J. Phycol. **47,** 25–35 (2011)

DOI: 10.1111/j.1529-8817.2010.00942.x

INTERSTRAIN VARIABILITY IN PHYSIOLOGY AND GENETICS OF *HETEROSIGMA AKASHIWO* (RAPHIDOPHYCEAE) FROM THE WEST COAST OF NORTH AMERICA[1]

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High levels of intraspecific variability are often associated with HAB species, and this variability is likely an important factor in their competitive success. *Heterosigma akashiwo* (Hada) Hada ex Y. Hara et M. Chihara is an ichthyotoxic raphidophyte capable of forming dense surface-water blooms in temperate coastal regions throughout the world. We isolated four strains of *H. akashiwo* from fish-killing northern Puget Sound blooms in 2006 and 2007. By assessing numerous aspects of biochemistry, physiology, and toxicity, we were able to describe distinct ecotypes that may be related to isolation location, source population, or bloom timing. Contrasting elements among strains were cell size, maximum growth and photosynthesis rates, tolerance of low salinities, amino acid use, and toxicity to the ciliate grazer *Strombidinopsis acuminatum* (Fauré-Fremiet). In addition, the rDNA sequences and chloroplast genome of each isolate were examined, and while all rDNA sequences were identical, the chloroplast genome identified differences among the strains that tracked differences in ecotype. *H. akashiwo* strain 07A, which was isolated from an unusual spring bloom, had a significantly higher maximum potential photosynthesis rate (28.7 pg C · cell^{-1} · h^{-1}) and consistently exhibited the highest growth rates. Strains 06A and 06B were not genetically distinct from one another and were able to grow on the amino acids glutamine and alanine, while the other two strains could not. Strain 07B, which is genetically distinct from the other three strains, exhibited the only nontoxic effect. Thus, molecular tools may support identification, tracking, and prediction of strains and/or ecotypes using distinctive chloroplast gene signatures.

Key index words: **genetic variability; growth; *Heterosigma akashiwo*; interstrain differences; microzooplankton toxicity; nitrogen sources; salinity**

Abbreviations: **alpha, photosynthetic efficiencies; dNTPs, deoxynucleoside triphosphates; dpm, disintegrations per minute; E_k, saturating irradiance for photosynthesis; ITS, internal transcribed spacer regions; N, nitrogen; P_{max}, maximum potential photosynthesis rate; rbcL, LSU of the RUBISCO gene; rbcS, SSU of the RUBISCO gene; S, salinity**

H. akashiwo is a bloom-forming raphidophyte distributed in temperate coastal waters throughout the world (Chang et al. 1990, Honjo 1993, Band-Schmidt et al. 2004). Although not associated with human illness, *H. akashiwo* blooms have caused massive fish kills, usually of caged fish (Chang et al. 1990, Honjo 1993, Horner et al. 1997, Khan et al. 1997 and references therein). Although there remains no generally agreed upon mode of ichthyotoxicity for this species, much research has focused on the production of reactive oxygen species (Twiner and Trick 2000), mucus (Nakamura et al. 1998), and neurotoxins (Khan et al. 1997) as potential causes. Diverse environmental factors, including temperature, salinity, and nutrient availability, have been associated with variable *H. akashiwo* toxicity (Chang and Page 1995, Ono et al. 2000, Haque and Onoue 2002a,b, de Boer et al. 2004, Brown et al. 2006). It is likely that toxicity plays a role in HAB dominance in the water column, and *H. akashiwo* can be toxic to planktonic grazers, including ciliates and rotifers (Verity and Stoecker 1982, Egloff 1986, Clough and Strom 2005). However, some zooplankton feed and grow on the raphidophyte (Jeong and Latz 1994, Jeong et al. 2003, 2005, Demir et al. 2008), while others avoid ingesting it under certain conditions (Tomas and Deason 1981, Colin and Dam 2002, Graham and Strom 2010). Evidently, there are a variety of mechanisms by which *H. akashiwo* interacts with potential grazers, and strain variability (see below), environmental effects on toxicity, and differential sensitivity of target species probably all contribute to this variety.

[1]Received 30 March 2010. Accepted 4 August 2010.
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In the Pacific Northwest region of the United States, blooms of *H. akashiwo* are often associated with episodes of high river runoff (Taylor and Haigh 1993). Blooms may initiate at the mouths of rivers where salinities are highly variable and much decreased compared with oceanic salinities (Taylor and Haigh 1993); the ability of *H. akashiwo* to thrive in a wide range of salinities should provide a competitive advantage in these habitats (Tomas 1978, Hosaka 1992, Haque and Onoue 2002a, Zhang et al. 2006). However, in other regions, such as Narragansett Bay on the U.S. northeastern coast, *H. akashiwo* blooms are not consistently associated with low salinities (Smayda 1998). Furthermore, in both Narragansett and the Delaware Inland Bays, summer and fall blooms occur under contrasting temperature and salinity conditions (Tomas 1980b, Zhang et al. 2006, Handy et al. 2008). Such disparities have led to the idea that *H. akashiwo* comprises a variety of ecotypes (Watanabe et al. 1982, Smayda 1998). In support of this idea, interstrain differences have been observed in a number of ecologically important physiological processes. These processes include salinity and temperature optima for growth (Smayda 1998 and references therein), growth rate (Connell and Cattolico 1996), urea utilization (Hosaka 1992), resting cell production (Han et al. 2002), swimming speed (Bearon et al. 2004), hydrogen peroxide production (Twiner et al. 2001), microzooplankton nutrition (Clough and Strom 2005), toxin profiles, and ichthyotoxicity (Khan et al. 2005).

Recent research has shown that high levels of genetic diversity can exist within a single phytoplankton-bloom population, as well as among populations that bloom at different times in the same region (Rynearson and Armbrust 2005, Iglesias-Rodriguez et al. 2006, Rynearson et al. 2006). This diversity may be key to competitive success under variable environmental conditions. Few studies, however, have examined interstrain variability across a broad range of physiological attributes, although successful species must simultaneously deal with a spectrum of environmental pressures and survival challenges. We examined the strain variability of four recent *H. akashiwo* isolates from northern Puget Sound: two strains isolated from the same bloom in June 2006, and two strains isolated from separate bloom events in 2007. Our study identified substantial phenotypic variability among the four isolates and determined that differences among phenotypes were associated with differences in the chloroplast genome. Our results suggest that Pacific Northwest waters do harbor distinct *H. akashiwo* ecotypes.

MATERIALS AND METHODS

Cultures. Four strains of *H. akashiwo* were isolated from northern Puget Sound: *H. akashiwo* 06A (CCMP2808) and 06B (CCMP2809) were collected on 29 June 2006, from Guemes Channel, Washington, USA (13.8°C, salinity = 27.4); *H. akashiwo* 07A (CCMP3149) was collected on 30 May 2007, from Lummi Bay, Washington, USA (collection temperature and salinity unknown); and *H. akashiwo* 07B (CCMP3150) was collected on 16 July 2007, from East Sound Washington, USA (15.6°C, salinity = 27.8; Fig. 1). Algae were maintained in f/2 medium without added silicate (Guillard and Ryther 1962) at a salinity of 30, incubated at 15°C and 100–160 μmol photons $\cdot$ $m^{-2} \cdot s^{-1}$ (12:12 light:dark [L:D] cycle). Three heterotrophic protist species were isolated from northern Puget Sound, Washington, USA: tintinnids *Metacylis* sp. (SPMC125) and *Favella ehrenbergii* (SPMC133), and the aloricate choreotrich *S. acuminatum*

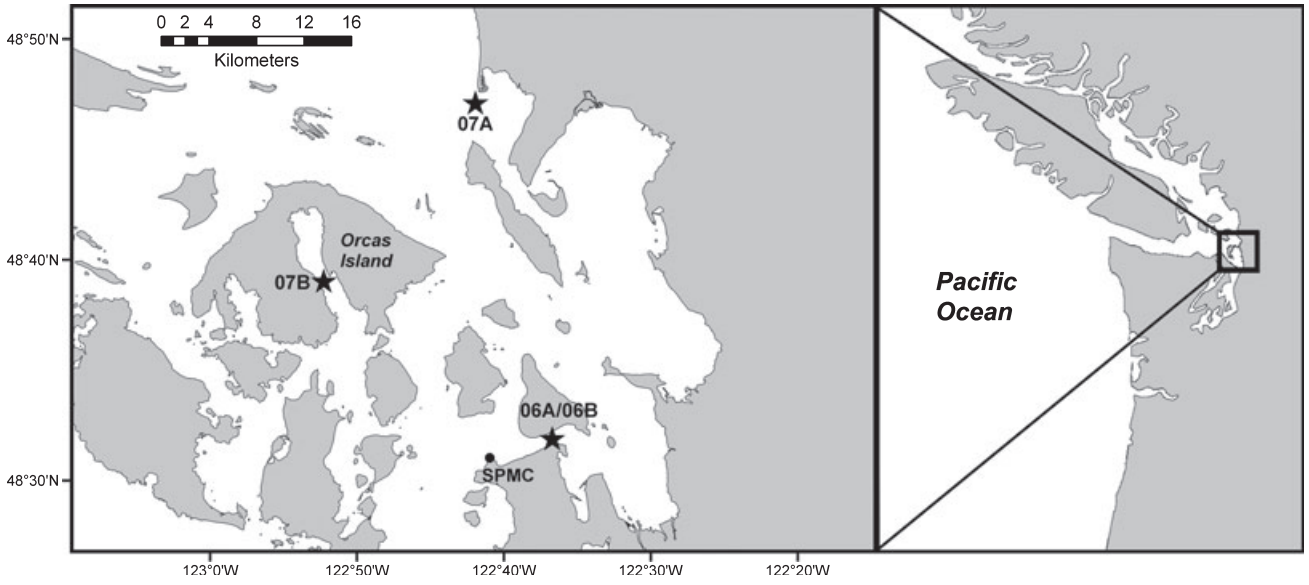

FIG. 1. Map of the San Juan Island Archipelago in coastal inland waters of Washington State (left panel), shown in relation to the Pacific Northwest region of the United States and Canada. Stars indicate the collection locations of four *Heterosigma akashiwo* strains used in this study.

(SPMC114). Heterotrophs were maintained on phytoplankton prey mixtures at 15°C in ''ciliate medium'' (Gifford 1985), which was 0.2 μm autoclaved filtered seawater (salinity = 30) enriched with a dilute trace-metal mixture, and kept in dim light at 0.5–4 μmol photons · m^{-2} · s^{-1}.

Biochemical measurements. Biovolume was estimated by preserving *H. akashiwo* cells in early-to-midstationary phase with $HgCl_2$ (0.2% final concentration), then photographing 20 cells of each strain using ImageJ (version 1.40g; National Institute of Health, Bethesda, MD, USA). Mercuric chloride was used for cell preservation because we have observed chloroplast ''shedding'' over a range (<0.1%–10%) of acid Lugol's concentrations. While mercuric chloride has been shown to cause slightly more cell shrinkage than acid Lugol's solution (Woelfl and Whitton 2000), it acts as an excellent preservative while maintaining cellular structure (Pace and Orcutt 1981). Cellular carbon (C) and nitrogen (N) content were determined by filtering measured culture volumes onto precombusted (450°C, 4 h) 13 mm GF/F filters (Whatman International Ltd., Maidstone, UK), which were then dried for 24–48 h at 50°C. Filters were stored in a desiccator until analysis on a Flash 1112 Elemental Analyzer (CE Instruments; Hindley Green, Wigan, UK) with standards made from aspartic acid and atropine. For chl analysis, 10 mL of each culture was filtered in duplicate onto glass fiber filters (GF/F, 25 mm). Chl samples were extracted in 90% acetone (−20°C, dark, 24 h) and analyzed fluorometrically (Turner Designs 10AU, Sunnyvale, CA, USA) using the acidification method (Welschmeyer 1994).

Measurement of photosynthetic parameters. ^{14}C-labeled sodium bicarbonate (Moravek Biochemicals Inc., Brea, CA, USA) was used to generate photosynthesis–irradiance (*P–E*) curves to determine maximum potential photosynthesis (P_{max}), photosynthetic efficiencies (alpha), and saturating irradiance for photosynthesis (E_k). ^{14}C bicarbonate (2 μCi) was added to 8 mL culture of each strain (in triplicate), then distributed as 0.5 mL aliquots into eight glass scintillation vials (7 mL volume) and placed in a temperature-controlled photosynthetron (CHPT Mfg. Inc., Georgetown, DE, USA), which housed two 250 W Quartzline® lamps (Bulb Direct, Pittsford, NY, USA) and was maintained at 15°C. Average total activity was determined by subsampling 0.1 mL from two randomly selected vials from each of the four strains and adding 5 mL Ecoscint scintillation cocktail (National Diagnostics, Atlanta, GA, USA) buffered with 0.75 mL 0.1 M NaOH. Vials were exposed to eight light levels over a range of irradiances (0–697 μmol photons · m^{-2} · s^{-1}) and allowed to incubate for ~0.5 h. Incubations were terminated by acidification of each sample with 0.5 mL 1 N HCl. After 24 h, vials were neutralized with 0.5 mL 1 N NaOH. Scintillation cocktail (Ecoscint, 5 mL) was added, and disintegrations per minute (dpm) were measured using a Packard 1900 TR scintillation counter (Meriden, CT, USA). For each vial, dpm were converted to photosynthesis rates (μg C · mL^{-1} · h^{-1}) according to Parsons et al. (1984). P_{max} and alpha were derived by fitting the photosynthesis rates and corresponding irradiance (*E*) to the hyperbolic tangent function of Jassby and Platt (1976) using Sigma Plot 8.0 (Systat Software, Chicago, IL, USA). Saturating irradiance for photosynthesis (E_k), which represents the optimal irradiance for photosynthesis (Sakshaug et al. 1997), was calculated from P_{max}/α.

The effect of salinity on H. akashiwo *growth.* We inoculated two *H. akashiwo* batch cultures (salinity = 15 and 30), both grown in 150 mL polycarbonate bottles, to avoid osmotic shock when transferring *H. akashiwo* grown in ambient seawater (salinity = 30) to lower salinity media. The 15 salinity culture was made using a 1:1 dilution of 30 salinity f/2 medium and medium made from ultrapure water enriched with f/2 nutrients. The batch cultures were placed in an incubator for ~2 weeks, and cells were allowed to grow and acclimate. *H. akashiwo* cells from the batch culture with a salinity of 15 were then used to initiate salinity treatments of 0, 5, 10, and 15; similarly, the batch culture with a salinity of 30 was used to initiate the 20, 25, and 30 psu treatments. Once treatments were established at an initial *H. akashiwo* concentration of 2,000 cells · mL^{-1} in 200 mL Erlenmeyer flasks, initial salinity measurements were made. Subsamples (30 mL) from each salinity treatment were immediately transferred to quadruplicate 50 mL glass culture tubes, and in vivo fluorescence readings were taken daily for 2 weeks using a Turner 10AU fluorometer. Cultures were maintained on a 12:12 L:D cycle at 15°C at 93–159 μmol photons · m^{-2} · s^{-1}. The maximum growth rate for each replicate was estimated from the linear portion of ln (fluorescence) versus time.

N-source growth experiments. To determine the effect of different N sources on the growth of *H. akashiwo*, strains were grown in a three-stage, batch culture procedure. (i) Less than 0.5 mL of *H. akashiwo* culture maintained in f/2 medium was inoculated in 100 mL standard (176 μM N) f/10 medium and grown to early stationary phase (10 d). All the f/10 medium used in this and subsequent stages of N-source experiments was prepared using N-depleted seawater (preparation method described below) to control N concentrations and ensure N limitation of *H. akashiwo* growth. (ii) Using this f/10-grown culture as an inoculum, triplicate batch cultures of each strain were initiated in low-N (15 μM) f/10 medium. Culture volumes were 310 mL, and initial *H. akashiwo* concentrations were 500 cells · mL^{-1}. In vivo fluorescence was measured by removal of 4 mL culture each day at 12:30 h (± 1.5 h) using a Turner 10AU fluorometer, until cultures were N depleted and in early stationary phase (9 d, except 8 d for 07A). (iii) Subsamples (30 mL) from each low-N culture above were then placed in 35 mL glass tubes and spiked with 0.1 mL of aqueous solution containing an N source to be tested. This procedure yielded triplicate treatments for each strain and N source. Initial N concentrations were 50 μM for all N sources. A control (no N) treatment was also used, which received a 0.1 mL spike of ultrapure water. Subsequent growth measurements and specific growth rate calculations were as described by Strom and Bright (2009).

Nitrogen-depleted seawater for the above-described media was obtained by placing locally collected seawater in high light (100 μmol photons · m^{-2} · s^{-1}) to promote phytoplankton blooms; these stripped out all measurable nitrate. N-deplete seawater was then passed through a 0.2 μm filter and enriched with f/10 nutrients to standard (176 μM N) or low (15 μM N) nitrate levels. Preliminary experiments were performed to ensure phytoplankton-bloom water did not cause deleterious effects on phytoplankton growth; cultures grown in bloom and nonbloom f/10 had nearly identical growth rates of 0.60 d^{-1} (±0.01) and 0.59 d^{-1} (± 0.01), respectively.

H. akashiwo *toxicity to protist grazers.* Three species of protist grazers were tested for their survival response to the four strains of *H. akashiwo*: tintinnids *F. ehrenbergii* and *Metacylis* sp., and the aloricate choreotrich *S. acuminatum*. *F. ehrenbergii* was separated from its maintenance prey by sieving through a 20 μm mesh, followed by resuspension in fresh ciliate medium. *Metacylis* sp. and *S. acuminatum* were separated from their prey by reverse concentration, in which most of the medium surrounding the ciliates was siphoned off using an 11 and 20 μm mesh, respectively; ciliates were then resuspended in prey-free ciliate medium. Ciliates were separated from their maintenance prey 3 h (*F. ehrenbergii* and *Metacylis* sp.) or 5 h (*S. acuminatum*) before experiments commenced.

Algal prey were grown in batch culture (see Cultures) and used during mid- to late-exponential phase. *H. akashiwo* was added to achieve a concentration of 10,000 cells · mL^{-1}, the highest concentration measured during a 2006 Puget Sound *H. akashiwo* bloom that resulted in fish mortality (Rensel 2007).

Phytoplankton prey species used as a positive control (*Isochrysis galbana* for *Metacylis* sp., *Heterocapsa triquetra* for *F. ehrenbergii* and *S. acuminatum*) were added at levels known to saturate feeding rates (400 μg C · L^{-1}). Phytoplankton were enumerated using a hemocytometer (*I. galbana*) or a Sedgewick-Rafter chamber (*H. akashiwo* and *H. triquetra*; SPI Supplies, West Chester, PA, USA).

Ciliate density was estimated by counting live cells in replicate droplets of known volume under an Olympus SZ-40 dissecting microscope (Olympus Optical Company, Tokyo, Japan); ciliates were then added to quadruplicate polycarbonate bottles (incubation volumes of 50 mL for *S. acuminatum*, 130 mL for *F. ehrenbergii*) or polystyrene tissue flasks (45 mL incubation volume for *Metacylis* sp.). Average initial ciliate concentrations were as follows: *Metacylis* sp., 10.1; *S. acuminatum*, 6.3; and *F. ehrenbergii*, 3.0 cells · mL^{-1}. Six diet treatments were used: (i) unfed (=ciliate medium only), (ii) positive control, (iii) *H. akashiwo* 06A, (iv) *H. akashiwo* 06B, (v) *H. akashiwo* 07A, and (vi) *H. akashiwo* 07B. Bottles were incubated at 15°C with a 12:12 L:D cycle at 3.3–8.3 μmol photons · m^{-2} · s^{-1} and at a salinity of 30 (*Metacylis* sp. and *F. ehrenbergii*) or 20 (*S. acuminatum*). Previous experiments had shown that *H. akashiwo* is toxic to *S. acuminatum* at a salinity of 20, but not at 30. A salinity of 20 was achieved by adding ultrapure water to 0.2 μm autoclaved filtered seawater.

Because the agent of toxicity to planktonic grazers is unknown for *H. akashiwo*, we chose to define toxicity based on the biological response of co-occurring species. Toxicity was defined as survival less than that of the unfed control (Clough and Strom 2005). Ciliate survival was calculated from cell loss; ciliates disappear soon after death, making cell loss a suitable measurement of mortality. Samples (20 or 60 mL, depending on the ciliate species) were taken initially and after 24 h and were fixed with acid Lugol's solution (10% final concentration). Each preserved sample was transferred to a 10 mL settling chamber and enumerated using inverted microscopy (transmitted light illumination; Wild M40, Wild Heerbrugg, Gais, Switzerland). Percent survival was calculated as follows:

$$[(\text{final ciliates} \cdot \text{mL}^{-1})/(\text{initial ciliates} \cdot \text{mL}^{-1})] \times 100 \quad (1)$$

Determination of rDNA and chloroplast gene sequences for H. akashiwo. DNA was extracted from each of the four strains of *H. akashiwo*: 06A, 06B, 07A, and 07B. Cells were collected on a 1.0 μm pore-size polycarbonate filters (GE Osmonics Labstore, Minnetonka, MN, USA) and stored frozen at −70°C until lysed in 0.7 mL cetyltrimethylammonium bromide (CTAB) buffer (100mM Tris-HCl [pH 8], 1.4 M NaCl, 20mM EDTA, 2% [w/v] CTAB, 0.4% [v/v] β-mercaptoethanol, 1% [w/v] polyvinylpyrollidone; Dempster et al. 1999), and DNA was extracted as described in Coyne et al. (2001). The 18S rDNA through the 5.8S rDNA region (including the ITS1 [internal transcribed spacer1] region) was amplified by PCR in 50 μL reactions containing 50 ng of template DNA, 0.2 mM deoxynucleoside triphosphates (dNTPs), 0.5 μM of primer Stram 9F (5′ CTGCCAGTAGTCATACGCTC 3′), 0.5 μM of primer Raph-ITS-R2 (5′ AAGTGCCAGGTGCGTTCGAA 3′ [modified from Connell 2002]), 2.5 mM $MgCl_2$, 1× Taq polymerase buffer (Sigma Chem. Co., St. Louis, IL, USA), and 0.5 units Jump-Start Taq Polymerase (Sigma Chem. Co.). The reaction consisted of 33 cycles of 30 s at 94°C, 30 s at 56°C, and 2.5 min at 72°C, followed by a 5 min extension at 72°C.

Approximately 4 kb of the *Heterosigma* chloroplast genome was also amplified from DNA extracted from duplicate cultures of each strain, using primers described in Akase et al. (2004). This region included the LSU of the RUBISCO gene (rbcL), SSU of the RUBISCO gene (rbcS), and a portion of the cfxQ gene, along with intergenic regions. DNA was amplified in 50 μL reactions containing 50 ng of template DNA, 0.2 mM dNTPs, 0.5 μM of primer PlrbcLU.2F (5′ GTGCGATTCATTACTGTAG 3′), 0.5 μM of primer PlrbcS.1R (5′ CGCACCAATGTTACGACT 3′; modified from Akase et al. 2004), 2.5 mM $MgCl_2$, 1× Taq polymerase buffer (Sigma Chem. Co.), and 0.5 units Jump-Start Taq Polymerase (Sigma Chem. Co.). The reaction consisted of 30 cycles of 30 s at 94°C, 30 s at 50°C, and 2 min at 72°C, followed by a 5 min extension at 72°C.

All PCR products were purified using the GenElute PCR Cleanup kit (Sigma Chem. Co.) and sequenced using Big Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystem, Foster City, CA, USA).

Statistical analysis. An analysis of variance (ANOVA) was used to determine significant differences among treatments, and Tukey's honestly significant difference was used to determine significant pair-wise differences between treatments (SPSS version 16.0, Chicago, IL, USA). For all statistical analyses, $\alpha = 0.05$.

RESULTS

The four strains of *H. akashiwo* isolated from northern Puget Sound were indistinguishable in their biochemical composition and in most photosynthetic parameters. Carbon densities ranged from 162 to 179 pg C · $cell^{-1}$, C:N ratios from 5.2 to 5.6, chl content from 4.9 to 5.6 pg chl · $cell^{-1}$, photosynthetic efficiencies from 0.12 to 0.13 pg C · $cell^{-1}$ · h^{-1}/μmol photons · m^{-2} · s^{-1}, and E_k values from 165 to 216 μmol photons · m^{-2} · s^{-1} (Table 1). The strains did differ in their maximum photosynthetic rates. Strain 07A, which was isolated during a spring 2007 bloom, had a P_{max} 27%–32% higher than the other three strains (P_{max} = 28.7 vs.

TABLE 1. Biochemical and photosynthetic parameters for four Pacific Northwest *Heterosigma akashiwo* strains.

Heterosigma strain	Biovolume	C density	C:N	pg chl · $cell^{-1}$	P_{max}	Alpha	E_k
06A	3,071 (1,082)[a]	162 (22)[a]	5.5 (0.49)[a]	5.6 (0.2)[a]	19.9 (2.6)[a]	0.12 (0.01)[a]	169 (18)[a]
06B	3,964 (1,434)[a,b]	179 (18)[a]	5.4 (0.45)[a]	5.3 (0.2)[a]	21.0 (0.7)[a]	0.13 (0.01)[a]	168 (13)[a]
07A	3,295 (1,902)[a]	175 (28)[a]	5.2 (0.54)[a]	4.9 (0.6)[a]	28.7 (1.7)[b]	0.13 (0.01)[a]	216 (23)[a]
07B	4,850 (2,189)[b]	163 (22)[a]	5.6 (0.01)[a]	5.1 (0.4)[a]	19.4 (2.3)[a]	0.12 (0.03)[a]	165 (35)[a]

Shown are biovolume (μm^3), carbon (C) density (pg C · $cell^{-1}$), C:N ratio, chl content (pg chl · $cell^{-1}$), maximum potential photosynthesis rate (P_{max}, pg C · $cell^{-1}$ · h^{-1}), photosynthetic efficiencies (alpha, pg C · $cell^{-1}$ · h^{-1}/μmol photons · m^{-2} · s^{-1}), and light saturation parameter (E_k, μmol photons · m^{-2} · s^{-1}). Common letters within each parameter indicate no statistical difference among strains ($P > 0.05$). Standard deviation is shown in parentheses; $n = 3$ for all, except for biovolume ($n = 20$) and chl content and 07A and 07B C:N ($n = 2$).

19.4–21.0 pg C · cell^{-1} · h^{-1}; $P = 0.003$). Although the differences were not significant, 07A also had the highest optimal irradiance for photosynthesis with an E_k of 216 μmol photons · m^{-2} · s^{-1} as compared to a range of 165–169 μmol photons · m^{-2} · s^{-1} for the other three strains. Isolates also differed in size. Strain 07B, which was obtained from a small fjord, had a significantly larger biovolume (4,850 μm^3) compared to 07A (3,295 μm^3) and 06A (3,071 μm^3; $P = 0.026$ and 0.008, respectively).

Growth rates were measured over a wide range of salinities (0–30) for each isolate. Northern Puget Sound *H. akashiwo* grew between salinities of 5 and 30, although all strains showed reduced growth rates at a salinity of 5 (Fig. 2); none of the isolates grew at 0 (data not shown). Interstrain differences were seen in the maximum growth rates achieved and in the response to low and high salinities. Strains 06B and 07A had the highest maximum growth rates of 0.66 and 0.60 d^{-1}, respectively (rates averaged across salinities 10–25 for 06B, and 10–30 for 07A; see Fig. 2). Lower maximum growth rates of 0.46 d^{-1} (salinity range 15–30) and 0.48 d^{-1} (salinity range 10–30) were measured for 07B and 06A, respectively. Strain 06B was the only one to show a slight growth-rate depression at the highest salinity of 30, while 07B was unique in its limited ability to grow at a salinity of 10.

All four *H. akashiwo* strains grew well on nitrate, ammonium, and urea (Fig. 3). None of the four strains grew on hypoxanthine, ethanolamine, and acetamide (data not shown). Growth rates in the varying N sources were lower than those grown over a range of salinities (Fig. 2; see above paragraph). This trend was most likely due to the relatively high *H. akashiwo* concentrations at the beginning of the N-source experiments, combined with low levels of N (f/10 nutrients with 50 μM N, compared to the f/2 medium [882 μM] used to test the effect of decreasing salinity). For 07A, there was no significant difference in growth among the five N sources shown in Figure 3 ($P = 0.152$); growth rates for this strain ranged from 0.55 d^{-1} (nitrate) to 0.26 d^{-1} (urea). The data suggest that 07B grew best on organic N sources. Highest growth rates occurred in the urea and alanine treatments (0.27 d^{-1}), and only the ammonium-treatment cultures exhibited growth rates that were significantly different from urea ($P = 0.024$) and alanine ($P = 0.027$). A striking result was the inability of the two 2006 strains (06A and 06B) to grow on glutamine and alanine. Growth rates on nitrate, ammonium, and urea for these strains were quite similar (0.25–0.29 d^{-1}).

Interstrain differences in toxicity were observed in our experiments with planktonic ciliates, but only with one of the three ciliate species. *H. akashiwo* strain 07B was nontoxic to the ciliate *S. acuminatum* (Fig. 4a); that is, survival in the presence of 07B was no different than in the unfed treatment. For the other three *H. akashiwo* strains, even though all treatments had >100% survival, the percent survival was significantly less than in the unfed treatment ($P < 0.004$), demonstrating toxicity. All four strains of *H. akashiwo* were toxic to the tintinnids *Metacylis* sp. and *F. ehrenbergii* (Fig. 4, b and c, respectively), with no significant differences observed among the four strains ($P = 0.084$ and 0.619). The strength of the toxic effect, however, depended on ciliate species. *H. akashiwo* was more toxic to *F. ehrenbergii* (19%–28% survival) than to *Metacylis* sp. (25%–47%

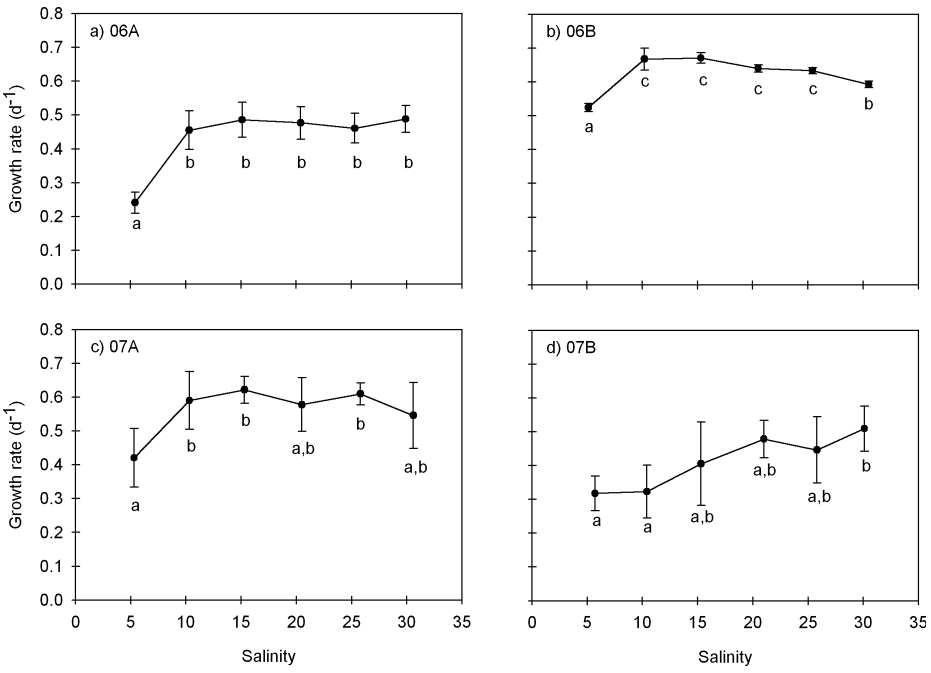

FIG. 2. Growth rates (d^{-1}) of *Heterosigma akashiwo* strains 06A (a), 06B (b), 07A (c), and 07B (d) over a range of salinities. Common letters denote no statistical difference among strains ($P < 0.05$). Error bars: ±1 standard deviation; $n = 4$.

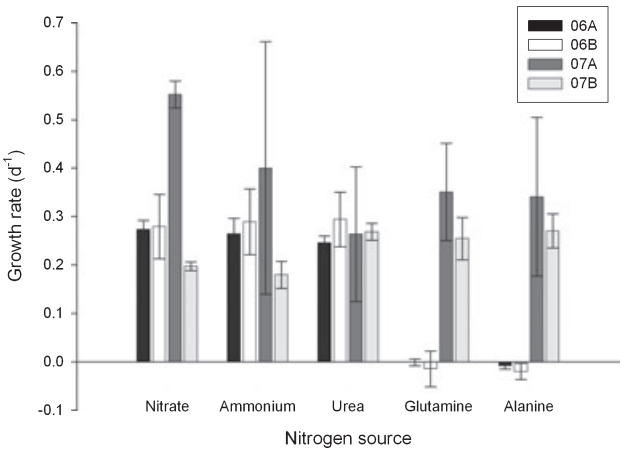

FIG. 3. Growth rates (d^{-1}) of four *Heterosigma akashiwo* strains on different nitrogen sources. Error bars: ±1 standard deviation; $n = 3$. *H. akashiwo* did not grow on hypoxanthine, ethanolamine, and acetamide (data not shown).

survival). There was no difference between the unfed and control treatments for *S. acuminatum* and *Metacylis* sp., but *F. ehrenbergii* did significantly better when fed *I. galbana* in the control treatment (166% survival), compared to the unfed treatment (99% survival, $P < 0.001$).

A portion of the 18S rDNA was sequenced from each *H. akashiwo* strain yielding 335 bp from the 5′ end and 413 bases from the 3′ end of the of the 18S rDNA. In addition, the entire ITS1 region (175 bases) and 112 bases from the 5′ end of the 5.8 rDNA were sequenced. The sequences obtained were identical for all four strains. Strain-specific sequence differences were found in the chloroplast genome, however (Fig. 5). DNA sequences from duplicate cultures of each strain were identical to each other. Between strains, however, three sequence differences were identified: two within the intergenic region between the trnL and rbcL genes, and a third site within the intergenic region between the rbcS and cfxQ genes. The first site may be used to distinguish strain 07B from the other three strains (C vs. T; Fig. 5a). Alignment with other chloroplast sequences available through GenBank also demonstrates that *H. akashiwo* strain 07B is genetically distinct at this site from other strains that have been sequenced. The second site distinguishes strains 07B and 07A from strains 06A and 06B (C vs. T; Fig. 5a). Interestingly, the chloroplast genome sequence from strain 06B appears to be polymorphic at this site as well: both C and T are evident in the sequence trace file at this position, suggesting that all four strains may be distinguished by sequence differences at these two sites. A third sequence difference was identified in the intergenic region between rbcS and cfxQ. This site also distinguishes *H. akashiwo* strains 07A and 07B from 06A and 06B (ATTTA vs. TAAAT; Fig. 5b). All sequences were deposited in GenBank

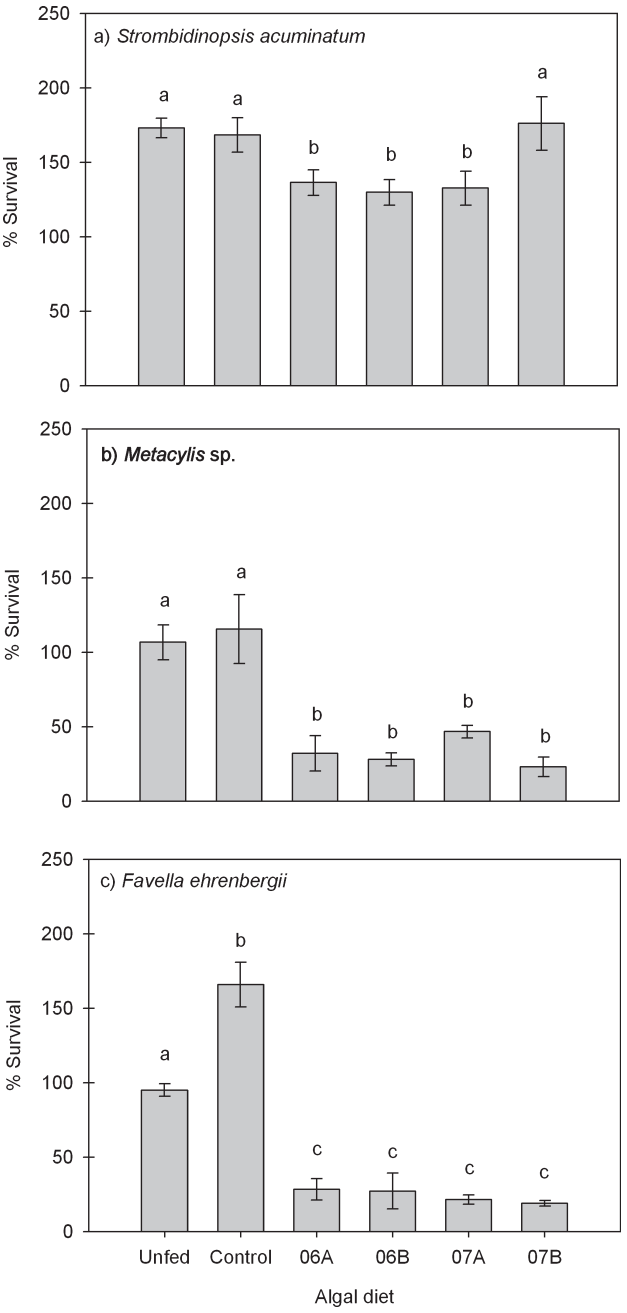

FIG. 4. Percent survival of ciliate grazers (a) *Strombidinopsis acuminatum*, (b) *Metacylis* sp., and (c) *Favella ehrenbergii*. Common letters indicate no statistical difference ($P < 0.05$). Error bars: ±1 standard deviation; $n = 4$.

under accession numbers HM439791–HM439794 and HM570037–HM570046.

DISCUSSION

We found substantial phenotypic variation among four Pacific Northwest *H. akashiwo* strains. Strains differed in numerous important ecophysiological properties, including cell size, maximum photosynthesis

a)
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CGGTGTTTACTTCCTTAAGTAAAACATTTGAGGTAAAATCAGCTCGCCAT K95-Ho
CGGTGTTTACTTCCTTAAGTAAAACATTTGAGGTAAAATCAGCTCGCCAT NIES-5
CGGTGTTTACTTCCTTAAGTAAAACATTTGAGGTAAAATCAGCTCGCCAT 07A
CGGTGTTTACTTCTTTAAGTAAAACATTTGAGGTAAAATCAGCTCGCCAT 07B
CGGTGTTTACTTCCTTAAGTAAAACATTTGAGGTAAAATCAGCTCGCCAT 06A
CGGTGTTTACTTCCTTAAGTAAAACATTTGAGGTAAAATCAGCTCGCCAT 06B

AGAGGTGAACTATCTTTTTTTACAAGTTATAAAGATTACAATTATGAGAT K95-Ho
AGAGGTGAACTATCTTTTTTTACAAGTTATAAAGATTACAATCATGAGAT NIES-5
AGAGGTGAACTATCTTTTTTTACAAGTTATAAAGATTACAATTATGAGAT 07A
AGAGGTGAACTATCTTTTTTTACAAGTTATAAAGATTACAATTATGAGAT 07B
AGAGGTGAACTATCTTTTTTTACAAGTTATAAAGATTACAATCATGAGAT 06A
AGAGGTGAACTATCTTTTTTTACAAGTTATAAAGATTACAATCATGAGAT 06B
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b)
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ATTCTGGTGCTTTTAAATAAAGCACCAGAAT   K95-Ho
ATTCTGGTGCTTTATTTAAAAGCACCAGAAT   NIES-5
ATTCTGGTGCTTTTAAATAAAGCACCAGAAT   07A
ATTCTGGTGCTTTTAAATAAAGCACCAGAAT   07B
ATTCTGGTGCTTTATTTAAAAGCACCAGAAT   06A
ATTCTGGTGCTTTATTTAAAAGCACCAGAAT   06B
```

FIG. 5. Alignment of *Heterosigma akashiwo* strains K95-Ho, NIES-5, 07A, 07B, 06A, and 06B. Nucleotides that differ from *H. akashiwo* strain K95-Ho are boxed. (a) Alignment of *trnl-rbcl* intergenic spacer region, nucleotides 671–770 (from *H. akashiwo* strain K95-Ho chloroplast sequence, GenBank accession number AB176659). (b) Alignment of *rbcS-cfxQ* intergenic spacer region, nucleotides 3,804–3,834 (from *H. akashiwo* strain K95-Ho chloroplast sequence, GenBank accession number AB176659).

and growth rates, salinity tolerance, and N use (Table 2). In addition, one strain showed reduced toxicity to a ciliate predator. Strain differences have been previously observed for this alga, including for the following parameters investigated in this study: growth rate (Connell and Cattolico 1996), urea utilization (Hosaka 1992), and microzooplankton toxicity (Clough and Strom 2005). However, few if any studies have simultaneously investigated interstrain variability in a range of characteristics and processes, as is necessary to assess ecotype and relative competitive ability. Instead, the idea that *H. akashiwo* as a species comprises multiple ecotypes has arisen either through post hoc comparison of studies done by different investigators (e.g., Watanabe et al. 1982) or through observation of the diversity of environments that support *H. akashiwo* blooms (Tomas 1980a,b, Honjo 1993, Zhang et al. 2006). Smayda (1998) documented the worldwide temperate-zone distribution of the species and noted the diversity of environments supporting blooms. He placed these environments into three categories (physically, chemically, and biologically dominated) and suggested that distinct *H. akashiwo* ecotypes thrive in each habitat type. The spectrum of ecophysiological differences that we observed (Table 2), and their relationship with differences in chloroplast genome sequence, fully supports the idea that even within a geographic region, strongly contrasting ecotypes with different competitive strategies are present within the *H. akashiwo* population.

The summer 2007 isolate (07B) was notably larger than the other three, but the strains did not differ in bulk biochemical properties including C density, C:N ratio, and chl content. Strain 07A, which was isolated from an unusually early 2007 *H. akashiwo* bloom, had a P_{max} that was significantly higher than the three other strains. High growth potential generally characterized this strain, which also achieved high growth rates in salinity tolerance and N-source experiments. Carbon density and photosynthetic parameters measured for the four Pacific Northwest isolates were comparable to those determined by Tomas (1980a,b) for a Narragansett Bay isolate of *H. akashiwo* (then called *Olisthodiscus luteus*). While our P_{max} rates were approximately three times higher than those published for the Delaware Bay isolate CCMP2393 (Fu et al. 2008), the photosynthetic efficiencies were similar, as was cellular carbon content.

Our Pacific Northwest *H. akashiwo* isolates showed the broad salinity tolerance that is characteristic of the species (Tomas 1978, Connell and Jacobs 1999, Haque and Onoue 2002a, Zhang et al. 2006) and of raphidophytes generally (Marshall and Hallegraeff 1999, Haque and Onoue 2002b). Not all isolates, however, were equally tolerant of low salinities. Most showed maximal growth rates between salinities of 10 and 30 with only modest reductions at 5, but 07B growth rates at 10 were also reduced. The idea that different *H. akashiwo* populations are adapted to lower and higher salinity regimes has arisen from laboratory studies showing different salinity maxima for growth (Tomas 1978, Watanabe et al. 1982, Haque and Onoue 2002a) and from field observations of bloom occurrence under contrasting salinity conditions (Honjo 1993 and references therein, Zhang et al. 2006). Our data indicate that, even within the Pacific Northwest

TABLE 2. Comparison of characteristics of four northern Puget Sound *Heterosigma akashiwo* strains.

Feature	06A	06B	07A	07B
Size and composition				Larger
P–E parameters			High P_{max}	
Salinity – growth response		High max μ	High max μ	Low μ at S = 10
N source – growth response	No growth: amino acids	No growth: amino acids	High max μ (on NO_3)	Grew well on DON
Toxicity				Not toxic to *Strombidinopsis acuminatum*

Only distinctive features and responses are noted. Max, maximum; μ, growth rate; S, salinity; DON, dissolved organic nitrogen.

region, subpopulations with differing tolerance of low salinity are present.

The role of broad halotolerance and low-salinity waters in promoting *H. akashiwo* blooms is not entirely clear, in part because interstrain variation has not always been considered in designing laboratory studies or interpreting field data. Salinity tolerance can promote bloom formation in several ways, each of which will be influenced by strain variation in physiology and behavior. First, the ability of *H. akashiwo* to grow at near-maximal rates across a broad range of salinities may give the species a competitive advantage in estuaries with strong salinity gradients. For example, blooms of *H. akashiwo* have been associated with periods of high runoff from the Fraser River in the Strait of Georgia, just to the north of our study region (Taylor and Haigh 1993). Combined with its ability to respond rapidly to nitrate input that often occurs with freshwater runoff (Coyne 2010), *H. akashiwo* may have a distinct advantage over other algal species that are less tolerant of low-salinity waters. However, when considering the worldwide distribution and incidence of *H. akashiwo* blooms, there is not a consistent association with salinity stratification, a discrepancy that has been attributed to the existence of multiple ecotypes (reviewed by Smayda 1998). *H. akashiwo* cells can aggregate in surface waters, a behavior that has often been associated with the presence of a low-salinity surface layer. This behavior was hypothesized by Kempton et al. (2008) to be the cause of a novel fish-killing *H. akashiwo* bloom that occurred in 2003, after freshwater from the Santee River was discharged into a South Carolina coastal bay. While *H. akashiwo* has been shown to form dense accumulations in low-salinity surface layers in the laboratory (Hershberger et al. 1997, Bearon et al. 2006), this accumulation phenomenon is also observed in cultures maintained with uniform salinity (K. Fredrickson, personal observation) or only slight, continuous salinity gradients (Bearon et al. 2004). In East Sound, Washington, a surface layer bloom of *H. akashiwo* was documented in association with a low-salinity surface layer, but not as low as is suggested by laboratory studies (Menden-Deuer et al. 2010). Strain variation in swimming behavior (Bearon et al. 2004) and its relationship to salinity structure, along with the potentially confounding influence of photoperiod on behavior (Handy et al. 2005), may explain some of these conflicting observations. A third way in which broad halotolerance may promote bloom formation is by providing a refuge from zooplankton predation. Pacific Northwest microzooplankton species able to prey on *H. akashiwo*, like *Favella* sp. and *S. acuminatum* (Graham and Strom 2010), were not tolerant of salinities below 15–20; this is a much-reduced halotolerance compared with that of *H. akashiwo* (data to be presented elsewhere). This could lead to a gap in the development of a grazer response to *H. akashiwo* blooms (i.e., an interruption of the successional sequence) as plankton communities move through estuarine salinity gradients. Furthermore, low salinities appear to increase *H. akashiwo* toxicity to some target species (Haque and Onoue 2002a), a response that is also likely to be strain specific.

We observed marked differences in the ability of Pacific Northwest *H. akashiwo* isolates to grow on organic N. While all four strains were able to grow on nitrate, ammonium, and urea, only the two 2007 isolates (07A and 07B) could grow on glutamine and alanine, while the other two strains could not (Fig. 3). N utilization by *H. akashiwo* is known to be variable, with strains showing varying preferences for nitrate, ammonium, and urea; furthermore, preferences within a given strain can vary with irradiance (Chang and Page 1995, Wood and Flynn 1995, Zhang et al. 2006, Herndon and Cochlan 2007). Based on within-strain comparisons, none of our isolates (all grown at $\sim$100 μmol photons $\cdot$ $m^{-2} \cdot s^{-1}$) showed significant differences in growth among nitrate, ammonium, and urea, the three major N sources utilized by marine phytoplankton. This finding agrees generally with those for an earlier (1996) Pacific Northwest isolate, which grew only slightly faster on ammonium versus nitrate and urea (Herndon and Cochlan 2007). While differences were not statistically significant, our data do suggest that 07A grew faster on nitrate than on other N sources, while 07B grew fastest on organic N (urea and amino acids). Further testing will be necessary to confirm these trends. An among-strain comparison shows that 07A had a considerably higher nitrate-based growth rate than the other three strains, consistent with this strain's higher growth rates in salinity tests as well as its higher P_{max} (Table 2). *H. akashiwo*'s ability to grow on multiple N sources offers a competitive advantage throughout the water column, which is beneficial to an organism that is able to migrate vertically to different depths (Yamochi and Abe 1984, Mackenzie 1991). The use of various organic N sources may also facilitate bloom persistence, as nitrate is stripped from the water column during biomass accumulation and regenerative processes become increasingly important. Interstrain variations in N use, including the forms of N that can be utilized and the growth rates supported by a given form, have been reported for other harmful bloom-forming phytoplankton species (Lartigue et al. 2009, Strom and Bright 2009). This plasticity in N use undoubtedly contributes to the range of environments supporting *H. akashiwo* and other harmful phytoplankton blooms.

We observed interstrain differences in *H. akashiwo* toxicity as well as in the response to environmental conditions. Strain 07B was not toxic to the ciliate *S. acuminatum*, while the three other strains were (Fig. 4a). *S. acuminatum* is a common member of the regional microzooplankton assemblage, and this isolate of the ciliate was actually collected from an *H. akashiwo* bloom. This information may explain its

reduced sensitivity to *H. akashiwo* in comparison with the other tested ciliate species, *Metacylis* sp. and *F. ehrenbergii*. An important point is that the assessment of toxicity in *H. akashiwo* and other harmful algal species depends critically on the choice of target species. Highly sensitive target species showed a uniformly severe toxicity response to all four *H. akashiwo* strains; only the use of a less-sensitive target species revealed interstrain variability in toxicity. Interstrain variability in toxicity is likely to play a role in when and where different *H. akashiwo* phenotypes are able to bloom. Our study adds to a growing body of evidence that suggests there is no single microzooplankton grazer response to Pacific Northwest *H. akashiwo* strains; instead, although several local grazer species can eat and grow on *H. akashiwo*, some avoid it, while others are killed by it (Clough and Strom 2005, Graham and Strom 2010). The observed species and strain-specific responses demonstrate the complex interactions that allow the development and subsequent decline of *H. akashiwo* blooms in this study region. Furthermore, variations in toxicity are a crucial component of the full suite of attributes that define the ecological niche of a given strain (Table 2).

In agreement with previous studies (Connell 2000, Akase et al. 2004, Ki and Han 2007), we found no genetic distinction among the four Pacific Northwest strains when we examined portions of the nuclear 18S, ITS1, and 5.8S rDNA sequences. However, analysis of selected regions within the chloroplast genome indicated strain-specific sequences among the four strains. Strain 07B, which was isolated from a July 2007 *H. akashiwo* bloom that occurred in East Sound, Washington, was genetically distinct from the other three strains, while both isolates from 2007 (07A and 07B) were different than the two isolated from a single June 2006 *H. akashiwo* bloom. These patterns of genetic relatedness closely resemble the ecophysiological differences that we detected, in that the two 2006 isolates were similar or identical in almost every assay, while the two 2007 isolates differed both from each other and from the 2006 isolates (Table 2). Chloroplast genome signatures identified in our study appear to map onto other genetic differences that underlie important physiological functions. Polymorphisms in these same chloroplast genome regions allowed Akase et al. (2005) to assign 30 Japanese *H. akashiwo* isolates to five groups. While the groupings showed no geographic coherence, they did relate to susceptibility to an *H. akashiwo* virus. Taken together, the Akase et al. (2005) findings and our results raise the exciting possibility that chloroplast genome signatures can be used to define and track ecotypes of *H. akashiwo* in natural waters.

In summary, we found substantial interstrain variation among the four Pacific Northwest *H. akashiwo* strains used in this study. Observed strain difference may be related to isolation location, source population, or bloom timing: Strain 07A was isolated from an early bloom and showed consistently high growth potential, particularly on nitrate. Strain 07B, which was isolated from a small fjord during summer, had larger cell size, lower levels of toxicity, less tolerance for low salinities, and a tendency to grow well on dissolved organic N. Strains 06A and 06B, which were isolated from the same massive 2006 bloom, were unable to grow on amino acids. Examination of additional strains will be necessary to determine the extent to which these combinations of characteristics represent persistent ecotypes. However, the association between ecophysiological diversity and chloroplast gene signatures should provide an avenue for tracking and study of different *H. akashiwo* populations. High levels of genetic and phenotypic variability within regional *H. akashiwo* populations likely contribute to the ability of this species to form persistent blooms.

We thank S. Menden-Deuer for East Sound bloom sampling and isolations, K. Bright for culturing assistance, and K. B. and J. Miller for Figure 1. This research was supported by NOAA ECOHAB grant NA06NOS4780248 to S. S., EPA-STAR ECOHAB grant R83-3221 to K. J. C., and NSF grant IOS-0745102 to K. J. C.

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