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The effects of salinity on the cellular permeability and cytotoxicity of *Heterosigma akashiwo*[1]

[2]Christopher E. Ikeda: Romberg Tiburon Center for Environmental Studies, San Francisco State University, 3150 Paradise Drive, Tiburon, California 94920, USA

William P. Cochlan: Romberg Tiburon Center for Environmental Studies, San Francisco State University, 3150 Paradise Drive, Tiburon, CA 94920, USA

Cayla M. Bronicheski: Department of Biology, Western University, London, Ontario, N6A 5B7, Canada

Vera L. Trainer: Northwest Fisheries Science Center, National Marine Fisheries Service, National Oceanic and Atmospheric Administration, 2725 Montlake Blvd. E., Seattle, WA 98112, USA

Charles G. Trick: Department of Biology, Western University, London, Ontario, N6A 5B7, Canada

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[2]Author for correspondence: email: christopher.ikeda@gmail.com
phone: 1 (415) 338-3708; Fax: 1 (415) 435-7120

Running Title: Salinity & toxicity in *H. akashiwo*

ABSTRACT:

A laboratory study using the fish-killing raphidophyte *Heterosigma akashiwo* was conducted to examine its capability to grow at salinities below oceanic, and to test the perceived relationship between reduced salinities and increased cytotoxicity. A non-axenic strain of *H. akashiwo* isolated from the U.S. Pacific Northwest was exposed to a combination of three salinity (32, 20 and 10) and five temperature (14.7, 18.4, 21.4, 24.4 and 27.8ºC) conditions. Our results demonstrate that cell permeability and cytotoxicity are strongly correlated in unialgal cultures of *H. akashiwo*, which both increased as salinity decreased from 32 to 10. Furthermore, over a broad median range of salinities (10 and 20), neither temperature nor specific growth rate were correlated with cytotoxicity. However, in cultures grown at the salinity of 32, both temperature and specific growth rate were inversely proportional to toxicity; this relationship was likely due to the effect of contamination by an unidentified species of *Skeletonema* in those cultures. The presence of *Skeletonema* sp. resulted in a cytotoxic response from *H. akashiwo* that was greater than the response caused by salinity alone. These laboratory results reveal the capability of *H. akashiwo* to become more toxic not only at reduced salinities but also in competition with another algal species. Changes in cell permeability in response to salinity may be an acclimation mechanism by which *H. akashiwo* is able to respond rapidly to different salinities. Furthermore, due to its strong positive correlation with cytotoxicity, cellular permeability is potentially associated with the ichthyotoxic pathway of this raphytophyte.

Key index words: cellular permeability; *Heterosigma akashiwo*; cytotoxicity; ichthyotoxicity; salinity; temperature

Abbreviations: DIC, dissolved inorganic carbon; ESAW, enriched seawater artificial water; ESNW, enriched seawater natural water; N, nitrogen; NO_3^-, nitrate; NO_2^-, nitrite; P, phosphorus; PPFD, photosynthetic photon flux density; RFU, relative fluorescence unit; μ, specific growth rate

INTRODUCTION:

In estuaries, variations in phytoplankton growth and community composition are linked to the connate differences in salinity, temperature, and the availability of light and nutrients. One estuary system with an exceptionally complex mosaic of environmental factors is the Salish Sea – the inland waters of southwestern British Columbia, Canada, and northwestern Washington, USA. This region has a long history of blooms of the photosynthetic flagellate *Heterosigma akashiwo* (Y. Hada) Y. Hada ex Y. Hara et M. Chihara, a marine raphidophyte responsible for economically devastating mortalities of cultivated (pen-raised) finfish here and in aquaculture operations worldwide, and lesser (but less quantified) losses of free-ranging finfish (e.g., Strom et al., 2013 and references therein). The complexity of environmental conditions in the Salish Sea is generated through the different patterns and magnitudes of supply of oceanic water from the Pacific through the Strait of Juan de Fuca and the deposition of freshwater from major river systems such as the Fraser River (Rensel et al. 2010). A surficial flux of seawater travels through the Strait of Juan de Fuca, influences northern Puget Sound, and then travels north to supply the Strait of Georgia. Some of the water of marine origin enters into central and southern Puget Sound through Admiralty Inlet (Khangaonkar et al. 2011), replenishing the saline water supply. Similarly, multiple freshwater sources supply the Salish Sea system, with the largest river supply being the Fraser River in British Columbia. It discharges its freshwater into the southern portion of the Strait of Georgia, and the freshwater then travels south to northern Puget Sound. Ultimately, the mingling of different water sources provides a mosaic of conditions based on temperature, salinity, nutrient composition, and supply that interact to initiate blooms of the potentially devastating species, *H. akashiwo*.

In the Salish Sea, the occurrence and magnitude of *H. akashiwo* blooms have been linked to changes in salinity. In particular, blooms of *H. akashiwo* have coincided with increased river flows from snowmelt in northern Puget Sound, with particularly large blooms detrimental to fish aquaculture operations occurring during the years with seasonally earlier and larger river flows from the Fraser River (Rensel et al. 2010). In the northern regions of Puget Sound, salinity is the major factor determining water column stratification due to the greater depth of the channel, but of lesser importance in the

shallower regions of central and southern Puget Sound where the temperature is the dominant factor controlling stratification (Rensel 2007).

Ecophysiological studies have revealed that salinity can affect *H. akashiwo* in a variety of ways, including cell growth, motility and ichthyotoxicity. Laboratory cultures of *H. akashiwo* can grow over a wide range of salinities, ranging from 0 to 30 (Strom et al. 2013); unhindered specific growth rates of *ca.* 0.6 d^{-1} were observed between salinities of 10 – 30, and decreased growth rates (*ca.* 0.2 d^{-1}) were reported at the lowest salinities and freshwater conditions (salinities of < 10 to 0). Haque and Onoue (2002) reported substantial cell growth at salinities above those normally found in the ocean, although the growth rate at a salinity of 40 (0.48 d^{-1}) was lower than the maximal growth rate observed at a salinity of 25 (0.68 d^{-1}). Salinity changes also drive changes in important physiological parameters such as motility (Bearon et al. 2006), sinking (Powers et al. 2012) and toxicity (Haque and Onoue 2002, Sutton-Quaid et al. 2014). Bearon et al. (2006) demonstrated that *H. akashiwo* could migrate through a strong halocline of 28 to 8 at a vertical speed of 17 $\mu m \cdot s^{-1}$, versus the unhindered speed of 45 $\mu m \cdot s^{-1}$ measured below the halocline. *H. akashiwo*'s broad tolerance to salinity has been suggested to play an important role in its bloom formation by allowing the cells to avoid predation by migrating to salinity conditions inhibitory to the survival of its predators (Harvey and Menden-Deuer 2012, Strom et al. 2013). In addition, cell growth at salinities below 32 promoted more ichthyotoxic cells (Haque and Onoue 2002). Together these results suggest that lower salinities in natural systems such as the Salish Sea could not only provide refuge from predation, but also promote more ichthyotoxic events.

Even with the research of Haque and Onoue (2002), our knowledge is woefully incomplete with respect to the relationship between salinity and ichthyotoxicity. First, their laboratory study only examined salinity conditions above 20, whereas salinities above and below this are frequently observed both seasonally and spatially in the Salish Sea (Sutherland and MacCready 2011). Second, only non-native strains to the Salish Sea were tested in their study, and the relative toxicity of *H. akashiwo* varies markedly among strains (Smayda et al. 2004, Cochlan et al. 2014). Therefore, it is unclear whether toxicity is regulated by environmental factors specific to a geographical region or is solely a function of strain variability. Third, ichthyotoxicity was estimated from the measured

concentration of brevetoxin-like compounds, and quantified using HPLC analysis (Khan et al. 1997). This is problematic given that these compounds from *H. akashiwo* have since been shown not to alter the sodium influx of cultured cell lines, as is typical for brevetoxins, but instead appear to alter calcium homeostasis, indicative that the putative toxin is a unique bioactive metabolite (Twiner et al. 2005, Trainer et al. 2015).

While increased cell growth, decreased motility and to some degree increased cellular ichthyotoxicity have been observed for *H. akashiwo* at salinities below oceanic levels, the mechanism of *H. akashiwo*'s response to reduced salinities is not yet fully understood. This paper examines the effect of salinity on the toxicity of the coastal flagellate *H. akashiwo* with the aim of enhancing our ability to predict ichthyotoxic blooms and their associated risks as a function of a change in the physico-chemical environment. The underlying thesis of this research is that growth rate is not a direct factor involved in ichthyotoxicity, but that toxicity is related to changes in cell membrane permeability. We hypothesize that a reduction in salinity will cause cells to alter membrane permeability as a means to reduce the stress associated with increased turgor pressure caused by a decrease in the solute content of their external environment. Such changes in cell permeability can be measured by the relative accumulation of SYTOX® Green nucleic acid stain within the cell. Although this fluorochrome traditionally has been used to measure the cell viability of *H. akashiwo* (Lawrence et al. 2006, Tobin et al. 2013), it has been suggested that SYTOX® Green can accumulate in viable eukaryotic cells with compromised cell membranes (Veldhuis et al. 2001).

This study describes laboratory experiments designed to test the effects of salinity on a non-axenic strain of *H. akashiwo*, isolated from the Salish Sea. The objectives of this research were: 1) to determine if cell growth varies as a function of salinity dilution as observed by others for both native and non-native strains to the Salish Sea, 2) to quantify the effect of decreased salinity on cellular permeability, 3) to determine if a combinatorial effect on cytotoxicity exists between salinity and temperature, and 4) to evaluate the relationship between cellular permeability and cytotoxicity.

MATERIALS AND METHODS:

153 *Cell culturing and experimentation*. One non-axenic strain of the raphidophycean
154 flagellate *H. akashiwo* (NWFSC-513), isolated by B. Bill from Clam Bay, WA, USA in
155 2010, was used in this study. This strain was maintained in 50-mL borosilicate glass
156 tubes using 0.2-µm filtered seawater with a salinity of 30 collected from Puget Sound,
157 WA, USA. The seawater was enriched with ESNW medium (Harrison et al. 1980, Berges
158 et al. 2001 and subsequent Corrigendum 2004) as outlined by Anderson et al. (2005) with
159 the following modifications: copper, as $CuSO_4 \cdot 5H_2O$, and manganese, as $MnSO_4 \cdot H_2O$,
160 were added to the seawater to achieve final concentrations of 3.93×10^{-9} M and 2.42×10^{-6} M, respectively. Selenium, as Na_2SeO_3, was prepared as a separate trace metal stock,
162 and added to achieve a concentration of 6.36×10^{-9} M, and all vitamins were added from
163 separate stocks. Silicon was not added, and nitrogen ($NaNO_3$) and phosphorus ($NaHPO_4$)
164 were added at the reduced concentrations of 50 and 5 µM, respectively to ensure that
165 inorganic carbon did not limit photosynthetic growth (Howard et al. 2007). Cultures were
166 grown at 12 ºC, at a PPFD of 75 µmol photons $\cdot$ $m^{-2} \cdot$ s^{-1} provided by a series of 15 W
167 GE fluorescent bulbs (#F15T8; General Electric, Fairfield, CT, USA), and maintained in
168 a 14:10 h light:dark cycle. All culturing and manipulations were performed using
169 glassware, plasticware and equipment cleaned with 10 % (v/v) HCl acid followed by
170 three rinses in ultra-pure water (18.2 MΩ. $\cdot$ cm, Milli-Q®; EMD Millipore Corporation,
171 Billerica, MA, USA).
172 In preparation for the experiment, isolate NWFSC-513 was grown for four days,
173 equating to *ca.* ≥ 4.5 generations, at three salinity (32, 20, 10) and five temperature (14.7,
174 18.4, 21.4, 24.4 and 27.8°C) treatments. During this period, duplicate cultures (n = 2)
175 were grown in 50-mL borosilicate (Pyrex®) culturing tubes, equipped with Nalgene®
176 polyethylene caps. Each culture tube contained 40-mL of culture.
177 To produce media at a range of salinities (i.e., 32, 20, 10), a 20-L polypropylene
178 carboy of natural seawater was collected from East Sound, WA (salinity = 31.5) using
179 10-L Niskin Bottles (General Oceanics, Miami, FL, USA). Salinity was determined using
180 a hand-held salinity refractometer with automatic temperature compensation (Fisher
181 Scientific™, Pittsburgh, PA, USA), which was calibrated with ultra-pure water. This
182 seawater was first filter-sterilized using a 0.2-µm filtration capsule (Whatman® PolyCap
183 150 TC; GE Healthcare Bio-science, Pittsburgh, PA, USA), prior to any salinity

manipulations, and enriched according to ESNW as previously outlined. The highest salinity was established by the salinity naturally occurring in East Sound, while the lower salinities (20 and 10) were created by diluting this seawater with Milli-Q® ultra-pure water in order to maintain similar ionic ratios among the salinity treatments. To adjust the dissolved inorganic carbon (DIC) removed during dilution, additional $NaHCO_3$ was added to the 20 and 10 salinity treatments to achieve a final concentration of 2.07×10^{-3} M, according to the modified enriched seawater artificial seawater (ESAW) medium (Anderson et al. 2005).

Temperature and light conditions for the experiments were regulated using an insulated aluminum temperature gradient bar (TBG) enclosed within a controlled light box, as described by Bill et al. (2016). The TGB maintains a stable and uniformly distributed temperature gradient within an insulated aluminum bar by mixing chilled water from one end of the bar with heated water from a 400W cartridge heater at the opposite end. A series of holes drilled along the length of the TGB house the 50-mL borosilicate glass culture tubes which were illuminated from below using six soft-white fluorescent tubes (Plusrite®, Ontario, CA), and maintained in a 14:10 h light: dark cycle. The photosynthetic photon flux density (PPFD), measured with a 4-π collector (QSL-100 Quantum scalar irradiance meter; Biospherical Instruments Inc., San Diego, CA, USA) immersed in medium-filled culture vessels, averaged 350 ± 112 μmol photons · m^{-2} · s^{-1}, and was experimentally determined to be saturating for the growth of NWFSC-513 (see Results: Fig. 1). Salinity treatments were haphazardly organized within each temperature range on a daily basis to avoid any systematic bias caused by slight variability in PPFD across the TGB. During the pre-experimental period, growth rates were estimated from daily measurements conducted at 14:00 h of in vivo fluorescence (as a proxy for biomass), using a Turner Designs 10-AU fluorometer (Turner Designs, Sunnyvale, CA, USA).

Duplicate, pre-experimental cultures were grown for four days at each salinity and temperature condition. These pre-experimental cultures were then combined together and used to inoculate the experimental cultures, which were exposed to the same salinity and temperature conditions as the pre-experimental cultures. The experimental cultures were grown in quadruplicate (n = 4), 50-mL borosilicate (Pyrex®) culturing tubes (40-mL of culture per tube) that contained fresh media for each treatment. Cultures were allowed to

enter the stationary growth phase resulting from N depletion, and grown for another three days to ensure that the cultures were well within the stationary growth phase. Sampling occurred every day at 14:00 h during the exponential and stationary growth phase for in vivo fluorescence, cell permeability, and cell density of the experimental cultures, whereas only in vivo fluorescence was measured in the pre-experimental cultures.

Samples for macronutrients, dissolved inorganic carbon (DIC), and cellular toxicity were taken only once, as they require relatively large volumes for analysis. These samples were taken on the last day to ensure that the cells were nutrient depleted, and therefore, most likely to be toxic (Cochlan et al. 2014). Samples for macronutrients and DIC were collected from only two of the quadruplicate culture tubes (i.e., A and C). Samples for cytotoxicity analyses were collected from the remaining two culture tubes (i.e., B and D), and combined with the residual sample volume left in culture tubes A and C. In summary, two samples for each treatment were analyzed for toxicity.

Macronutrients. Unfiltered samples for DIC analysis were injected by syringe into pre-combusted (450 °C for 6 h), 20-mL glass scintillation vials. To minimize contamination from atmospheric CO_2 during injection, silicon tubing was used to transfer the sample from the syringe to the bottom of the vial. DIC samples were immediately preserved with 300 µL of 5 % (w/v) mercuric chloride ($HgCl_2$), and sealed with an acid-washed, inverted poly-cone cap to ensure no headspace within the sample vials. Samples were kept in the dark at room temperature (*ca.* 20ºC) until analysis with an acid-sparging non-dispersive infrared (NDIR) gas analyzer (Friederich et al. 2002).

Nutrient samples for nitrate plus nitrite ($NO_3^- + NO_2^-$) and phosphate (PO_4^{3-}) were collected in 15-mL polypropylene conical tubes (Falcon® 352097; Becton Dickson, Franklin Lakes, NJ, USA), previously soaked in Milli-Q® ultra-pure water for at least 24 h. Sub-samples (12 mL) were collected from two of the quadruplicate cultures (i.e., tubes A and C), to yield two samples for nutrient analysis per experimental treatment. Once the nutrient samples were collected, they were immediately stored at -20°C until analysis. Samples were thawed overnight at room temperature (*ca.* 20°C) prior to analysis using a Lachat Instruments flow injection automated ion analyzer (8000 Series; Hach Co., Loveland, CO, USA), following the methods of Smith and Bogren (2001) for $NO_3^- + NO_2^-$, and Knepel and Borgen (2002) for PO_4^{3-}.

246 *Cell density*. Samples (0.5 mL) for cell density were collected from each culture,
247 transferred into 12 x 75 mm borosilicate test tubes (14-961-26; Fisher Scientific,
248 Pittsburgh, PA, USA), and immediately preserved with Lugol's iodine solution to a final
249 concentration of 0.5 % (v/v). After three inversions to mix the preservative with the
250 sample, 50-µL sub-samples were analyzed within 1 min by flow cytometry (BD Accuri
251 C6 flow cytometer; BD Bioscience, San Jose, CA, USA). Incubation times longer than 1
252 min were avoided as excessive exposure to Lugol's iodine solution will cause *H.*
253 *akashiwo* cells to shrivel and lyse resulting in inaccurate measures of cell density
254 (Herndon et al. 2003). We identified the *H. akashiwo* population by creating a histogram
255 plot and 'gating' the population that contained high chlorophyll *a* fluorescence (i.e., > 10^6
256 RFU), using the BD Accuri C6 Analysis software (BD Bioscience, San Jose, CA, USA).
257 A 90 % attenuation filter (#653177; Becton Dickinson and Company, San Jose, CA,
258 USA) was used to reduce the chlorophyll *a* fluorescence signal generated from the high
259 chlorophyll content in *H. akashiwo* cells. The cellular density (cells · mL^{-1}) of *H.*
260 *akashiwo* was calculated using the following equation:
261
262 $$\text{Cell density} = \frac{g}{s} * 1000 \quad (1)$$
263
264 Where *g* is the total count within the 'gated' region, and *s* is the volume (µL) sub-
265 sampled by the flow cytometer.
266 Specific growth rates were calculated from least-squares linear regression analysis
267 of the exponential phase of cell growth, determined from plots of the natural log of cell
268 density (or in vivo fluorescence) versus elapsed time for each culture, and using the
269 exponential growth equation (Guillard 1973):
270
271 $$K_e = \frac{\ln(N_1 / N_o)}{t_1 - t_o} \quad (2)$$
272
273 Where K_e is the growth rate (d^{-1}), and N_1 and N_o are the cell densities (or in vivo
274 fluorescence) at time 1 (t_1) and time 0 (t_0), respectively. The average growth rates of
275 replicate cultures, determined during the exponential growth phase, are reported.

Cell permeability. The permeability of *H. akashiwo* cells was determined using the fluorochrome SYTOX® Green (S7020; Invitrogen, Grand Island, NY, USA), and measured by flow cytometry according to a modified protocol by Tobin et al. (2013). This cyanine stain has a high affinity for nucleic acids and fluoresces when bound to the cellular DNA of cells with compromised cell membranes, thereby providing a measure of cell viability (Veldhuis et al. 2001). It was used here to quantify changes in cell permeability by measuring the relative fluorescence per cell. A 50 µM working solution of SYTOX® was prepared in ultra-pure water, and stored at -20°C until use. Initially, the background fluorescence of 50 µL of live cells was measured with the flow cytometer at the excitation and emission wavelengths for SYTOX® Green (488/523 nm, respectively). After the background fluorescence had been measured, 5 µL of the SYTOX® Green working solution was then added to unstained cell samples (final concentration of 0.6 µM). The samples were then inverted four times and incubated at room temperature (20 °C) for 15 min in the dark. A 50-µL subsample of the incubated SYTOX® Green-stained cells was subsequently assayed with the flow cytometer using the same excitation/emission wavelengths of 488/523 nm.

The BD Accuri C6 Analysis software was used to identify cells with compromised membranes (i.e., cells stained with SYTOX® Green), by using a bivariate scatter plot of SYTOX® Green fluorescence versus chlorophyll *a*. The bivariate plot was divided into four quadrants. Cells that appear in the lower right quadrant of the bivariate plot indicate the background fluorescence of non-stained cells, whereas cells that appear in the upper right quadrant indicate cells that have been stained with SYTOX® Green. The line separating the lower right quadrant from the upper right quadrant was determined daily by placing this line above the background fluorescence of non-stained cells before the addition of SYTOX® Green. To correct for the natural background fluorescence of *H. akashiwo* at the excitation and emission wavelengths of SYTOX® Green, the fluorescence of stained samples was subtracted from the corresponding fluorescence of unstained samples. The corrected SYTOX® Green fluorescence was then averaged among the biological replicates (n = 4) over the exponential and stationary portions of the growth phase for each treatment. As a consequence, cell permeability is reported as the average (n = 4) background-corrected SYTOX® Green fluorescence for

stained cells for each experimental treatment during the exponential growth phase and the average (n = 4) during the stationary growth phase.

SYTOX® Green fluorescence values represent viable cells only during the exponential growth phase. During this phase, the entire *H. akashiwo* population monitored by flow cytometry fluoresces significantly greater than the fluorescence measured for unstained cells (i.e., the background). This is a population response to the stain rather than a selective response that's generally observed with non-viable cells. Therefore, changes in SYTOX® Green fluorescence during the exponential growth phase indicate viable cells with compromised cell membranes. However during the stationary growth phase, changes in the SYTOX® Green fluorescence are assumed to represent both viable and non-viable cells, since these cells have become stressed by nutrient limitation.

Cytotoxicity analysis. Cytotoxicity was measured using a modified version of the gill cell assay (GCA; Dorantes-Aranda et al. 2011). This assay measures the viability of a rainbow trout gill cell line (RTgill-W1) after exposure to *H. akashiwo* extracts. Gill cell viability was determined by measuring the increase in fluorescence due to the digestion of resazurin to resorufin, which only occurs in viable gill cells. Resazurin is a non-toxic, non-fluorescent, cellular permeable compound that is blue in color. Upon entering the gill cells, resazurin is reduced to resorufin, a compound that is red in color and highly fluorescent. Viable gill cells continuously convert resazurin to resorufin through mitochondrial metabolic activity, increasing the overall fluorescence and color of the medium surrounding the cells. In summary, the increase in fluorescence of resorufin is a quantitative measure of cell proliferation.

The modified GCA described here is adapted from tested and published methods originally developed to evaluate the toxicity of water samples, including effluents from oil refineries (Schirmer et al. 2001) and paper mills (Dayeh et al. 2003a). This cell culture assay has been shown to offer advantages over the use of live fish (Fent 2001, Dayeh et al. 2002, Castano et al. 2003) that include reductions in cost, analytical time, and volume required for the assay. The GCA was adapted for use with raphidophyte cultures by Dorantes-Aranda et al. (2011), and has been shown to accurately reflect the lack of cytotoxicity in cultures considered to be nontoxic, as demonstrated with laboratory

cultures of the dinoflagellate *Akashiwo sanguine*a and the diatom *Pseudo-nitzschia pungens* (Trainer et al., unpublished data).

To measure the cytotoxicity of the *H. akashiwo* cultures reported in this study, samples were obtained by combining two of the four replicate cultures to generate *ca.* 41 mL of sample per replicate, which was then filtered onto a 25-mm glass-fiber filter (Whatman GF/F; GE Healthcare Life Sciences, Piscataway, NJ, USA), and stored at -20ºC. These samples were then transported to the National Oceanic Atmospheric Administration (NOAA) Northwest Fisheries Science Center (NWFSC) in Seattle, WA, for methanol extraction and cytotoxicity analysis.

The intracellular cytotoxin was extracted by adding 4 mL of methanol (LC-MS grade) to 12-mL glass tubes containing the filtered *H. akashiwo* cells. Cells were removed from the filter by gentle agitation for 2 min (or until cells were re-suspended) and centrifuged at *ca.* 1,700*g* for 15 min. The supernatant was then removed with a disposable glass pipette, and placed into a 4-mL amber, speed vac vial. The cytotoxin contained in the supernatant was concentrated using a centrifugal evaporator (Oligo Prep OP120 concentrator; Savant Instruments Inc., Saroor Nagar, Hyderabad, India) to remove the methanol until 1 mL of extract remained. The remaining 1 mL of methanol was dried using a flow of ultra-high purity nitrogen gas (grade 4.8), followed by re-suspension in 1 mL of Leibovitz's L-15 medium (Leibovitz 1963; ATCC® 30-2008™, ATCC, Manassas, VA, USA) to a final cell density of 5.0×10^5 cells $\cdot$ mL^{-1}. This solution was vortexed for 1 min, then stored at -20 ºC to preserve the cytotoxin until analysis using the GCA.

To measure cytotoxicity using the GCA, the previously collected cytotoxin extracts were thawed, sonicated to break up the cellular matter, and centrifuged (3,500 g) for 10 min to partition the intracellular contents into the supernatant. One hundred µL of the extract, or control solution (see below), was added to a flat bottom 96-well microplate (353072; Corning, Tewksbury, MA, USA) containing the live rainbow trout gill cell line (ATCC® CRL-2523™; ATCC, Manassas, VA, USA) at *ca.* 90 % confluence, which was then incubated in the dark for 6 h at 19 °C. After incubation, the solution was removed and replaced with 100 µL of L-15/ex medium (Schirmer et al. 1997) containing 5% AlamarBlue® dye (DAL1025; Invitrogen, Grand Island, NY, USA), which is the trade name for resazurin. The plated samples were then incubated for an additional 2 h before

gill cell viability was determined using a Synergy 2 microplate reader (Biotek, Winooski, VT, USA) that measures the change in fluorescence (excitation: 540 nm, emission: 600 nm) due to the digestion of AlamarBlue® dye in samples that contained the *H. akashiwo* extract relative to controls (negative control = no cells; positive control = 0.3 % H_2O_2 to confirm gill cell kills on each plate). Cytotoxicity values are reported as percentage of non-viable gill cells (i.e., 1 minus the percentage of viable gill cells), as shown by the equation below:

$$\text{Toxicity (\%)} = \left[1 - \left(\frac{\left(\text{RFU}_{ex.\ cells} - \text{RFU}_{ex.\ no\ cells}\right)}{\text{Average}\ \left(\text{RFU}_{con\ cells} - \text{RFU}_{control\ no\ cells}\right)}\right)\right] \text{ x } 100 \qquad (3)$$

Where $\text{RFU}_{ex.\ cells}$ is the fluorescence of gill cells after exposure to *H. akashiwo* extracts in the L-15/ex medium, $\text{RFU}_{ex.\ no\ cells}$ is the fluorescence of only *H. akashiwo* extracts in the L-15/ex medium. $\text{RFU}_{con.\ cells}$ is the fluorescence of gill cells in the L-15/ex medium, and $\text{RFU}_{control\ no\ cells}$ is the fluorescence of the L-15/ex medium without any cells (i.e., no gill cells or *H. akashiwo* cells).

Statistical analysis. All statistical analyses were performed using either KaleidaGraph™ (Synergy Software, Reading, PA, USA) or RStudio (Version 0.97.551; Boston, MA, USA). Data were normally distributed, as determined by the Shapiro-Wilk test, thus permitting use of parametric statistical analyses. The level of significance (α) was set to 0.05 for all statistical tests employed in this study (i.e., Student's *t*-test, Pearson product-moment correlation coefficient and two-way ANOVAs). The significance of the reported *p*-values for the correlation coefficient, was determined by Student's *t*-test. Unless otherwise stated, variability is reported as plus/minus one standard deviation of the mean.

RESULTS:

Macronutrients. Initial samples for DIC analysis for each experimental condition were collected prior to the addition of cells to the media, and on the last sampling day The initial DIC concentrations were 2,112 and 2,046 µM C for the 20 and 10 salinity treatments, respectively, which are similar to the initial (un-enriched) DIC concentration

of 1,994 µM C at a salinity of 32. On the last sample day, all DIC concentrations were >1,400 µM C. As temperature increased from 14.7 to 27.8 ºC, the concentration of DIC decreased for all salinities. The initial nutrient concentrations were 47.6, 48.1, and 47.8 µM NO_3^- + NO_2^-, and 4.9, 5.1, and 4.8 µM PO_4^{3-} for the 32, 20 and 10 salinity treatments, respectively. During the stationary growth phase, concentrations of NO_3^- + NO_2^- were at or below the limit of detection (0.05 µM) and PO_4^{3-} ranged between 0.1 to 2.4 µM, indicating that N-depletion was responsible for the induction of stationary growth, as neither PO_4^{3-} nor DIC concentrations were low enough to limit phytoplankton growth.

Growth rates. The specific growth rates (µ) for pre-experimental and experimental *H. akashiwo* cultures for each salinity and temperature condition are reported in Figure 2. In vivo fluorescence as a function of time was used to determine these rates for the pre-experimental cultures, whereas both *in vivo* fluorescence measurements and cell counts from flow cytometry were used for the experimental cultures. No statistical differences were observed between the growth rates determined by *in vivo* fluorescence and those determined by flow cytometry, and only the latter are reported for the experimental cultures.

In general, the specific growth rates of pre-experimental cells increased as a function of temperature at all three salinities, with the slowest growth observed at the highest salinity (Fig. 2A). The growth rates of cells cultured at salinities of 10 and 20 were very similar at equivalent temperatures, and were on average *ca.* 20 % faster than cells grown at the salinity of 32. The growth rates of the experimental cultures (Fig. 2B) were slower than the growth rates of pre-experimental cultures. For all three salinities, the cultures grown at the warmer temperatures resulted in faster specific growth rates than those reported for the cooler temperatures; a 2.4-fold increase (0.7 ± 0.04 d^{-1} to 1.7 ± 0.18 d^{-1}) was reported at the salinity of 10 as temperature increased from 14.7 to 27.8 °C, whereas cultures grown at salinities of 20 and 32 increased 1.4-fold (1.0 ± 0.076 to 1.4 ± 0.148 d^{-1}) and 2.7-fold (0.7 ± 0.023 to 1.94 ± 0.143 d^{-1}), respectively.

A two-way ANOVA reveals that both temperature ($F_{3,36} = 51.49$, $p < 0.0001$) and salinity ($F_{2,36} = 38.53$, $p < 0.0001$) significantly impacted cell growth for the experimental cultures. But the combined interactive effects between temperature and

429 salinity on the specific growth rate were non-significant ($F_{6,36}$ = 2.06, p = 0.083). The
430 statistical analyses of specific growth rates as a function of temperature and salinity did
431 not include data from 27.8 °C. The growth rates reported for this temperature represented
432 cell growth over a 2-d period, whereas growth rates were determined over 3 or more days
433 for all the other growth temperatures. As a consequence, our degree of confidence in the
434 accuracy of the specific growth rates at this highest temperature was lower than for the
435 other rates, and therefore, they were not included.
436 *Cellular permeability*. The fluorochrome SYTOX® Green was employed to
437 estimate the degree of cell membrane permeability of *H. akashiwo* during both the
438 exponential (nutrient-replete) and stationary (nutrient-depleted) growth phases for all
439 treatments. During the exponential phase, the most permeable cells (indicated by the
440 greatest SYTOX® Green fluorescence per cell) were the cultures grown at the salinity of
441 10, followed by the cultures grown at salinities of 20 and 32 (Fig. 3A). The average slope
442 of cellular permeability versus temperature for the cultures (n = 4) grown at salinities of
443 32 (-1,021 ± 444; p = 0.022), 20 (-3,424 ± 2,210; p = 0.008) and 10 (-8,441 ± 1,279; p =
444 0.013) were all negative, indicating a significant decrease in cellular permeability as
445 temperature increased from 14.7 to 27.8 ºC. Compared to the salinity of 32, the decrease
446 in cellular permeability was greatest at the salinities of 10 and 20, respectively.
447 During the stationary growth phase, the average cellular permeability of the
448 quadruplicate cultures was still greatest at the salinity of 10, followed by 20 and 32,
449 respectively (Fig. 3B). However during this growth phase, only cultures grown at the
450 salinity of 32 showed a significant decreasing trend in cell permeability with increasing
451 temperature (slope = -4,035 ± 3118; p = 0.043). At the salinities of 10 and 20, average
452 cell permeability increased as temperature increased from 14.7 to 27.8 ºC (slope = 2,046
453 ± 1,830 and 907 ± 4873, respectively), but neither of these slopes were significantly
454 different from zero (p = 0.097 and p = 0.65, respectively).
455 *Cellular toxicity*. Measurements of cytotoxicity produced by *H. akashiwo* (Fig. 4),
456 were collected only during the stationary phase of growth. In general, cytotoxicity was
457 greatest at the salinity of 32 for the temperatures of 14.7, 18.4 and 21.4 °C, followed by
458 cultures grown at salinities of 10 and 20 over the entire temperature range. The average
459 cytotoxicity did not vary significantly with the incubation temperature for cultures grown

460 at the salinities of 20 (slope = -0.2 ± 1.0; p = 0.88) and 10 (slope = -0.6 ± 0.5; p = 0.35).
461 However, the cytotoxicity of cultures grown at the salinity of 32 significantly decreased
462 (slope = -5.6 ± 0.3; p = 0.001) by 3 fold when cells were grown at the warmer
463 temperatures of 24.4 and 27.8 °C compared to the cooler temperatures (14.7, 18.4 and
464 21.4 °C).

465 Unlike the other salinity treatments, cultures grown at the salinity of 32 at the
466 three lower temperatures (14.7, 18.4 and 21.4 ºC) were contaminated with an unidentified
467 species of the diatom genus *Skeletonema*. Uni-algal conditions of *H. akashiwo* were
468 maintained at the two higher temperatures at a salinity of 32, and in all the other culture
469 treatments. To ensure that the measured cytotoxicity in the contaminated cultures was
470 due to *H. akashiwo* and not *Skeletonema* a uni-algal culture of *Skeletonema costatum* was
471 examined for cytotoxicity using the GCA. Gill cell viability was ≥ 100 % after exposure
472 to extracts of *S. costatum* collected from its stationary growth phase (Trainer et al.
473 unpublished data) indicating the non-toxic nature of this *Skeletonema* species. Based on
474 this result, it is unlikely that unidentified species of *Skeletonema* in the contaminated
475 cultures of the present study independently contributed to any measured cytotoxicity
476 reported here. For uni-algal cultures of *H. akashiwo,* the cellular toxicity generally
477 decreased with increasing salinity. The average cytotoxicity of uni-algal cultures,
478 measured at the two highest temperatures (24.4 and 27.8 ºC), varied inversely with
479 salinity, and cells grown at the salinity of 32 were 1.5- and 2.1-fold less toxic than cells
480 grown at salinities of 20 and 10, respectively.

481 The relationship between *H. akashiwo* cell density and cytotoxicity varied
482 according to the salinity treatment. At the salinity of 32 (Fig. 5A), a relationship was
483 found between *H. akashiwo* cell density and toxicity at each temperature treatment; cell
484 density is inversely related to cellular toxicity, and the 18.3-fold decrease in cell density
485 from 40,515 (27.8 ºC) to 2,205 cells · mL^{-1} (14.7 ºC) was accompanied by a 2.9-fold
486 increase in toxicity. For salinities of 20 and 10 (Fig. 5, B & C), no relationship was
487 detected between toxicity and cell density, regardless of the incubation temperature.
488 Given that this method used a constant number of *H. akashiwo* cells for every assay, the
489 toxicity measured is effectively normalized to cell density, commonly referred to as
490 either cell toxin quota or cellular toxin concentration. Therefore the observed relationship

between cell density and toxicity for *H. akashiwo* cultures grown at the salinity of 32 was likely the result of competition with the *Skeletonema* cells.

DISCUSSION:

The euryhaline nature of *H. akashiwo* is well-documented by several investigators who report cell growth at a range of salinity conditions from freshwater (Strom et al. 2013) to salinities greater than those found in oceanic waters (salinity of 40; Haque and Onoue 2002). The reduction in salinity due to freshwater inputs by river sources has been suggested as a precursor to ichthyotoxic bloom development in the Salish Sea (Rensel et al. 2010) and Bulls Bay, South Carolina, USA (Kempton et al. 2008). However, few studies have examined changes in toxicity as a function of salinity for *H. akashiwo* (e.g., Haque and Onoue 2002), and no research to date has explained the physiological modifications that *H. akashiwo* cells undergo in order to migrate through drastically different salinity conditions, as observed by Bearon et al. (2006). Therefore, this study examined the effects of salinity on the specific growth rate, cellular permeability, and cytotoxicity of *H. akashiwo*.

Growth rates. During the four-day acclimation period, the specific growth rates determined for pre-experimental cultures (Fig. 2A) were generally faster than experimental culture growth rates (Fig. 2B). This decrease in the specific growth rate from the pre-experimental to the experimental period is unusual, as the specific growth rate was expected to increase after cells acclimated to the experimental conditions. Cell growth at low salinities can reduce the efficiency of photosystem I by inhibiting the enzyme reactions of the CO_2-fixation cycle (Gilmour et al. 1984). Although Gilmour et al. (1984) did not report any changes in chloroplasts per cell at reduced salinities, it is possible that the number of chloroplasts increased to compensate for the decreased efficiency of photosystem I. If true, then the rate of change in the number of chloroplasts per cell for the pre-experimental cultures may not have been the same as for the experimental cultures, resulting in faster specific growth rates for the pre-experimental cultures.

Specific growth rates of experimental cultures were influenced by both temperature and salinity (Fig. 2A). Growth rates increased with increasing temperatures

(14.7 - 24.4 °C) from 0.7 to 1.1 d^{-1} for cultures grown at the salinity of 10; 1.0 to 1.5 d^{-1} for cultures grown at the salinity of 20; and 0.7 to 1.2 d^{-1} for cultures grown at the salinity of 32. The fastest specific growth rates, regardless of incubation temperature, were obtained from cultures grown at the salinity of 20, followed by cultures grown at the salinities of 10 and 32, respectively. This pattern of maximal growth rates at reduced salinities observed here and previously by others (Tomas 1978, Haque and Onoue 2002, Kempton et al. 2008), suggests preference for growth of *H. akashiwo* cells in brackish water conditions, although others have reported specific growth rates of *H. akashiwo* to increase with salinity (Fredrickson et al. 2011, Strom et al. 2013), or either increase or decrease depending on the location of isolation (Martinez et al. 2010).

Specific growth rates as a function of temperature and salinity, determined from a variety of *H. akashiwo* strains, are presented in Table 1. The growth rates from the present study (0.7 - 1.4 d^{-1}) are comparable to the rates reported by Tomas (1978), Honjo and Tabata (1985), Wood and Flynn (1995), Ono et al. (2000) and Herndon et al. (2007) but are greater than all of the other cited studies (Watanabe et al. 1982, Haque and Onoue 2002, Kempton et al. 2008, Martinez et al. 2010, Fredrickson et al. 2011, Strom et al. 2013). Although an isolate's ability to acclimate to different salinity conditions is assumed to contribute to the observed variability in growth rates, methodological variability among studies cannot be discounted. In particular, the PPFDs available for photosynthesis were < 200 µmol photons $\cdot$ m^{-2} $\cdot$ s^{-1} for the previously mentioned studies, but 350 µmol photons $\cdot$ m^{-2} $\cdot$ s^{-1} was used for the present study. This greater PPFD was experimentally determined to be saturating, but not photo-inhibitory to the cell growth of our *H. akashiwo* isolate (see Results: Fig. 1), and likely contributed to the generally faster growth rates reported here. In the Salish Sea, PPFDs > 200 µmol photons $\cdot$ m^{-2} $\cdot$ s^{-1} are routinely observed from May to August – the typical blooming months for *H. akashiwo* in this region. During this time period in 2013 – the year of the present study, surface PPFD ranged from *ca.* 500 - 1,600 µmol photons $\cdot$ m^{-2} $\cdot$ s^{-1} (from the Friday Harbor Laboratory Weather Station located on San Juan Island in the Strait of Juan de Fuca). Given that *H. akashiwo* characteristically accumulates at or near the sea surface (e.g., Imai 2003), one might expect in situ rates to exceed those reported for most laboratory

552 studies, and instead approach rates reported for outdoor cultures of this flagellate (e.g.,
553 exponential rates up to 2.4 d^{-1} in Sagami Bay, Japan; Honjo and Tabata, 1985).
554 The length of the acclimation period used in the previous studies was highly
555 variable (Table 1). Acclimation period is defined as the length of time required for the
556 rate of growth to not change after multiple transfers into fresh media (Wood et al. 2005).
557 The intent is to reduce the effect(s) of preconditioned trait(s) established from previous
558 growth conditions, and thus minimize any bias that may influence observations not
559 attributable to the experimental conditions (Lakeman et al. 2009). The current study
560 acclimated cells for a period of 4 d (*ca.* ≥ 4.5 generations), which is longer than the
561 acclimation periods reported by Haque and Onoue (2002) and Martinez et al. (2010), but
562 shorter than those reported by Tomas (1978) and Strom et al. (2013). Only Kempton et al.
563 (2008) reported acclimated growth rates according to the definition provided by Wood et
564 al. (2005).
565 Therefore, the specific growth rates observed in the present study may not be
566 directly comparable to the growth rates reported by others, due to a range of cellular
567 physiological states resulting from acclimation period variability. This may produce cells
568 that are either partially or fully acclimated to their respective experimental conditions.
569 However, the occurrence and duration of *H. akashiwo* blooms are generally observed
570 over relatively short time spans (Honjo 1994). Therefore, the short-term acclimation
571 period observed in the present study can be considered ecologically relevant by defining
572 partially acclimated cultures as representative of natural assemblages accustomed to
573 inconsistent environmental conditions.
574 *Cellular permeability*. The ability of *H. akashiwo* to migrate through strong
575 haloclines has been reported by Bearon et al. (2006) and Tobin et al. (2011). However,
576 change in cell permeability, as a physiological adaptive response to salinity, has not been
577 previously reported for this raphidophyte. During the exponential growth phase, cellular
578 permeability was inversely proportional to salinity at all temperatures (Fig. 3A).
579 Relatively large decreases in cellular permeability with increased temperature (14.7 to
580 27.7 ºC) were observed for cultures grown at salinities of 10 and 20, but not for cultures
581 grown at a salinity of 32. Since cells were maintained at a salinity of 32 prior to the start
582 of this study, no extensive physiological modifications (i.e., cell permeability changes)

583 would be expected or were observed for the experimental cultures grown at a salinity of
584 32. For cultures grown at salinities of 10 and 20, it is unknown whether longer
585 acclimation periods would have resulted in cell permeability values similar to those
586 reported for cultures grown at 32. If true, then the elevated cell permeability reported for
587 cultures grown at salinities of 10 and 20 are only part of the acclimation process and do
588 not represent the adapted state. During the stationary growth phase (Fig. 3B), cell
589 permeability followed the general pattern observed with exponential growing cells;
590 permeability was greatest at a salinity of 10, and decreased with increasing salinity to 32.
591 However, cell permeability during this growth phase may not only be a function of the
592 salinity, but also may reflect the decreased ability of stressed cells to maintain their
593 cellular structure.

594 SYTOX® Green is generally used as an indicator for the presence of non-viable
595 bacterial (Lebaron et al. 1998) and algal (Veldhuis and Kraay 2000) cells, based on the
596 intracellular accumulation of this stain due to compromised membranes. Two distinct
597 populations (i.e., non-viable cells with high SYTOX® Green fluorescence, and viable
598 cells with low SYTOX® Green fluorescence) were demonstrated in cultures of *H.*
599 *akashiwo* infected with either a double-stranded DNA virus or a single-stranded RNA
600 virus by Lawrence et al. (2006). In the present study, we observed intracellular
601 accumulation of SYTOX® Green during the exponential growth phase, and these cells
602 fluoresced well above the background fluorescence of similarly growing control cells
603 (those without the added stain). Our results show that intracellular accumulation of
604 SYTOX® Green is a relatively constant response for *H. akashiwo* independent of cellular
605 viability, and a depressed physiological state is not a necessary requirement for the
606 accumulation of SYTOX® Green in this alga. Veldhuis et al. (2001) reported similar
607 findings for various other phytoplankton species during exponential growth, and found
608 that cells stained with SYTOX® Green routinely fluoresced 2- to 3-fold greater than the
609 background fluorescence of control (unstained) cells.

610 However, Velduis et al. (1997) reported a 38 % decrease in SYTOX® Green
611 fluorescence as salinity increased from 10 to 30; a result based on samples containing live
612 cells. We examined the direct effect of salinity on SYTOX® Green fluorescence in the
613 absence of cells, and found that although fluorescence was greatest at the salinity of 32, it

decreased slightly with decreasing salinity (i.e., 5.2 and 1.6 % decreases for the salinities of 20 and 10, respectively). This together with the weak correlation between fluorescence and salinity ($r = 0.11$, $p = 0.78$) suggests that the decreases in fluorescence reported by Velduis et al. (1997) may reflect intracellular accumulations of SYTOX® Green due to compromised membrane integrity as a function of increased salinity.

The change in cellular permeability with salinity may be linked to the osmotic acclimation process described by Kirst (1989), which is defined by two phases. The first phase is characterized by rapid intracellular water flux in response to the osmotic gradient, which is not metabolically controlled, and occurs over short time spans (5 - 10 sec). The direction of the water flux is in response to the intra- and extracellular solute concentration(s). At low extracellular solute concentrations, relative to intracellular concentrations, water influxes into the cell, whereas at high extracellular concentrations there is an efflux of water from the cell. The second phase occurs over longer time spans (40 - 120 min) and is a metabolically controlled process that involves the internal regulation of ions (e.g., K^+, Na^+, Cl^-) and organic osmolytes to re-adjust the intracellular water potential to a preferred turgor pressure state.

The ability of a cell to maintain turgor pressure without damage to the cell structure is related to the elasticity of the cell wall, which is measured as the elastic modulus, ϵ (Philip 1958). High ϵ values indicate increased rigidity of the cell wall (Zimmermann 1978). For *H. akashiwo*, the lack of a thick cell wall (Imai et al. 1993) suggests that these cells have low ϵ values. This might prevent excessive pressure on the cell wall during the first phase of osmotic acclimation, as defined by Kirst (1989). Low ϵ values may also provide cells with higher resistance to short-term fluctuations in salinity (Zimmermann 1978), which would help explain *H. akashiwo's* ability to rapidly migrate through strong haloclines. The changes in cell permeability reported in the present study may further reduce turgor pressure during the first phase of the osmotic acclimation process and help prevent structural damage.

Cellular toxicity. Cytotoxicity increased with decreasing salinity (Fig. 4); uni-algal cultures grown at a salinity of 10 were on average 2.1-fold more toxic than uni-algal cultures grown at a salinity of 32. This follows the pattern previously reported for other laboratory studies of ichthyotoxic raphidophytes, including *Chattonella* spp. (Haque and

Onoue 2001), *Fibrocapsa japonica* (de Boer et al. 2004) and *H. akashiwo* (Haque and Onoue 2002). Our results also support the relationship observed between reduced salinity conditions in the Salish Sea, resulting from earlier and larger outflows of the Fraser River, and large, economically damaging blooms of *H. akashiwo* (Rensel et al. 2010). However, Rensel et al. (2010) did not report any ichthyotoxicity measures, and their suggestion of enhanced toxicity under low salinity conditions is based on field observations of the timing and extent of *H. akashiwo* blooms, and their impact on local mariculture operations.

In the present study, samples for cytotoxicity assays were not collected during the exponential growth phase due to limited culture volume and because minimal cytotoxicity values have been reported using the GCA for three strains isolated from the Salish Sea, including the present strain of *H. akashiwo,* (Cochlan et al. 2014). Consequently, it was decided to collect toxicity samples once cultures entered their stationary (unbalanced) phase of growth where cytotoxicity of cultures is expected to be greatest. To determine if toxicity is correlated with its growth rate, as observed by Ono et al. (2000), we compared our cytotoxicity results from the stationary growth phase to the growth rates determined from exponentially growing cells (Fig. 6). For cultures grown at a salinity of 32 (Fig. 6A), increased temperature resulted in corresponding increases in specific growth rate, but these cultures (14.7, 18.4, 21.4°C) were contaminated with an unidentified species of the diatom *Skeletonema.* Neither temperature nor specific growth rate appear to affect the cellular toxicity of cultures grown at salinities of 20 and 10 (Fig. 6, B & C). Given that none of the uncontaminated cultures grown at salinities of 32 (24.4 and 27.8 °C), 20 and 10 demonstrate a relationship between cytotoxicity and growth rate or temperature, suggests that co-growth with *Skeletonema* sp. was responsible for the considerable increase in cytotoxicity, accompanying a decrease in growth rate observed for the lower temperature cultures grown at 32.

Ono et al. (2000) reported that slower-growing cells (i.e., $< 0.6\ d^{-1}$; between 10-20 ºC) were more ichthyotoxic than faster-growing cells (i.e., $> 0.6\ d^{-1}$; between 25-30 ºC). These authors suggest that the reduction in ichthyotoxicity observed in the faster-growing cultures resulted from better growth conditions at the warmer incubation temperatures, whereas cooler temperatures resulted in increased cellular stress reflected in slower

676 growth rates and increased ichthyotoxicity. While Ono et al. (2000) and our present study
677 each report a positive relationship between temperature and specific growth rate, we did
678 not observe a significant relationship between toxicity and either growth rate or
679 temperature. This difference may be due to a number of factors, including assay
680 methodology (i.e., ichthyotoxicity vs. cytotoxicity assays), sampling during the
681 exponential versus the stationary phase of cell growth, and strain variability (Japanese vs.
682 North American strains) of *H. akashiwo* used by in two studies.
683 The coincidental occurrence of *H. akashiwo* and *Skeletonema* in the cultures
684 relates well with the natural bloom events of these two species during spring and summer
685 months that has led several authors (e.g., Pratt 1966, Yamasaki et al. 2007, Xu et al.
686 2010) to suggest that *H. akashiwo* uses an allelopathic compound to establish population
687 dominance over *Skeletonema* species. In these studies, the initial cell densities were
688 found to be a critical factor in the establishment of population dominance. Yamasaki et al.
689 (2007) reported that when cultures of *H. akashiwo* were inoculated at lower cell densities
690 than *Skeletonema costatum*, the resulting growth of *H. akashiwo* was negativity affected.
691 This may explain the inverse relationship observed in the present study between *H.*
692 *akashiwo* cell density and cytotoxicity for cultures grown at a salinity of 32, and the lack
693 of a relationship between cell density and toxicity for the uni-algal cultures grown at the
694 lower salinities (i.e., 10 and 20). Since a measurable increase in cytotoxicity was found in
695 contaminated cultures of *H. akashiwo*, compared to the uncontaminated cultures, the
696 ichthyotoxin reported for this raphidophyte might in fact be the same compound used for
697 allelopathy by *H. akashiwo*. Pratt (1966) reported that filtrates from *H. akashiwo* cultures
698 were the source of the allelochemical, and indicated that this compound may be a tannin-
699 like ectocrine. This was later expanded by Yamasaki et al. (2009), who described these
700 filtrate compounds as allelopathic polysaccharide-protein complexes (APPCs) with a
701 molecular size > 10^6 Da. Yamasaki et al. (2009) further proposed that these APPCs
702 produced from *H. akashiwo* function as glycoproteins that bind to the cell surface of *S.*
703 *costatum* to induce an allelopathic affect. The importance of direct cell contact in the
704 transmission of this compound also has been suggested in competition experiments
705 between *H. akashiwo* and *Akashiwo sanguinea* (Qiu et al. 2012). The transfer of this
706 allelopathic compound from *H. akashiwo* to other cells may be facilitated by the presence

of acidic and sulfated carbohydrates contained within the extracellular polysaccharide structure surrounding the cell (Yokote et al. 1985, Lopes et al. 2012), which may affect cellular adhesion and aggregation (Lopes et al. 2012). Therefore, this polysaccharide compound may be tightly associated with the cell surface, and its toxic effect (whether as an allelochemical or ichthyotoxin) will likely require direct cell contact.

Cellular permeability and cytotoxicity. The current study found a strong positive relationship ($r = 0.77$, $p = 0.003$) between cellular permeability and cytotoxicity for uncontaminated *H. akashiwo* cultures (Fig. 7). Increased permeability could enable cytoplasmic compounds, even large ones as proposed by Yamasaki et al. (2009) to be passively released from uni-algal culture cells. In *H. akashiwo* cultures contaminated with *Skeletonema* sp., the increase in cytotoxicity, irrespective of cellular permeability, implies an active release of this compound. Although speculative, this compound may serve dual physiological purposes in response to two distinct stressors – salinity and algal competition.

For ichthyotoxic raphidophytes, including *H. akashiwo*, the identity of the ichthyotoxin(s) and the associated mechanism(s) responsible for the mortality of finfish are enigmatic at best. Four ichthyotoxic pathways have been proposed, these include: (1) extracellular production of reactive oxygen species (ROS) by *H. akashiwo* that either damage gill cells directly, resulting in the suffocation of impacted fish (Yang et al. 1995, Twiner and Trick 2000) or function synergistically with other compounds to induce a more complex ichthyotoxic pathway (Twiner et al. 2001, Marshal et al. 2003, Dorantes-Aranda et al. 2015); (2) production of mucus by fish due to the presence of *H. akashiwo* cells. Fish mucus has been shown to increase ROS production by raphidophytes in a concentration dependent manner that could enhance ROS-mediated toxicity (Nakamura et al. 1998, Kim and Oda 2010), and potentially explain the lesions observed on affected fish gills (Chang et al. 1990); (3) production of free fatty acids by raphidophytes (Marshal et al. 2003) may function as hemolytic compound(s) that cause the lysis of fish erythrocytes (Ling & Trick 2010); and (4) production of an intracellular neurotoxin by *H. akashiwo* that negatively affects the cardiovascular system of fish (Endo et al. 1992, Landsberg 2002).

737 Historically, such neurotoxins have been characterized as being brevetoxin-like
738 compounds due to isolated organics from *H. akashiwo* having a molecular weight similar
739 to a brevetoxin (Khan et al. 1997, Ono et al. 2000). However, isolated organics from *H.*
740 *akashiwo* do not cause sodium channel activation, a characteristic of brevetoxins, but
741 instead cause sodium channel inhibition similar to a saxitoxin or tetrodotoxin (Astuya et
742 al. 2015), or cause apoptotic cell death by altering intracellular calcium hemostasis
743 (Twiner et al. 2005). Therefore, it is possible that this ichthyotoxin is a bioactive
744 metabolite unique from other known phycotoxins, and finfish mortality may be the result
745 of a combination, or successional sequence, of the pathways identified to date.
746 In light of the previously described studies, the findings from our study suggest
747 that *H. akashiwo* toxicity may be triggered by specific stressors (e.g., salinity and algal
748 competition), and the toxic response can vary markedly depending on the stressor. This
749 may partially explain the observed variability in ichthyotoxicity for different strains
750 (Smayda et al. 2006, Cochlan et al. 2014), and the change in toxicity from exponential
751 (Ono et al. 2000) to stationary (Cochlan et al. 2014) growth phases.
752 The strong correlation between cellular permeability and cytotoxicity reported
753 here for uni-algal cultures may be linked to changes in the extracellular accumulation of
754 polysaccharide structures surrounding the cell – termed the glycocalyx (Yokote et al.
755 1985). While the exact purpose of the glycocalyx is unknown, it has been suggested that
756 the acidic complex carbohydrates within the glycocalyx are linked to structural integrity,
757 selective absorption, filtration and ion-exchange (Honjo 1993). Scanning electron
758 microscopy (SEM) performed on the *H. akashiwo* strain used here show an extracellular
759 mesh-like structure surrounding the cell (results not shown). If the structure observed by
760 SEM is the glycocalyx, then it could be responsible for the observed changes in cellular
761 permeability by altering the intra- and extracellular flux of compounds. Furthermore, the
762 glycocalyx may be associated with ichthyotoxicity due to its ability to generate ROS
763 irrespective of the *H. akashiwo* cells (Kim et al. 2001). While it's doubtful that ROS
764 generated from the glycocalyx are the sole cause for finfish mortality, it is possible that
765 the glycocalyx is part of a larger ichthyotoxic mechanism and serves to regulate the
766 potency of neurotoxins (Twiner et al. 2001) or hemolytic compounds (Ling and Trick
767 2010).

Salinity and temperature will change in many coastal regions in response to global warming, and these abiotic changes also will likely impact the growth and toxicity of *H. akashiwo* found in the Salish Sea. By the year 2050, surface water temperatures of this estuarine region are predicted to increase by a maximum of 3 ºC during April to October, and the lowest salinity levels are expected to occur *ca.* 30 days earlier than presently observed during the growing season (Moore et al. 2015). In the present study, the temperature was found to have no direct measurable effect on cytotoxicity. However the earlier seasonal timing of lowered salinity conditions, together with increased ambient temperatures may result in the occurrence of earlier blooms of this fish-killing raphidophyte in the Salish Sea. Given that lower salinity conditions have been suggested by others (e.g., Strom et al. 2012) to provide *H. akashiwo* with a refuge from predation, and that toxicity was found here to be greatest at lower salinities, one can hypothesize that the general cytotoxic activity expressed in *H. akashiwo* would enable this population to outcompete other cells in the refuge. On a broader environmental scale, predicted changes in salinity and temperature may result in longer and more widespread blooms of *H. akashiwo* in the Salish Sea with potentially greater negative impacts on local fin-fish aquaculture operations.

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REFERENCES:

Anderson, R.A., Berges, J.A., Harrison, P.J. & Watanbe, M.M. 2005. Appendix A – recipes for freshwater and seawater media. *In* Anderson, R.A. [Ed.] *Algal Culturing Techniques*. Elsevier Academic Press, London, UK, pp. 429-532.

Astuya, A. Ramírez, A.E., Aballay, A., Araya, J., Silva, J., Ulloa, V. & Fuentealba, J. 2015. Neurotoxin-like compounds from the ichthyotoxic red tide alga *Heterosigma akashiwo* induce a TTX-like synaptic silencing in mammalian neurons. *Harmful Algae* 47: 1-8.

Bill, B.D., Moore, S.K., Hay, L.R., Anderson, D.M. & Trainer, V.L. Effects of temperature and salinity on the growth of *Alexandrium* (Dinophyceae) isolates from the Salish Sea. *J. Phycol.* doi: 10.1111/jpy.12386.

Bearon, R.N., Grunbaum, D. & Cattolico, R.A. 2006. Effects of salinity structure on swimming behavior and harmful algal bloom formation in *Heterosigma akashiwo*, a toxic raphidophyte. *Mar. Ecol.-Prog. Ser.* 306: 153-163.

Berges, J.A., Franklin, D.J. & Harrison, P.J. 2001. Evolution of an artificial seawater medium: Improvements in enriched seawater, artificial water over the last two decades. *J. Phycol.* 37: 1138-1145.

Butron, A., Madariaga, I. & Orive, E. 2012. Tolerance to high irradiance levels as a determinant of the bloom-forming Heterosigma akashiwo success in estuarine waters in summer. *Estuar. Coast Shelf S.* 107: 141-149.

Castaño, A., Bols, N.C., Braunbeck, T., Dierickx, P., Halder, M., Isomaa, B., Kawahara, K., Lee, L.E.J., Mothersill, C., Part, P., Repetto, G., Sintes, J.R., Rufli, H., Smith, R., Wood, C. & Segner, H. 2003. The use of fish cells in ecotoxicology –The report and recommendations of ECVAM workshop 47, *Altern. Lab. Anim.* 31: 317-351.

Chang, F.H., Anderson, C. & Boustead, N.C. 1990. First record of a *Heterosigma* (Raphidophyceae) bloom with associated mortality of cage-reared salmon in Big Glory Bay, New Zealand. *New Zeal. J. Mar. Fresh.* 24: 461-469.

Cochlan, W.P., Trainer, V.L., Trick, C.G., Wells, M.L., Bill, B.D. & Eberhart, B.T.L. 2014. *Heterosigma akashiwo* in the Salish Sea: Defining growth and toxicity leading to fish kills. *In* Kim, H.G., Reguera, B., Hallegraeff, G.M., Lee, C.K., Han, M.S. & Choi, J.K. [Eds.]. *Harmful Algae 2012, Proceedings of the 15th International Conference on Harmful Algae*. International Society for the Study of Harmful Algae 2014, ISBN 978-87-990827-4-2.

Dayeh, V.R., Schirmer, K. & Bols, N.C. 2002. Development and testing of methods for applying industrial effluent directly to fish cell cultures in order to evaluate effluent toxicity. *Water Res.* 36: 3727-3738.

Dayeh, V.R., Lynn, D.H. & Bols, N.C. 2003. Assessment of metal toxicity to protozoa and rainbow trout cells in culture. *In* Spiers, G., Beckett, P. & Conroy, H. [Eds.]. *Sudbury 2003 Mining and the Environment Conference Programme,* Laurentian University Centre for Continuing education, Sudbury, ON, Canada, pp. 205-211.

de Boer, M.K., Tyl, M.R., Vrieling, E.G. & Rijssel, M.V. 2004. Effects of salinity and nutrient conditions on growth and haemolytic activity of *Fibrocapsa japonica* (Raphidophyceae). *Aquat. Microb. Ecol.* 37: 171-181.

Dorantes-Aranda, J.J., Waite, T.D., Godrant, A., Rose, A.L., Tovar, C.D., Woods, G.M. & Hallegraeff, G.M. 2011. Novel application of a fish gill cell line assay to assess ichthyotoxicity of harmful marine microalgae. *Harmful Algae* 10: 366-373.

Dorantes-Aranda, J.J., Seger, A., Mardones, J.I., Nichols, P.D. & Hallegraeff, G.M. 2015. Progress in understanding algal bloom-mediated fish kills: The role of superoxide radiacals, phycotoxins and fatty acids. *PLoS One* 10: e0133549. doi: 10.1371/journal.pone.0133549

Endo, M., Onoue, Y. & Kuroki, A. 1992. Neurotoxin-induced cardiac disorder and its role in the death of fish exposed to *Chattonella marina*. *Mar. Biol.* 112: 371-376.

Fent, K. 2001. Fish cell lines as versatile tools in ecotoxicology: assessment of cytotoxicity, cytochrome P450, an induction potential and estrogenic activity of chemicals and environmental samples. *Toxicol. In vitro* 15: 477-488.

Friederich, G.E., Walz, P.M., Burczynski, M.G. & Chavez, F.P. 2002. Inorganic carbon in the central California upwelling system during the 1997–1999 El Niño–La Niña event. *Prog. Oceanogr.* 54: 185-203.

Gilmour, D.J., Hipkins, M.F. & Boney, A.D. 1984. The effect of decreasing the external salinity on the primary processes of photosynthesis in *Dunaliella tertiolecta*. *J. Exp. Bot.* 35: 28-35.

Guillard, R.R.L. 1973. Division rates. *In* Stein. J.R. [Ed]. *Handbook of Phycological Methods*. Cambridge University Press, Cambridge, UK, pp. 290-311.

Haque, S.M. & Onoue, Y. 2001. Variation in toxin compositions of two harmful raphidophytes, *Chattonella antiqua* and *Chattonella marina*, at different salinities. *Environ. Toxicol.* 17: 113-118.

Haque, S.M. & Onoue, Y. 2002. Effects of salinity on growth and toxin production of a noxious phytoflagellate, *Heterosigma akashiwo* (Raphidophyceae). *Bot. Mar.* 45: 356-363.

Harrison, P.J., Waters, R.E. & Taylor, F.J.R. 1980. A broad spectrum artificial sea water medium for coastal and open ocean phytoplankton. *J. Phycol.* 16: 28-35.

Harvey, E.L.A. & Menden-Deuer, S. 2012. Predator-induced fleeing behaviors in phytoplankton: A new mechanism for harmful algal bloom formation. *PLoS One* 7: e46438. doi: 10.1371/journal.pone.0046438

Herndon, J., Cochlan, W.P. & Horner, R. 2003. *Heterosigma akashiwo* blooms in San Francisco Bay. *Interagency Ecol. Program San Francisco Estuary Newslett*. 16: 46-48.

Herndon, J. & Cochlan, W.P. 2007. Nitrogen utilization by the raphidophyte *Heterosigma akashiwo*: Growth and uptake kinetics in laboratory cultures. *Harmful Algae* 6: 260-270.

Hishida, Y., Ishimatsu, A. & Oda, T. 1997. Mucus blockade of lamellar water channels in yellowtail exposed to *Chattonella marina*. *Fisheries Sci.* 63: 315-316.

Honjo, T. 1993. Overview on bloom dynamics and physiological ecology of *Heterosigma akashiwo*. *In* Smayda, T.J. & Shimizu, Y. [Eds.]. Toxic Phytoplankton Blooms in the Sea: Proceedings of the Fifth International Conference on Toxic Marine Phytoplankton, Newport, Rhode Island, USA, pp. 33-41.

Honjo, T. 1994. The biology and prediction of representative red tides associated with fish kills in Japan. *Rev. Fish. Sci.* 2: 225-253.

Honjo, T. & Tabata, K. 1985. Growth dynamics of *Olisthodiscus luteus* in outdoor tanks with flowing coastal water and in small vessels. *Limnol. Oceanogr*. 30, 653-664.

Howard, M.D.A., Cochlan, W.P., Ladizinsky, N. & Kudela, R.M. 2007. Nitrogenous preference of toxigenic *Pseudo-nitzschia australis* (Bacillariophyceae) from field and laboratory experiments. *Harmful Algae* 6: 206-217.

Ikeda, C.E. 2014. Growth and ichthyotoxicity of laboratory cultures of the raphidophyte *Heterosigma akashiwo* from Puget Sound, WA. M.S. Thesis, San Francisco State University, San Francisco, 143 pp.

Imai, I., Itakura, S. & Itoh, K. 1993. Cysts of the red tide flagellate *Heterosigma akashiwo*, Raphidophyceae, found in bottom sediments of northern Hiroshima Bay, Japan.

Nippon Suisan Gakk. 59: 1669-1673.

Imai, I. & Itakura, S. 1999. Importance of cysts in the population dynamics of the red tide flagellate *Heterosigma akashiwo* (Raphidophyceae). *Mar. Biol.* 133: 755-762.

Imai, I. 2003. Life history of raphidophycean flagelates. In: Okaichi T. [Ed.]. *Red Tides.* Terra Scientific Publishing Company, Tokyo, Japan, pp. 111-127.

Ishimatsu, A., Sameshima, M., Tamura, A. & Tatsuya, O. 1996. Histological analysis of the mechanisms of *Chattonella*-induced hypoxemia in Yellowtail. *Fisheries Sci.* 62: 50-58.

Kempton, J., Keppler, C.J., Lewitus, A., Shuler, A. & Wilde, S. 2008. A novel *Heterosigma akashiwo* (Raphidophyceae) bloom extending from a South Carolina bay to offshore waters. *Harmful Algae* 7: 235-240.

Khan, S., Arakawa, O. & Onoue. Y. 1997. Neurotoxins in a toxic red tide of *Heterosigma akashiwo* (Raphidophyceae) in Kagoshima Bay, Japan. *Aquac. Res.* 28: 9-14.

Khangaonkar, T., Yang, Z., Kim, T. & Roberts, M. 2011. Tidally averaged circulation in Puget Sound sub-basins: Comparison of historical data, analytical model, and numerical model. *Estuar. Coast. Shelf S.* 93: 305-319.

Kim, D(-K.)., Okamoto, T., Oda, T., Tachibana, K., Lee, K.S., Ishimatsu, A., Matsuyama, Y., Honjo, T. & Muramatsu, T. 2001. Possible involvment of the glycocalyx in the ichthyotoxicity of *Chattonella marina* (Raphidophyceae): immunological approach using antiserum against cell surface structures of the flagellate. *Mar. Biol.* 136: 625-632.

Kim, D. & Oda, T. 2010. Possible factors responsible for the fish-killing mechanisms of the red tide phytoplankton, *Chattonella marian* and *Cochlodinium polykrikoides*. In: Ishimatsu, A. & Lie, H-.J. [Eds.]. Coastal Environmental and Ecosystem Issues of the

East China Sea. Terrapub, Tokyo, Japan, pp. 245-268.

Kirst, G. O. 1989. Salinity tolerance of eukaryotic marine algae. *Annu. Rev. Plant Biol.* 40: 21-53.

Knepel, K. & Bogren, K. 2002. *Determination of orthophosphate by flow injection analysis: QuickChem® Method 31-115-01-1-H*. Lachat Instruments. Milwaukee, Wisconsin, 14 pp.

Kok, J.W.K., Yeo, D.C.J. & Leong, S.C.Y. 2015. Growth and physiological responses of a tropical toxic marine microgalga *Heterosigma akashiwo* (Heterokontophyta: Raphidophyceae) from Singapore waters to varying nitrogen sources and light conditions. *Ocean Sci. J.* 50: 491-508.

Lakeman, M.B., Dassow, V. & Cattolico, R.A. 2009. The strain concept in phytoplankton ecology. *Harmful Algae* 8: 746-758.

Landsberg, J.H. 2002. The effects of harmful algal blooms on aquatic organisms. *Rev. Fish. Sci.* 10: 113-390.

Lawrence, J.E., Brussaard, C.P. & Suttle, C.A. 2006. Virus-specific responses of *Heterosigma akashiwo* to infection. *Appl. Environ. Microb.* 72: 7829-7834.

Lebaron, P., Catala, P. & Parthuisot, N. 1998. Effectiveness of SYTOX Green stain for bacterial viability assessment. *Appl. Environ. Microb.* 64: 2697-2700.

Leibovitz, A. 1963. The growth and maintance of tissue-cell cultures in free gas exchange with the atmosphere. *Am. J. Epidemiol.* 78: 173-180.

Ling, C. & Trick, C.G. 2010. Expression and standardized measurement of hemolytic activity in *Heterosigma akashiwo*. *Harmful Algae* 9: 522-529.

Lopes, D.C., Maurer, J.B.B., Stevan-Hancke, F.R., Proenca, L.A.O. & Zawadzki-Baggio, S.F. 2012. Chemical analysis of exopolysaccharide fractions and lipid compounds of the microalga *Heterosigma akashiwo* grown in vitro. *Bot. Mar.* 55: 565-580.

Martinez, R., Orive, E. Laza-Martinez, A. & Seoane, S. 2010. Growth response of six strains of *Heterosigma akashiwo* to varying temperature, salinity and irradiance conditions. *J. Plankton Res.* 32: 529-538.

Moore, S.K., Johnstone, J.A., Banas, N.S. & Salathé Jr., E.P. 2015. Present-day and future climate pathways affecting *Alexandrium* blooms in Puget Sound, WA, USA. *Harmful Algae* 48: 1-11.

Marshall, J-.A., Nichols, P.D., Hamilton, B., Lewis, R.J. & Hallegraeff, G.M. 2003. Ichthyotoxicity of *Chattonella marina* (Raphidophyceae) to damselfish (*Acanthochromis polycanthus*): the synergistic role of reactive oxygen species and free fatty acids. *Harmful Algae* 2: 273-281.

Nakamura, A., Okamoto, T., Komatsu, N., Ooka, S., Oda, T., Ishimatsu, A. & Muramatsu, T. 1998. Fish mucus stimulates the generation of superoxide anion by *Chattonella marina* and *Heterosigma akashiwo*. *Fisheries Sci.* 64: 866-869.

Ono, K., Khan, S. & Onoue, Y. 2000. Effects of temperature and light intensity on the growth and toxicity of *Heterosigma akashiwo* (Raphidophyceae). *Aquac. Res.* 31: 427-433.

Philip, J.R. 1958. The osmotic cell, solute diffusibility, and the plant water cconomy. *Plant Physiol.* 33: 264-271.

Powers, L., Creed, I.F. & Trick, C.G. 2012. Sinking of *Heterosigma akashiwo* results in increased toxicity of this harmful algal bloom species. *Harmful Algae* 13: 95-104.

Pratt, D.M. 1966. Competition between *Skeletonema costatum* and *Olisthodiscus luteus* in Narragansett Bay and in culture. *Limnol. Oceanogr.* 11: 447-455.

Qiu, X., Yamasaki, Y., Shimasaki, Y., Gunjikake, H., Honda, M., Kawaguchi, M., Matsubara, T., Nagasoe, S., Etoh, T., Matsui, S., Honjo, T. & Oshima, Y. 2012. Allelopathy of the raphidophyte *Heterosigma akashiwo* against the dinoflagellate *Akashiwo sanguinea* is mediated via allelochemicals and cell contact. *Mar. Ecol. Prog. Ser.* 446: 107-118.

Rensel, J.E. 2007. Fish kills from the harmful alga *Heterosigma akashiwo* in Puget Sound: Recent blooms and review. Prepared by Rensel Associates Aquatic Sciences for the National Oceanic and Atmospheric Administration Center for Sponsored Coastal Ocean Research (CSCOR), Washington, DC, 58 pp.

Rensel, J.E., Haigh, N. & Tynan, T.J. 2010. Fraser River sockeye salmon marine survival decline and harmful blooms of *Heterosigma akashiwo*. *Harmful Algae* 10: 98-115.

Schirmer, K., Chan, A.G.J., Greenberg, B.M., Dixon, D.G., & Bols, N.C. 1997 Methodology for demonstrating and measuring the photocytotoxicity of fluoranthene to fish cells in culture. *Toxicol. Vitr.* 11: 107-119.

Schirmer, K., Tom, D.J., Bols, N.C. & Sherry, J.P. 2001. Ability of fractionated petroleum refineryeffluent to elicit cyto- and photocytotoxic responses and to induce 7-ethoxyresorufin-o-deethylase activity in fish cell lines. *Sci. Total. Environ.* 271: 61-78.

Shikata, T., Matsunaga, S., Nishide, H., Sakamoto, S., Onituka, G. & Yamaguchi, M. 2015. Diurnal vertical migration rhythms and their photoresponse in four phytoflagellates causing harmful algal blooms. *Limnol. Oceanogr.* 60: 1251-1264.

Smayda, T.J. 2006. Harmful algal bloom communities in Scottish coastal waters:

Relationship to fish farming and regional comparisons - A review; Paper 2006/3. Scottish Executive, Scottish Environmental Protection Agency (SEPA) http://www.scotland.gov.uk/Publications/2006/02/03095327.

Smith, P. & Bogren, K. 2001. *Determination of nitrate and/or nitrite in brackish or seawater by flow injection analysis colorimetry: QuickChem® Method 31-107-04-1-E*. Lachat Instruments, Milwaukee, Wisconsin, 12 pp.

Strom, S.L., Harvey, E.L., Fredickson, K.A. & Menden-Deuer, S. 2013. Broad salinity tolerance as a refuge from predation in the harmful raphidophyte alga *Heterosigma akashiwo* (Raphidophyceae). *J. Phycol.* 49: 20-31.

Sutherland, D.A., MacCready, P. Banas, N.S. & Smedstad, L.F. 2011. A model study of the Salish Sea estuarine circulation. *J. Phys. Oceanogr.* 41: 1125-1143.

Sutton-Quaid, B. & Trick, C.G. 2014. Turbulence, shear stress, and toxicity in *Heterosigma akashiwo*. *In* Kim, H.G., Reguera, B., Hallegraeff, G.M., Lee, C.K., Han, M.S. & Choi, J.K. [Eds]. *Harmful Algae 2012, Proceedings of the 15th International Conference on Harmful Algae. International Society for the Study of Harmful Algae 2014*, ISBN 978-87-990827-4-2.

Tobin, E.D., Gunbaum, D. & Cattolico, R.A. 2011. Pelagic-benthic transition of the harmful alga, *Heterosigma akashiwo*: Changes in swimming and implications for benthic cell distributions. *Harmful Algae* 10: 619-628.

Tobin, E.D., Grunbaum, D., Patterson, J. & Cattolico, R.A. 2013. Behavioral and physiological changes during benthic-pelagic transition in the harmful alga, *Heterosigma akashiwo*: Potential for rapid bloom formation. *PLoS One* 8: e76663.

Tomas, C.R. 1978. *Olithodiscus luteus* (Chrysophyeae) I. Effects of salinity and temperature on growth, motility and survival. *J. Phycol.* 14: 309-313.

Trainer, V.L., Moore, L., Eberhart, B.T.L, Bill, B.D., Cochlan, W.P., Ikeda, C.E., Wells, M.L., Incardona, J., Linbo, T., Miles, C.O. & Trick, C.G. 2015. Characterizing toxic activity from Heterosigma akashiwo: a tale of two assays. *In* MacKenzie, A. L. [Ed]. Marine and Freshwater Harmful Algae. *Proceedings of the 16th International Conference on Harmful Algae, Wellington, New Zealand 27th-31st October 2014. Cawthron Institute, Nelson, New Zealand and International Society of Harmful Algae (ISSHA),* ISBN 978-87-990827-5-9.

Tomas, C.R. 1980. *Olisthodiscus luteus* (Chrysophyeae). IV. Effects of light intensity and temperature on photosynthesis, and cellular composition. *J. Phycol.* 16: 149-156.

Twiner, M.J. & Trick, C.G. 2000. Possible physiological mechanisms for production of hydrogen peroxide by the ichthyotoxic flagellate *Heterosigma akashiwo*. *J. Plankton Res.* 22: 1961-1975.

Twiner, M.J., Dixon, S.J. & Trick, C.G. 2001. Toxic effects of *Heterosigma akashiwo* do not appear to be mediated by hydrogen peroxide. *Limnol. Oceanogr.* 46: 1400-1405.

Twiner, M.J., Chidiac, P., Dixon, S.J. & Trick, C.G. 2005. Extracellular organic compounds from the ichthyotoxic red tide alga *Heterosigma akashiwo* elevate cytosolic calcium and induce apoptosis in Sf9 cells. *Harmful Algae* 4: 789-800.

Veldhuis, M.J.W., Cucci, T.L. & Sieracki, M.E. 1997. Cellular DNA content of marine phytoplankton using two new fluorochromes: Taxonomic and ecological Implications. *J. Phycol.* 33: 527-541.

Veldhuis, M.J.W. & Kraay, G.W. 2000. Application of flow cytometry in marine phytoplankton research: Current applications and future perspectives. *Sci. Mar.* 64: 121-134.

1108 Veldhuis, M., Kraay, G. & Timmermans, K. 2001. Cell death in phytoplankton:
1109 Correlation between changes in membrane permeability, photosynthetic activity,
1110 pigmentation and growth. *Eur. J. Phycol.* 36: 167-177.
1111
1112 Watanabe, M., Nakamura, Y., Mori, S. & Yamochi, S. 1982. Effects of physico-chemical
1113 factors and nutrients on the growth of *Heterosigma akashiwo* Hada from Osaka Bay.
1114 Japan. *Jpn. J. Phycol.* 30: 279-288.
1115
1116 Wood, A.M., Everroad, R.C. & Wingard, L.M. 2005. Measuring growth rates in
1117 microalgal cultures. *In* Anderson, R.A. [Ed.] *Algal Culturing Techniques*. Elsevier
1118 Academic Press, San Diego, pp. 269-285.
1119
1120 Wood, G.J. & Flynn, K.J. 1995. Growth of *Heterosigma carterae* (Raphidophyceae) on
1121 nitrate and ammonium at three photon flux densities: Evidence for N stress in nitrate-
1122 growing cells. *J. Phycol.* 31: 859-867.
1123
1124 Xu, D., Zhou, B., Wang, Y., Ju, Q., Yu, Q. & Tang, X. 2010. Effect of CO_2 enrichment
1125 on competition between *Skeletonema costatum* and *Heterosigma akashiwo*. *Chin. J.*
1126 *Oceanol. Limn.* 28: 933-939.
1127
1128 Yamasaki, Y., Nagasoe, S., Matsubara, T., Shikata, T., Shimasaki, Y., Oshima, Y. &
1129 Honjo, T. 2007. Allelopathic interactions between the bacillariophyte *Skeletonema*
1130 *costatum* and the raphidophyte *Heterosigma akashiwo*. *Mar. Ecol. Prog. Ser.* 339: 83-92.
1131
1132 Yamasaki, Y., Shikata, T., Nukata, A., Ichiki, S., Nagasoe, S., Matsubara, T., Shimasaki,
1133 Y., Nakao, M., Yamaguchi, K., Oshima, Y., Oda, T., Ito, M., Jenkinson, I.R., Asakawa,
1134 M. & Honjo, T. 2009. Extracellular polysaccharide-protein complexes of a harmful alga
1135 mediate the allelopathic control it exerts within the phytoplankton community. *Int. S.*
1136 *Microb. Ecol.* 3: 808-817.
1137
1138 Yang, C.Z., Albright, L.J. & Yousif, A.N. 1995. Oxygen-radical-mediated effects of the

toxic phytoplankter *Heterosigma carterae* on juvenile rainbow trout *Oncorhynchus mykiss*. *Dis. Aquat. Org*. 23: 101-108.

Yokote, M., Honjo, T. & Asakawa, M. 1985. Histochemical demonstration of a glycocalyx on the cell surface of *Heterosigma akashiwo*. *Mar. Biol.* 88: 295-299.

Zimmermann, U. 1978. Physics of turgor and osmoregulation. *Annu. Rev. Plant Physio.* 29: 121-148.

Table 1. Exponential growth rates (μ; d^{-1}) for *H. akashiwo* cells, grown in N-replete batch cultures. Growth rates are reported as the average ± 1 SD where available; gen. = generations and NR = not reported.

Isolate Origin	Acclimation Period	PPFD[a]	L:D cycle (h)	Temp (ºC)	Salinity	μ (d^{-1})	Reference
Bulls Bay, SC, USA	see below[b]	62	14:10	25.0	30 20 10	0.63 ±0.02 0.76 ±0.04 0.57 ±0.03	Kempton et al. (2008)
Kagoshima Bay, Japan	≥ 2 gen.	60	12:12	24.4	35 30 25 20	0.60 ±0.02 0.63 ±0.02 0.68 ±0.01 0.66 ±0.02	Haque and Onoue (2002)
Kalaloch, WA, USA	NR	110	24:0	15.0	30.5	0.82 ±0.04	Herndon and Cochlan (2007)[c]
Milford Sound, NZ	> 2 gen.	100	12:12	23.0	35 20	0.47 0.38	Martinez et al. (2010)
Narragansett Bay, RI, USA	*ca.* 14 days	132	12:12	20.0	30 20 10	0.9 1.2 1.3	Tomas (1978)[d]
Narragansett Bay, RI, USA	> 2 gen.	100	12:12	23.0	35 20	0.43 0.49	Martinez et al. (2010)
Nervion River, Spain	> 2 gen.	100	12:12	23.0	35 20	0.57 0.47	Martinez et al. (2010)
Oslo Fjord, Norway	> 2 gen.	100	12:12	23.0	35 20	0.45 0.42	
Osaka Bay, Japan	NR	139[e]	12:12	20.0	9-30	0.64	Watanabe et al. (1982)
Salish Sea, WA & BC	*ca.* 14 days	93-159	12:12	15.0	30 20 10	*ca.* 0.5-0.6 *ca.* 0.5-0.7 *ca.* 0.3-0.7	Fredrickson et al. (2011)[e]
	7 - 14 days	85-160	12:12	15.0	30	*ca.* 0.5-0.6	Strom et al.

					20 8-12	*ca.* 0.5-0.7 *ca.* 0.3-0.7	(2013)[f]
	≥ 4.5 gen.	350	14:10	18.4	32 20 10	0.7 ±0.05 1.1 ±0.01 1.0 ±0.13	Present study
	≥ 4.5 gen.	350	14:10	21.4	32 20 10	1.0 ±0.11 1.4 ±0.03 1.2 ±0.11	
Sagami Bay, Japan	NR	58	14:10	24.0	33-34	1.4-1.6	Honjo and Tabata (1985)[g]
Seto Inland Sea, Japan	≥ 2 gen.	60	12:12	20.0	30	*ca.* 1.1	Ono et al. (2000)[h]
Vigo, Spain	NR	350 200	12:12 12:12	18.0 18.0	23 23	*ca.* 0.9 *ca.* 0.6	Wood and Flynn (1995)[i]

[a]Photosynthetic photon flux density (PPFD) measured in µmol photons · m^{-2} · s^{-1}.

[b]Acclimated µ determined when no change in µ was observed after three consecutive growth phases.

[c]Used an artificial seawater medium (ESAW; Harrison et al. 1980) with salinity *ca.* 30.5; mean growth rates are for NO_3^--grown cultures reported in figure 2 of Herndon and Cochlan (2007).

[d]PPFD calculated by converting from langleys · min^{-1} using 1 langley · min^{-1} = 3485 µmol photons · m^{-2} · s^{-1} (Richardson et al. 1983); growth rates estimated from the rates reported in figure 3 of Tomas (1978).

[e]Growth rates estimated from the rates reported in figure 2 of Fredrickson et al. (2011).

[f]Growth rates estimated from the rates reported in figure 2A of Strom et al. (2013).

[g]PPFD calculated by converting from 1klx = 16.5 µmol photons · m^{-2} · s^{-1} (Richardson et al. 1983).

[h]Growth rates estimated from the rates reported in figure 3 of Ono et al. (2000).

[i]Used an artificial seawater medium (ESAW; Harrison et al. 1980) with salinity of ca. 23; mean growth rates are for NO_3^--grown cultures reported in figure 2 of Wood and Flynn (1995).

Fig. 1. Specific growth rates of *H. akashiwo* (NWFSC-513) as a function of PPFD at two growth temperatures. Rates are determined by *in vivo* fluorescence over time (7 days) for cultures grown at 20 ºC (■) and 15 ºC (□). Symbols represent the mean ± the range of duplicate cultures (n=2); no error bars indicate that errors are smaller than symbol width.

Fig. 2. Specific growth rates (d^{-1}) for pre-experimental (A) and experimental (B) cultures of *H. akashiwo* grown at three different salinities (32, 20 and 10) as a function of incubation temperature. Rates were determined by *in vivo* fluorescence or cell abundance

(measured with flow cytometry) over time for A and B, respectively. Error bars for pre-experimental cells are the range of duplicate (n=2) samples; experimental cells are ± 1 SD of quadruplicate (n=4) samples.

Fig. 3. Cell permeability for cultures of *H. akashiwo* grown at three different salinities (32, 20 and 10) as a function of incubation temperature. Permeability values were determined as the average cellular-bound SYTOX® Green fluorescence during the exponential (A) and stationary (B) portion of the growth curve. Error bars indicate ± 1 SD of quadruplicate cultures (n=4).

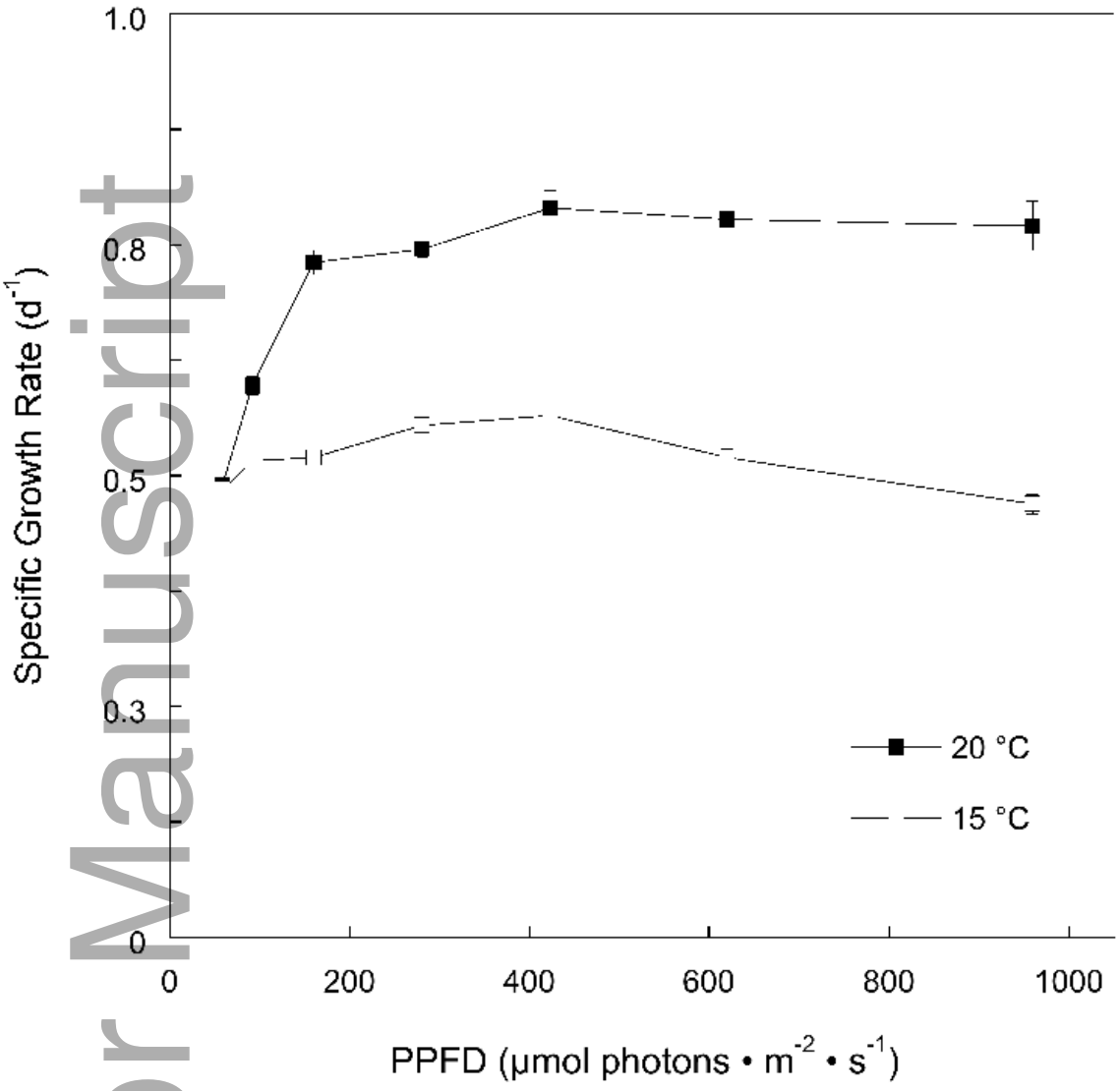
Fig. 4. Cellular toxicity of *H. akashiwo* for the three salinities (32, 20 and 10) as a function of incubation temperature. Cellular toxicity is reported as the percentage of non-viable gill cells as measured using the GCA. The open square (□) indicates an unusually high cytotoxicity measured for cells grown at the salinity of 20 and temperature of 21.4 °C, and is not averaged with its replicate culture's toxicity measurement. Error bars indicate the range (n=2) of duplicate cultures collected during the stationary growth phase.

Fig. 5. Cell density (cells · mL^{-1}) and cellular toxicity (%) for *H. akashiwo* cultures grown at the salinity of 32 (A), 20 (B), and 10 (C). Cell density is reported as the mean (n=4) ± 1 SD, and cytotoxicity values are the mean ± range of duplicates (n=2) samples. Values reported for cell density and cytotoxicity were collected during the stationary phase of growth.

Fig. 6. Comparison of specific growth rates (μ; d^{-1}) and cellular toxicity (%) for *H. akashiwo* cultures grown at the salinity of 32 (A), 20 (B), and 10 (C). Specific growth rates are reported as the mean ± 1 SD (n=4) of cultures measured during exponential

phase of growth, and toxicity values are the mean ± range of duplicates (n=2) during stationary phase of growth.

Fig. 7. Cellular toxicity as a function of cellular permeability. Results are the average cell permeability and average toxicity responses for cultures at each temperature/salinity treatment. Boxed data are the cultural responses for treatments contaminated with *Skeletonema* sp. at 14.7, 18.4, and 21.4 °C. Error bars reported for cell permeability are ± 1 SD (n=4); error bars for cellular toxicity indicate the range of duplicate (n=2) samples.



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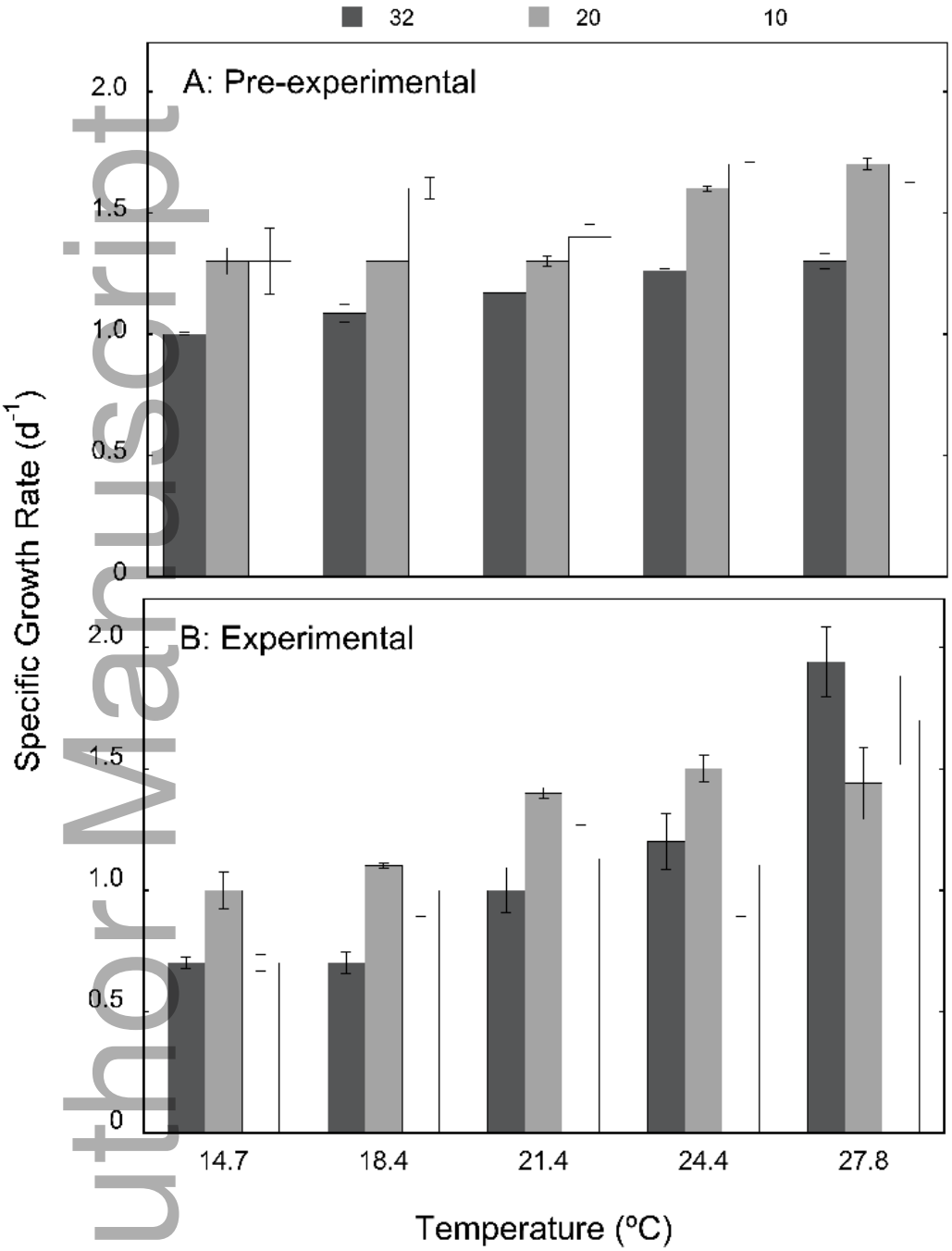

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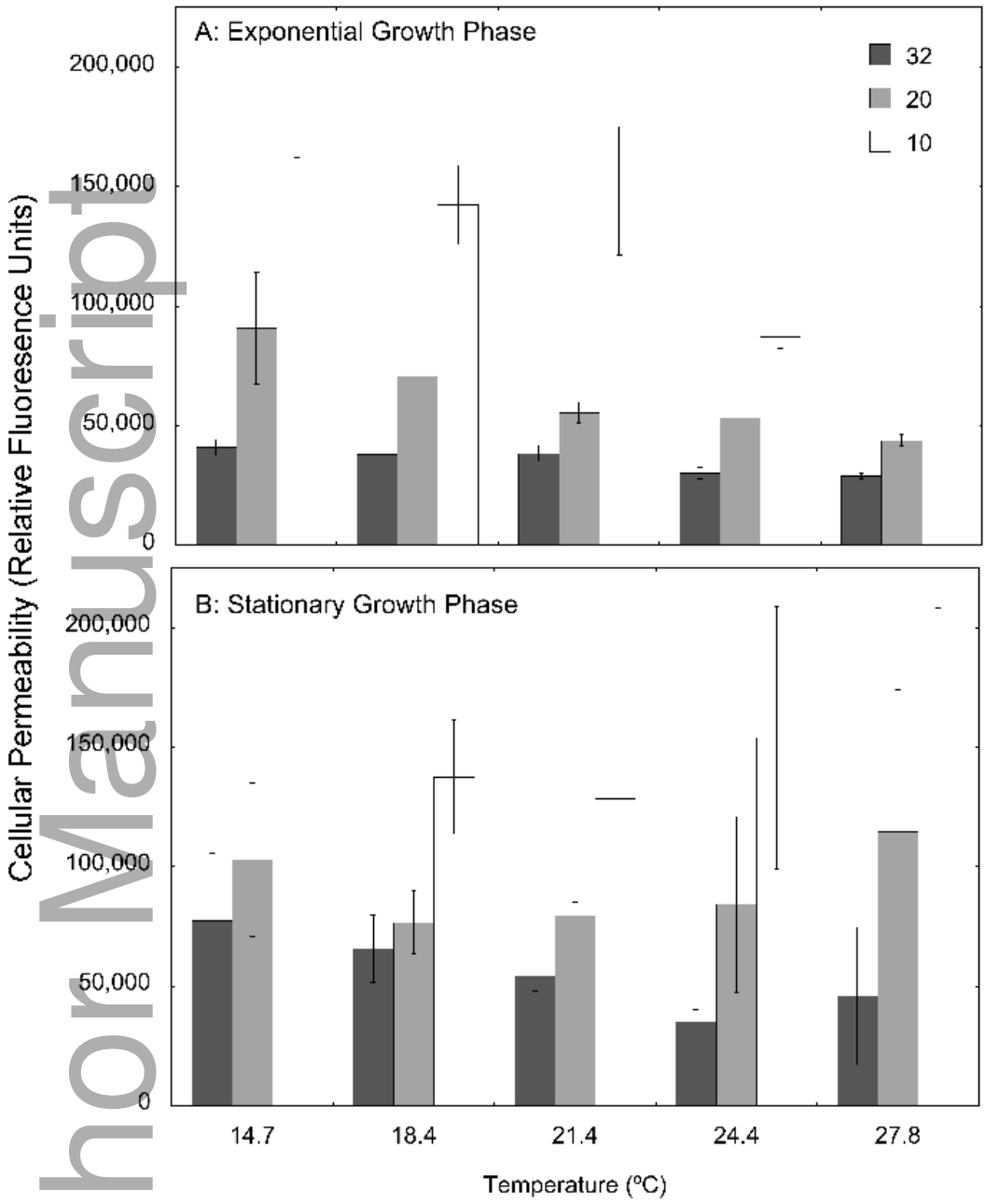

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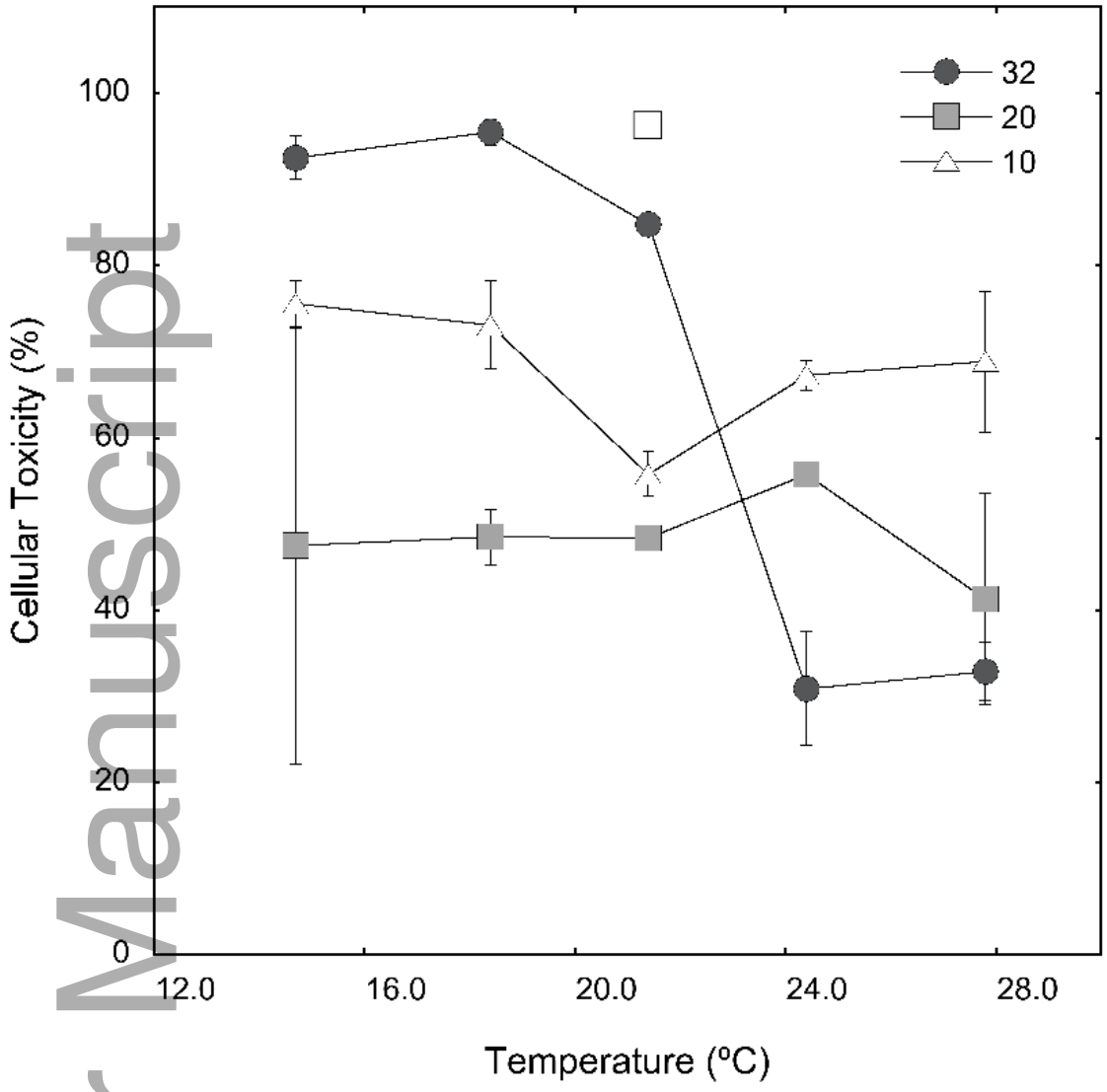

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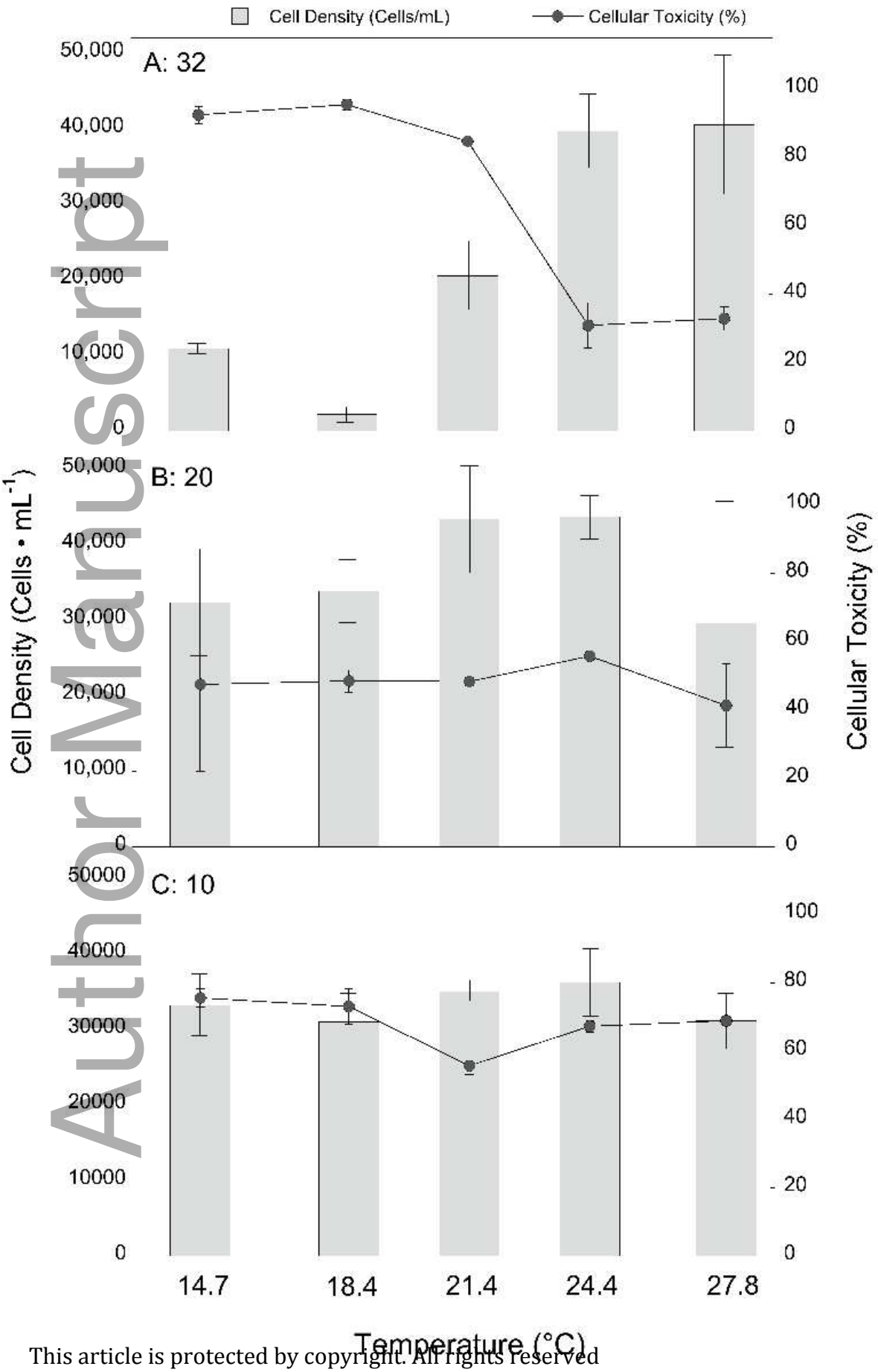

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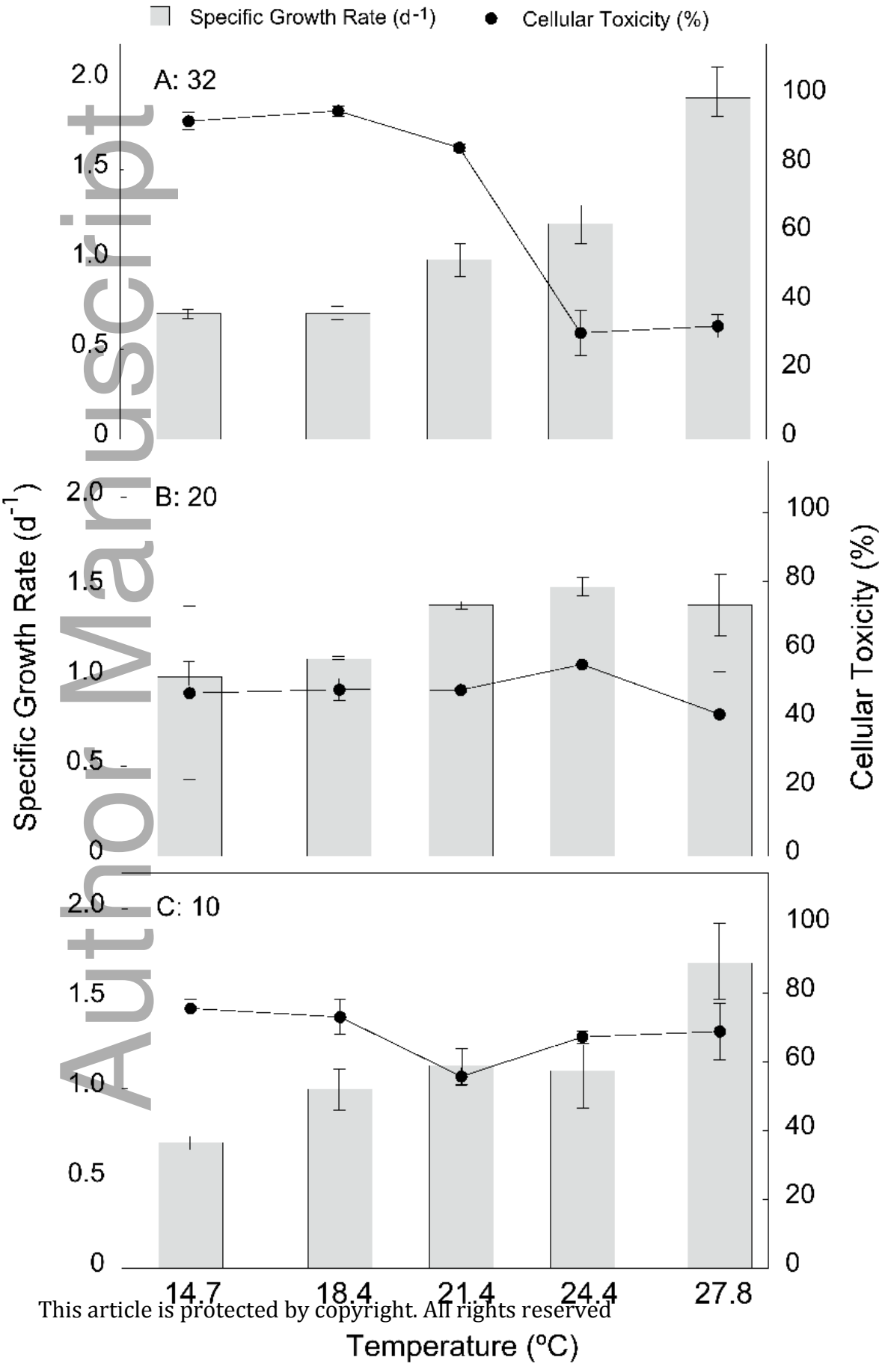

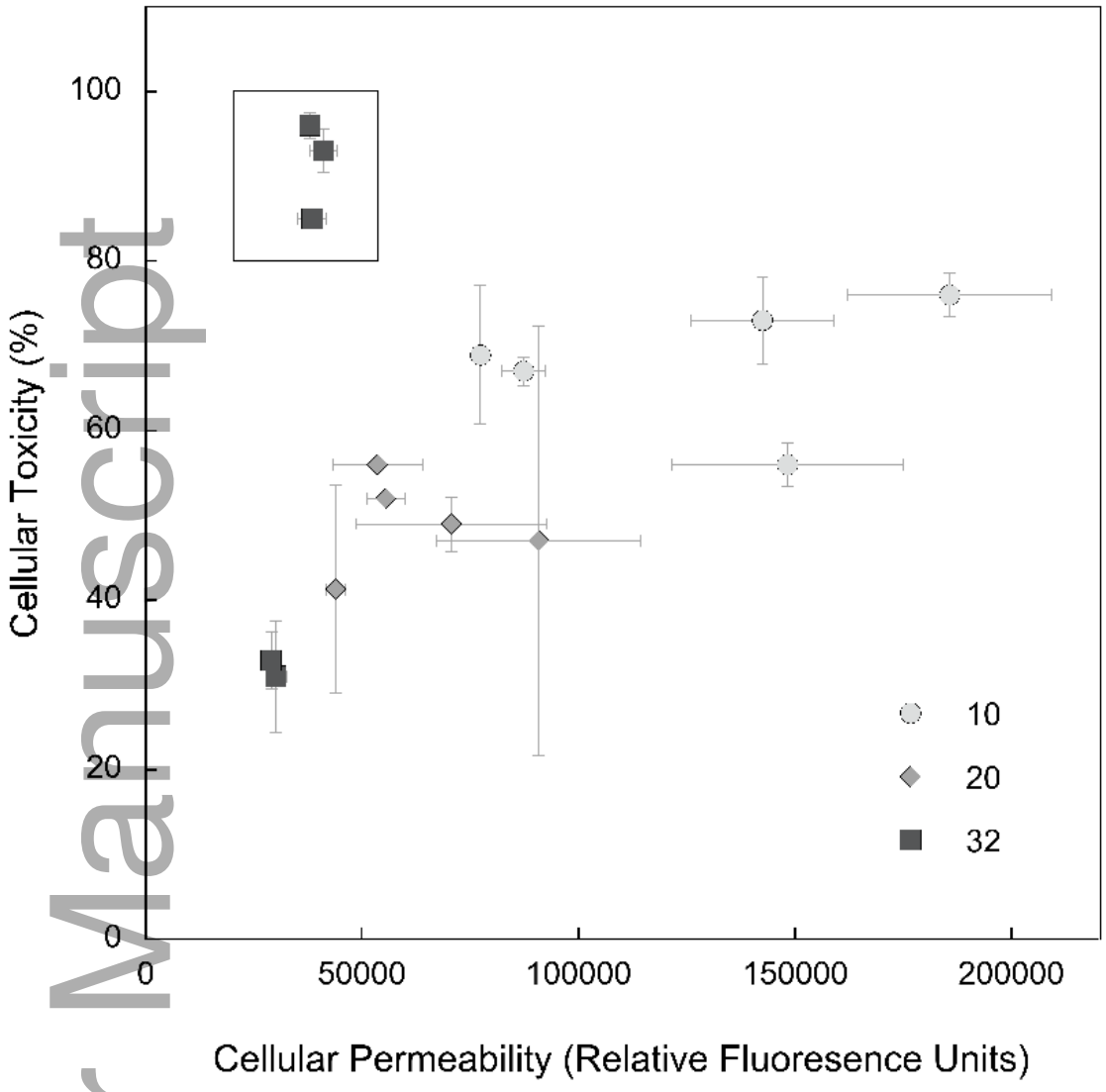

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