates from temperate coastal regions around the world (e.g., Tomas 1978, Watanabe et al. 1982, Hosaka 1992, Taylor and Haigh 1993, Zhang et al. 2006). Although strain-specific differences have been noted (Honjo 1993), it seems clear that *H. akashiwo* is a markedly euryhaline species. This has been hypothesized to provide a competitive advantage to *H. akashiwo* in mixed species assemblages (Smayda 1998). The broad halotolerance of raphidophytes has rarely, if ever, been viewed in a food web context. Here, we show that *H. akashiwo*'s wide salinity tolerance, in combination

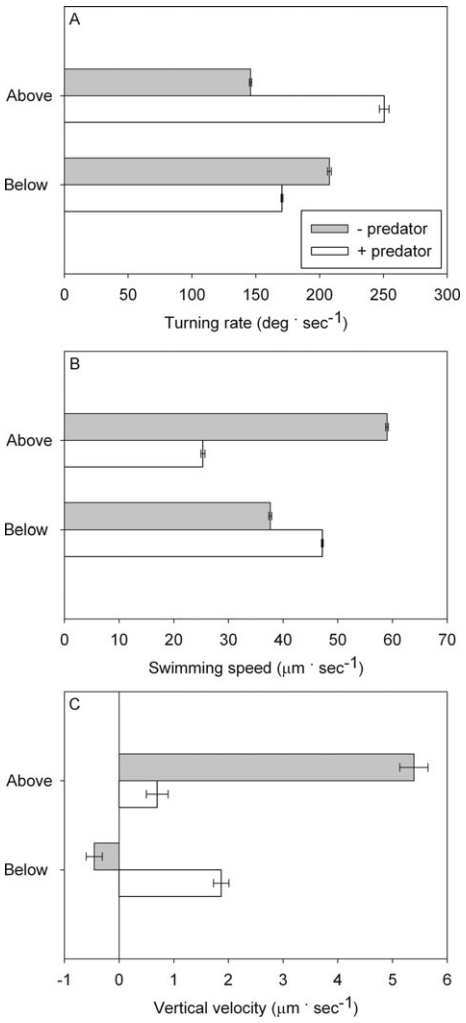

FIG. 6. Fleeing behavior of *Heterosigma akashiwo*: Turning rate (deg · sec^{-1}; a), swimming speed (μm · sec^{-1}; b), and vertical velocity (μm · sec^{-1}; c) of *H. akashiwo* in the absence (gray) and presence (white) of the ciliate predator, *Favella* sp., above and below the halocline. Negative vertical velocities indicate downward swimming. Data were averaged over the entire 6-h experiment time period. Error bars indicate one standard error of the mean.

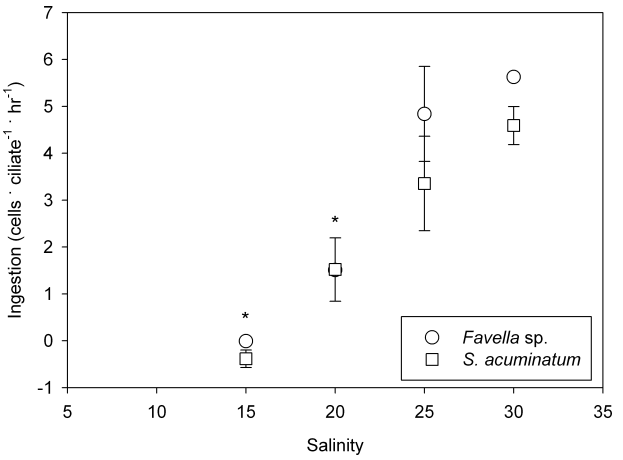

FIG. 7. Ingestion rates (prey cells ciliate^{-1} · hr^{-1}) for ciliates *Favella* sp. and *Strombidinopsis acuminatum* over a range of salinities (Means ± 1 SD, $n = 4$). * rate significantly lower than at 30 (Dunnett *t*-test, $P < 0.05$)

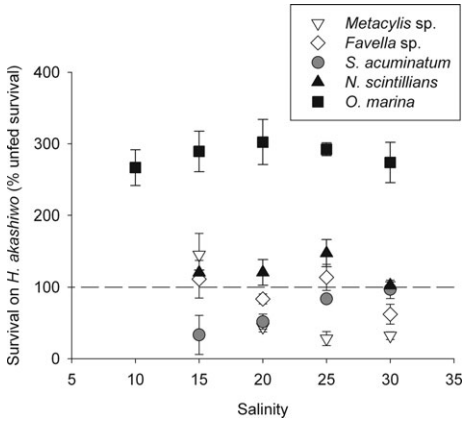

FIG. 8. Microzooplankton growth or mortality responses to *Heterosigma akashiwo* CCMP2809 as a function of salinity. Responses represent survival in the presence of *H. akashiwo* normalized to that in unfed treatments at equivalent salinities: [final cells · mL^{-1} with Ha]/[final cells · mL^{-1} unfed] • 100. Horizontal dashed line at 100% indicates no response to *H. akashiwo* (survival equal to that in unfed treatments). Dinoflagellates (black symbols): *Oxyrrhis marina* (squares) and *Noctiluca scintillans* (triangles). Ciliates (open or gray symbols): *Favella* sp. (diamonds), *Strombisinopsis acuminatum* (gray circles); *Metacylis* sp. (inverted triangles). *H. akashiwo* concentration 10^4 cells · mL^{-1} in all experiments.

with the response of protist predators to salinity variation, creates refuges through a number of mechanisms that act to reduce predation and promote net growth of *H. akashiwo* populations.

Our data demonstrate that *H. akashiwo* and several common and abundant estuarine predator species differ in their tolerance of low salinity conditions. This implies that predator and prey do not occupy the same wide spectrum of salinity habitats; in general, *H. akashiwo* may have access to vastly greater habitat 'space' in a typical estuary than any given predator species. The *H. akashiwo* strains and co-occurring predators studied here all were isolated from Salish Sea waters with salinities of ~30. However, while *H. akashiwo* strains grew at near-maximal rates at salinities as low as 6, protist predators (with the exception of *O. marina*) had substantially reduced survival at salinities below 20. This survival was measured in the absence of food, so represents a direct physiological effect of low salinity on the heterotrophic protists.

Heterosigma akashiwo blooms in the Salish Sea are hypothesized to initiate in low salinity embayments (Taylor and Haigh 1993); these localized populations are thought to seed larger, more extensive blooms as they are transported through the estuary. Based on our findings of a mismatch in *H. akashiwo* and predator salinity tolerance, we hypothesize that the "low

salinity" microzooplankton community characteristic of surface waters in embayments will be replaced by a "high salinity" microzooplankton community as water masses mix and move through the main body of the estuary. Evidence in support of such replacement along estuarine salinity gradients comes from studies of tintinnid ciliates. Although not typically a large component of the protist predator community, their biogeography is better understood than that of the more abundant aloricate ciliates or dinoflagellates. In the Chesapeake Bay, tintinnid species abundant in low salinity waters to the north are largely distinct from species dominant in the higher salinity southern region (Dolan and Gallegos 2001). In a Japanese lagoon system, only a few tintinnid species (all members of the genus *Tintinnopsis*) were abundant across the entire salinity gradient; all others (80% of studied species) were predominantly associated with either low (<20) or high (>20) salinities (Godhantaraman and Uye 2003). Rotifers were almost entirely restricted to salinities <20. Thus, while *H. akashiwo* can sustain near-maximal intrinsic growth rates at all but the lowest salinities encountered in the Salish Sea, an interruption in predation pressure is predicted during the transition from low (<15) to higher (>20) salinities. In addition, there may be an overall gradient in predation pressure from low to higher along the estuarine salinity gradient (e.g., Lehrter et al. 1999). Both of these phenomena will promote an increase in net growth of *H. akashiwo* populations.

Our acclimation experiments suggest that *H. akashiwo* could cope well with major transitions in salinity. Transfers involving a salinity decrease of 27 were survived by all three tested strains, and one (1914) grew at moderate rates immediately after such a transfer. In most cases, acclimation to lower salinities (15) provided an additional buffer against salinity stress, allowing moderate to rapid growth of two strains at very low salinity levels (3). In contrast, four of five studied predator species suffered complete mortality upon transfer through a salinity decrease of 20 units (e.g., from 30 to 10), and evidently would not have tolerated a low salinity (15) acclimation period at all due to negligible survival at that salinity. The coastal marine environment is dynamic, and has frequent intrusions of low salinity water masses (Yin et al. 1997, Halverson and Pawlowicz 2008). Due to the physiological mismatch in salinity tolerance between *H. akashiwo* and common protist predators, low salinity intrusions may provide a window of opportunity during which *H. akashiwo* populations can rapidly increase.

Acclimation also affected the distribution of *H. akashiwo* populations in vertical salinity gradients. Aggregation behavior affords several advantages to *H. akashiwo* populations in terms of escaping predation. These include the tendency to create layers of high cell concentration, which may enhance deterrent properties or increase toxic effects (Verity and Stoecker 1982, Clough and Strom 2005), and the ability to accumulate in low salinity waters inhospitable to some protist predators (see also Hershberger et al. 1997, Bearon et al. 2006). Conversely, aggregation would tend to increase prey encounter rates for tolerant predator species, potentially increasing *H. akashiwo* mortality rates. However, the percent of the population aggregating to low salinity waters was consistently greater for *H. akashiwo* than for any of the three protist predators studied here and, for some predator species, we observed additional negative effects of low salinities on feeding rates and toxicity (see below).

Remarkably, we show that the presence of a ciliate predator enhanced accumulation of *H. akashiwo* in predator-free low salinity waters (Fig. 6; see also Harvey and Menden-Deuer 2012). Compared to a predator-free control, predator exposure changed swimming behaviors in ways that resulted in prolonged residence (reduced dispersal) of algae populations above the halocline. These altered behaviors persisted even when predator exposure was distant in time (h) and space (cm). Predator-induced shifts in behavior imply that algal motility at low salinities is not simply reduced by osmotic stress, as suggested by Bearon et al. (2006), but that *H. akashiwo* remain able to modulate motility in response to predator exposure. In general, this apparent "fleeing" behavior reduces predator-prey encounter rates and thus predation pressure, increasing the potential for *H. akashiwo* to accumulate biomass and form blooms. The behavioral response also constitutes further evidence that predation selects for low salinity tolerance and associated physiological and behavioral capabilities in environments with strong salinity gradients.

Another instance of salinity-related plasticity in *H. akashiwo* was seen in the response to acclimation. In addition to previously discussed changes in growth potential, acclimated algae maintained a preference for their source salinity by preferentially aggregating to the equivalent salinity/depth in an experimental water column. In a dynamic estuary, *H. akashiwo* could potentially maintain position with respect to specific water masses or salinities, exerting considerable control over their distribution. Moreover, such aggregations could contribute to HAB formation that is independent of enhanced net growth rates. Overall, the physiological and behavioral responses we observed for *H. akashiwo* should deepen the predator – prey mismatch between this raphidophyte and its protist predators.

Negative effects of low salinity on feeding-related processes constitute a third mechanism providing a predation refuge for *H. akashiwo*. For the two studied ciliate species, low salinity conditions impaired feeding even more than they reduced survival: relative to salinities of 30, ingestion rates decreased somewhat at 25 and substantially at 20. These responses indicate that entrainment into low

salinity waters will have a doubly negative effect on some protist predators (and thus a doubly enhanced refuge for *H. akashiwo*), combining direct negative effects on survival with even greater relative reductions in feeding rate. We also examined whether low salinities affected the food quality or toxicity of *H. akashiwo*. In agreement with previous findings (e.g., Jeong et al. 2003, Clough and Strom 2005), *H. akashiwo* was not toxic to either dinoflagellate predator. Furthermore, there was no evidence for a salinity effect on food quality. Rather, negative effects of low salinity on *N. scintillans* appeared to be entirely due to direct effects on the dinoflagellate. In contrast, *H. akashiwo* was toxic to all three studied ciliates at almost all salinities. Although at least one ciliate can grow on *H. akashiwo* (Jeong et al. 2002), previous studies have also reported a tendency for the alga to be toxic to large ciliates (Verity and Stoecker 1982, Clough and Strom 2005). Of particular relevance to the low salinity refuge hypothesis is the response of the oligotrich ciliate *S. acuminatum*. *Heterosigma akashiwo* was not toxic to this predator at 30; toxicity then increased linearly with decreasing salinity. There is intriguing evidence from other studies that raphidophyte toxicity may be enhanced at low salinities. Highest *H. akashiwo* toxicity to sea bream was observed at 20, the lowest salinity tested (Haque and Onoue 2002) Similarly, hemolytic activity of *F. japonica* was highest at 15, again the lowest salinity tested (De Boer et al. 2004). Taken together, our results demonstrate that some protist predators will experience reduced growth (due to direct salinity effects) and increased toxicity in blooms of *H. akashiwo* at lower salinities. Together with reductions in predation rate, these responses will promote increased net growth of *H. akashiwo* in lower salinity regions (in either the vertical or horizontal sense) of the estuary.

Predator defenses are generally associated with a cost, leading to ecological and evolutionary tradeoffs between the benefits and costs of exhibiting defensive behavior (Lima 1998). In particular, the ability of *H. akashiwo* to adjust rapidly to salinity changes is thought to require energy expenditures that should decrease growth rates at extreme (high and low) salinities (Zhang et al. 2006 and references therein). However, our results and those of others demonstrate that some *H. akashiwo* strains can survive even in very low salinity waters with no measurable reduction in growth rate. In general, maximum growth rates of *H. akashiwo* are equivalent to maxima of other phytoplankton at comparable temperature and irradiance (e.g., as predicted by the data of Eppley (1972)). Although these observations do not exclude the possibility of a cost (e.g., reductions in cell size), we did not observe an obvious trade-off for *H. akashiwo*'s broad halotolerance. However, all of our experiments were conducted at saturating resource levels. Investigations under limiting light or nutrient levels may reveal a cost, restricting the broad halotolerance of *H. akashiwo* to a subset of environmental conditions and reducing the associated predation refuge. However, resource-saturated *H. akashiwo* apparently acquire a distinct advantage in exploiting low salinity waters, thereby accessing an expanded habitat within the estuary and finding refuge from at least some protist predators.

We examined salinity data from northern Salish Sea embayments, the purported source of large-scale *H. akashiwo* blooms in the region (Taylor and Haigh 1993). In the Salish Sea, large-scale *H. akashiwo* blooms mainly occur in late spring and early summer (late May–early July, Rensel et al. 2010). This coincides with the time of maximum runoff from the Fraser River (e.g., Halverson and

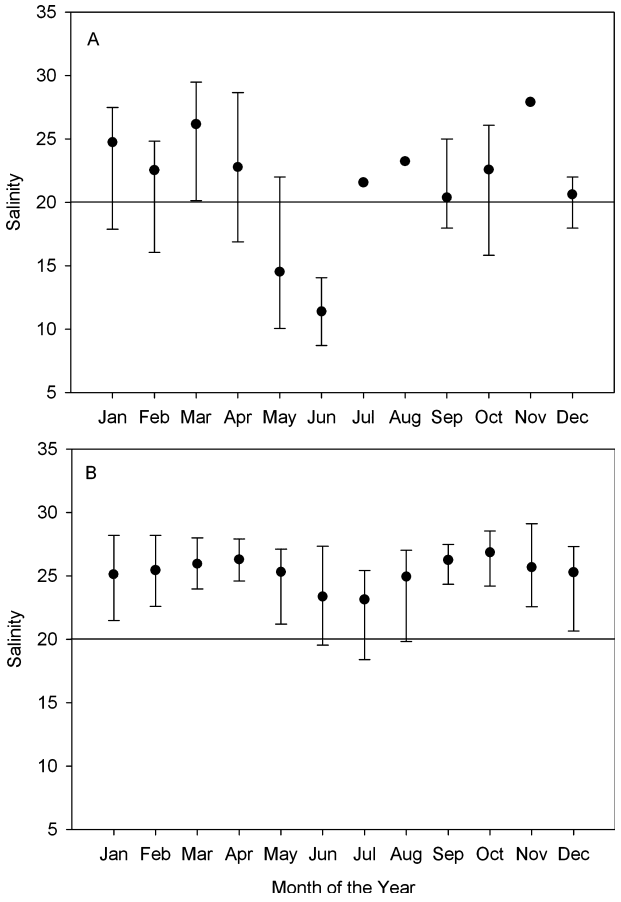

FIG. 9. Surface seawater salinities (monthly means and ranges) over the annual cycle for embayments in the northern Salish Sea (Fig. 1), where many *H. akashiwo* blooms originate. Horizontal lines at 20 show thresholds for salinity impairment of protist predation processes including survival and feeding as demonstrated in this study. (A) Burrard Inlet near the city of Vancouver, data collected 1969–1970, 2004–2005, and 2006–2007 from various shoreline locations. Salinity measured with a refractometer; data replotted from Held and Harley (2009, their Fig. 2) using DataThief III. (B) Departure Bay on the northeast coast of Vancouver Island, data collected ~daily 1980–2011 by Department of Fisheries and Oceans Laboratory, Nanaimo, British Columbia (salinity measured with a hydrometer; data source www.pac.dfo-mpo.gc.ca/science/oceans/data-donnees/lighthouses-phares/index-eng.htm)

Pawlowicz 2008), the largest source of fresh water to the Salish Sea estuary. Sea surface salinity in the northern embayments, the purported source of *H. akashiwo* seed populations (Taylor and Haigh 1993), is at its annual minimum during the May–July period (Fig. 9). These minima correspond to salinity levels that we found to disrupt predator–prey interactions through physiological and behavioral mechanisms. Especially, intriguing is the observation that years of earlier and higher Fraser River runoff – and presumably more extensive excursions of low salinity waters into embayments – are associated with widespread *H. akashiwo* blooms (Rensel et al. 2010). These environmental observations support the contention that broad salinity tolerance in *H. akashiwo* provides a predation refuge, and contributes to the formation of Salish Sea *H. akashiwo* blooms.

How general is the salinity tolerance – predation refuge model proposed here? Is bloom initiation in low salinity waters, and transit to higher salinity waters, a consistent feature of *H. akashiwo* blooms? Our own observations (Menden-Deuer et al. 2010) showed the occurrence of a *H. akashiwo* bloom in surface waters that had decreased by two salinity units (to 27) due to a rain storm. This bloom development was concurrent with a decrease in protistan grazing pressure. However, a subsequent, similar freshening of the water column did not induce a *H. akashiwo* bloom. We concluded that freshening events are a promoter rather than a predictor of *H. akashiwo* blooms. Elsewhere, periods of low – even unusually low – salinity have been associated with *H. akashiwo* blooms in Osaka Bay, Japan (Yamochi and Abe 1984) and off the coast of South Carolina, USA (Kempton et al. 2008). In Hakata Bay in Southern Japan, blooms were sometimes but not always associated with low salinity conditions (Shikata et al. 2008), and Honjo (1993) has reported that Japanese strains in general bloom over a wide range of salinities. One difficulty is that salinity data are not always included in reports of blooms. In addition, environmental measurements are sometimes collected only when blooms are full blown and causing harm. By this time, conditions responsible for bloom initiation and development are past so that environmental conditions correlated with high *H. akashiwo* cell densities may not be indicative of conditions promoting bloom formation.

Currently available data lend support for the hypothesis that broad halotolerance in *H. akashiwo*, and possibly other harmful raphidophytes, is a defensive adaptation that results in significantly lowered predation and thus enhances net population growth and bloom formation potential. Our data suggest a distinctly separate mechanism from hypotheses that link HAB formation solely to nutrient concentration and other factors associated with resource availability. Formal testing of our hypothesis will require a Lagrangian study that examines *H. akashiwo* physiology, growth rate and the various sources of mortality (including predation and viral lysis) as the population transitions through a salinity-structured estuary.

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