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# Extracellular organics from specific cultures of *Heterosigma akashiwo* (Raphidophyceae) irreversibly alter respiratory activity in mammalian cells

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**Abstract**

Raphidophyte blooms have been well documented in several coastal areas around the world. Centring raphidophyte-bloom research has been a focus evolving around issues of ichthyotoxicity, allelopathy and anti-predatory activity. However, the details of these phenomena such as the identity of the compounds and the mechanisms underlying these processes are poorly understood. One such raphidophyte, *Heterosigma akashiwo* (Hada) Hara et Chihara, has historically received much attention with regard to its ichthyotoxic and allelopathic properties. In this study, we collected extracellular organic compounds from cultures of nine *H. akashiwo* isolates and tested those exudates on two mammalian cell lines: rat osteoblastic sarcoma (UMR-106) and human embryonic kidney (HEK-293). A tetrazolium colourimetric assay was used to determine the activity of mitochondrial dehydrogenases. Exposure of the mammalian cell lines to exudates collected from cultures of *H. akashiwo* (strain 764) significantly increased activity in a concentration- and time-dependent manner. Exudate concentrations of as little as 0.3 mg ml$^{-1}$ elicited a stimulatory response in the mammalian cells. This is comparable to the range of exudate concentrations that were originally in the algal cultures (>0.1 mg ml$^{-1}$). Significant increases in activity were observed 12–24 h following continuous or 1 h (transient) exposure to the exudate. Production of the stimulatory bioactive exudate was not altered by nutrient-stressed *H. akashiwo* cultures (reduced iron, phosphate or nitrate). Collectively, these bioactive compound(s) consistently increased cellular activity 3–15-fold. Interestingly, of the nine isolates tested, four of them produced the stimulatory exudate, whereas four others did not produce the stimulatory compound(s) and isolate 560R produced a compound(s) that was inhibitory in nature. Thus, we have shown that cultures of *H. akashiwo* produce organic compounds that can alter the metabolic activity of mammalian cells. Future isolation and characterization of these bioactive compounds may determine them to have ecological relevance, potentially involved in the ichthyotoxic, allelopathic and/or anti-predatory behaviour of this alga.


*Keywords:* Exudates; HAB; HEK-293; *Heterosigma akashiwo*; Tetrazolium assay; UMR-106

## 1. Introduction

Harmful algal blooms (HABs) of the coastal raphidophyte *Heterosigma akashiwo* (Hada) Hara et Chihara have been the focus of many studies, primarily

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due to the devastating effects this alga has had on the aquaculture industry (Black et al., 1991). Not limited to ichthyotoxicity, *H. akashiwo* is also known for its anti-predatory activity (Uye and Takamatsu, 1990; Connell et al., 1997) and has also been shown to have allelopathic effects towards other algae, specifically diatoms (Pratt, 1966). Outbreaks of *H. akashiwo* have occurred in Canada (Taylor and Haigh, 1993), Japan (Honjo, 1993), New Zealand (Chang et al., 1993), the United States of America (Smayda, 1998), China (Tseng et al., 1993) as well as in Europe, Chile, and Bermuda (Honjo, 1993). Estimated annual losses by the aquaculture industry can exceed millions of dollars for many coastal areas (Black et al., 1991; Horner et al., 1991; Yang et al., 1995). Unfortunately, very little is known is about the ecotoxicology of this marine alga. As such, the ichthyotoxic mechanism by which raphidophytes such as *H. akashiwo*, *Chattonella* spp. and *Fibrocapsa* spp. employ is of particular interest due to the dramatic economical impacts of these algae, but has still remained a mystery. Proposed raphidophyte ichthyotoxic agents, which may also have allelopathic and/or anti-predatory activity include: (i) lectin-like polysaccharides that may cause asphyxiation by covering the surfaces of fish gills (Nakamura et al., 1998; Oda et al., 1998; Smayda, 1998); (ii) the production of reactive oxygen species (ROS) such as superoxide, hydrogen peroxide and hydroxy radicals (Oda et al., 1997; Twiner and Trick, 2000; Yang et al., 1995); and (iii) the production of an organic toxin that has been postulated to be brevetoxin (Khan et al., 1996, 1997). However, none of these agents have been undisputedly shown to be the ichthyotoxic agent. Toxicological studies using finfish incubations with toxic *H. akashiwo* suggest mixed results. In some studies, histological examination of gill tissue has observed deformities (Endo et al., 1985) whereas others have not observed such a phenomenon (Black, 2000). To complicate matters even more, not all blooms of *H. akashiwo* are constitutively toxic.

*H. akashiwo*-induced growth suppression, feeding suppression and/or mortality of bacteria (Tomas, 1980), fungi (Nagai et al., 1990), phytoplankton (Pratt, 1966), copepods (Uye and Takamatsu, 1990), bivalves (Connell et al., 1997) and finfish (Black, 2000) have been well documented for many years. Although identification of any bioactive extracellular compound(s) has yet to be definitively elucidated and characterized, it may be reasonable to suggest, as has been shown with many other toxic HAB species, that the above-mentioned toxicological phenomena associated with raphidophyte blooms may all be elicited by a chemically and functionally similar compound.

The purpose of this paper was to help elucidate *H. akashiwo* toxicity by investigating and characterizing the bioactivity of extracellular exudates collected from cultures of *H. akashiwo* in two mammalian cell types. Alteration of cellular activity induced by these exudates may lead to an understanding of the causative mechanism underlying fish kills or other ecological phenomena associated with this alga.

## 2. Materials and methods

### 2.1. Algal cultures and cell lines

Nine isolates of *H. akashiwo* and one isolate of *Tetraselmis apiculata*, which is known to be non-toxic to fish and marine invertebrates, were used in this study. Cultures of *H. akashiwo* (isolates 522R, 764, 625R, 560R, 102R, 630R) were obtained from the North East Pacific Culture Collection (NEPCC), Vancouver, Canada. Isolates Het PBS and Eddy were a gift from Dr. E.A. Black, Department of Fisheries and Oceans, Courtenay, Canada. The isolates from NEPCC and Dr. Black were originally collected from the British Columbia (Canada) coast with the exception of isolates 630R and 560R, which originated from Long Island Sound, USA. *H. akashiwo* isolate 302 (from Milford Sound, New Zealand) was purchased from the Provasoli-Guillard National Center for Culture of Marine Phytoplankton (CCMP), West Boothbay Harbor, USA. Cultures of *T. apiculata* 2562 were obtained from UTEX Culture Collection of Algae, Austin, Texas, USA.

All stock cultures were maintained in f/2 medium (–Si) (Guillard and Ryther, 1962) before transfer to artificial seawater media (ASM) (Harrison et al., 1980) supplemented with f/2 nutrients and vitamins. This transfer was done to ensure growth of the algae in nominally organic-free media. Nutrient supplemented f/2 stocks and replete ASM treatments contained $NaNO_3$ ($8.82 \times 10^{-4}$ M), $Na_2HPO_4$ ($3.6 \times 10^{-5}$ M), $Na_2EDTA$ ($1.17 \times 10^{-5}$ M), $FeCl_3$ ($1.17 \times 10^{-5}$), $CuSO_4$ ($4.00 \times 10^{-8}$ M), $ZnSO_4$ ($7.65 \times 10^{-8}$ M), $CoCl_2$ (4.20 ×

$10^{-8}$ M), $MnCl_2$ ($9.09 \times 10^{-7}$ M), $NaMoO_4$ ($2.60 \times 10^{-8}$ M), Vitamin $B_{12}$ ($3.68 \times 10^{-7}$ M), biotin ($2.04 \times 10^{-7}$ M), and thiamine ($2.96 \times 10^{-7}$ M). The effect of nutrient-stress on the production of extracellular exudates was tested by growing the isolates in reduced amounts of iron ($1.0 \times 10^{-8}$ M), nitrate ($4.4 \times 10^{-5}$ M) or phosphate ($1.8 \times 10^{-6}$ M). Upon transfer to ASM, isolates were grown in batch cultures (2–4 l) until late exponential or early stationary phase (10–12 days following inoculation) prior to exudate collection (as outlined below). Cells were grown without rotation at 18 °C under a continuous light flux of 65–80 μmol photons $m^{-2}$ $s^{-1}$.

Mammalian cell lines HEK-293 (human embryonic kidney) and UMR-106 (rat osteosarcoma) were maintained in Dulbecco's modified Eagle medium (DMEM) and alpha minimum essential medium (MEM), respectively. Both were supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS) and 1% (v/v) antibiotics (final concentrations 100 μg $ml^{-1}$ streptomycin, 100 U $ml^{-1}$ penicillin, 0.25 μg $ml^{-1}$ amphotericin B). Cells were subcultured at an inoculum to fresh medium ratio of 1:7 every 2–3 days by washing with Dulbecco's phosphate-buffered saline followed by release with a trypsin (0.5 g $l^{-1}$) and EDTA (0.2 g $l^{-1}$) solution. Cultures were maintained in humidified 5% $CO_2$: 95% air at 37 °C.

### 2.2. Exudate collection

Fractions of extracellular exudates from each isolate were obtained from batch cultures grown to early stationary phase by centrifugation (6000 × *g* for 10 min at 4 °C) and collection of the supernatant following filtration through a 0.45 μm pore size GF/C filter (Millipore™). Supernatant samples (400 ml) were concentrated via lyophilization into a ~30 ml salt and exudate slurry which was desalted via dialysis (molecular weight cutoff of 1 kDa). The 30 ml sample was twice flushed with 2 l of high purity Milli-Q© water (over 48 h at 4 °C) to desalt the exudate sample. The exudates were lyophilized to dryness, weighed as a crude organic fraction, and resuspended in 2% methanol (using Milli-Q© water). All culture glassware was acid-washed (1 N HCl) for >24 h and all polycarbonate bottles used to store organic fractions were washed and rinsed with distilled water, rinsed with methanol, rinsed with 1 N HCl, rinsed with Milli-Q© water and then filled with ultraviolet light-treated Milli-Q© water before use.

### 2.3. Cellular activity assay

For cellular activity assays, cells were inoculated in 100 μl of supplemented MEM at a density of 1–2 × $10^4$ cells per well in sterile, 96-well culture plates. After 12–24 h, to allow for attachment, the medium was aspirated and replaced with fresh, non-supplemented MEM (100 μl). To this, various amounts of filter-sterilized exudates (0.1–1.5 mg $ml^{-1}$) collected from the algal cultures were added for the appropriate course (up to 24 h). Controls included the addition of 2% methanol or addition of exudates from the non-toxic green alga, *T. apiculata*. Following exposure, the medium was replaced with fresh non-supplemented MEM (100 μl) prior to activity testing. Cellular activity was colourmetrically determined by the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay, which measures the activity of mitochondrial dehydrogenases (Mosmann, 1983). Briefly, 10 μl of MTT solution (5 mg $ml^{-1}$ in phosphate-buffered saline; 0.22 μm filter sterilized) was added to each well of a 96-well plate containing the attached cells in 100 μl MEM. Incubation proceeded for 4 h at 37 °C during which the tetrazolium ring of the dye was cleaved by mitochondrial dehydrogenases to form insoluble, blue formazan crystals. The overlying medium (and non-cleaved dye) was aspirated and replaced with 100 μl of an acidified isopropanol solution (0.04 N HCl in isopropanol) to dissolve the crystals. Following complete solubilization of the MTT crystals, the plates were read using a μQuant microplate reader (Bio-Tek Instruments Inc.) with KC4 software using a test wavelength of 562 nm and a reference wavelength of 690 nm. Cellular activity was determined from the ratio of absorbances at 562 and 690 nm. All data were expressed as a percentage of the control when no exudate was present. For consistency of cell number, protein quantification was done for each treatment using the Bio-Rad protein assay.

### 2.4. Statistical analysis

All data are expressed as mean ± S.E. ($n$ = 4–6 wells) and were compared statistically by one-way

analysis of variance (ANOVA) followed by Tukey multiple comparison tests ($P \leq 0.05$ being considered significant).

## 3. Results

### 3.1. Effect of exudate concentration on cellular activity of mammalian cells

HEK-293 and UMR-106 cells in non-supplemented MEM were exposed for 24 h to exudates (0, 0.1, 0.3, 0.5, 1, 1.5 mg ml$^{-1}$ final concentrations) collected from cultures of *H. akashiwo* (isolate 764). Following exposure to the exudates, the cellular activity of both cell lines increased significantly up to 8.7- and 2.7-fold the normalized, basal cellular activity for HEK-293 and UMR-106 cells, respectively (Fig. 1). Cellular activity of HEK-293 cells increased at exudate concentrations $\geq$0.3 mg ml$^{-1}$ whereas cellular activity of UMR-106 increased at concentrations $\geq$0.5 mg ml$^{-1}$.

### 3.2. Effect of exudate exposure time on cellular activity of mammalian cells

HEK-293 and UMR-106 cells in non-supplemented MEM were exposed to 0.5 mg ml$^{-1}$ exudate, collected from cultures of *H. akashiwo* (isolate 764), for various times periods (1, 12, 18, 24 h). Following exposure to the exudates, the cellular activity of both cells lines increased significantly up to 15.9- and 3.9-fold the normalized, basal cellular activity for HEK-293 and UMR-106 cells, respectively (Fig. 2). Cellular activity of HEK-293 cells increased after an exposure time of $\geq$18 h whereas cellular activity of UMR-106 increased after an exposure time of $\geq$12 h. To test if the increase in cellular activity was a delayed response

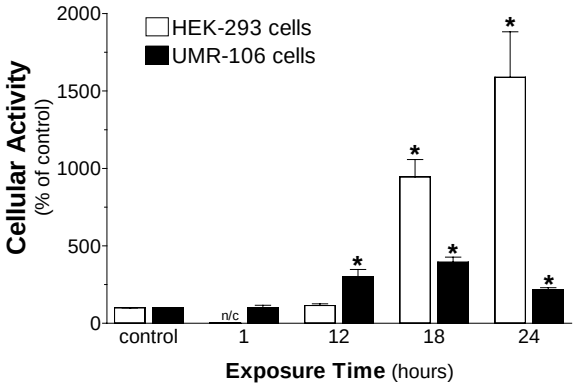


Fig. 2. Effect of exudate exposure time on cellular activity of HEK-293 and UMR-106 cells. Cells were exposed to exudates (0.5 mg ml$^{-1}$) collected from cultures of *H. akashiwo* (isolate 764) for various time periods (1, 12, 18, 24 h). Immediately following removal of exudate treatment, cellular activity was determined by the MTT assay. All data ($n = 6$ cell wells) are normalized to their respective control and expressed as a percentage. Significant differences relative to the control are shown by an asterisk ($*$) ($P < 0.05$). Exposure of HEK-293 cells for 1 h was not completed (n/c).

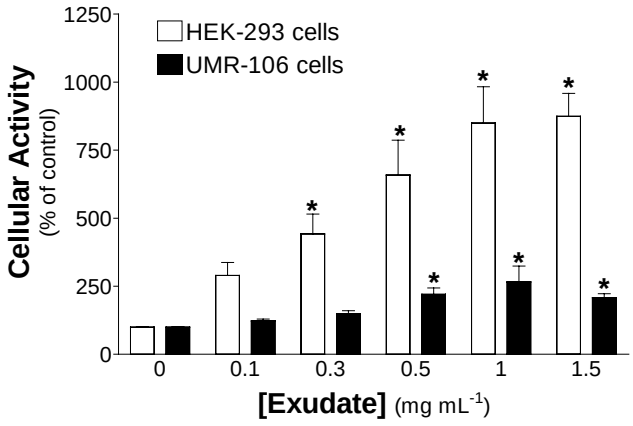


Fig. 1. Effects of *H. akashiwo* exudates on cellular activity of HEK-293 and UMR-106 cells. Cells were exposed for 24 h to various concentrations of exudates (0, 0.1, 0.3, 0.5, 1.0, 1.5 mg ml$^{-1}$) collected from cultures of *H. akashiwo* (isolate 764). Immediately following removal of exudate, cellular activity was determined by the MTT assay. All data ($n = 6$ wells) are normalized to their respective control and expressed as a percentage. Significant differences relative to the control are shown by an asterisk ($*$) ($P < 0.05$).

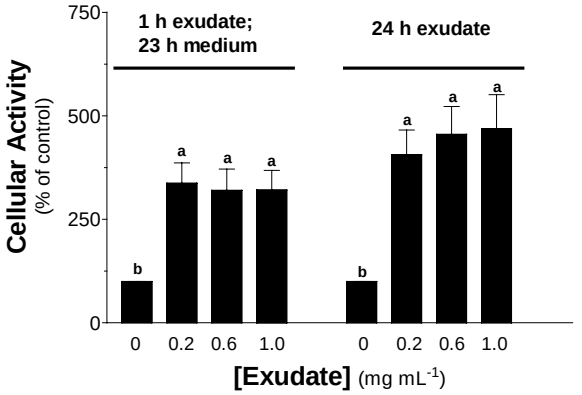


Fig. 3. Effects of exudate concentration and exposure time on cellular activity of UMR-106 cells. UMR-106 cells were exposed to various concentrations of exudate (0, 0.2, 0.6, 1.0 mg ml$^{-1}$) collected from cultures of *H. akashiwo* (isolate 764). Exposure time was either transient (1 h exudate followed by 23 h in exudate-free medium), or for 24 h exudate (protracted). Immediately following the 24 h treatment periods for both experiments, cells were assayed for cellular activity using the MTT assay. Parallel controls were done for each concentration and time treatment. All data ($n = 6$ cell wells) are normalized to their respective control and expressed as a percentage. Significant differences are shown by letters of significance ($P < 0.05$).

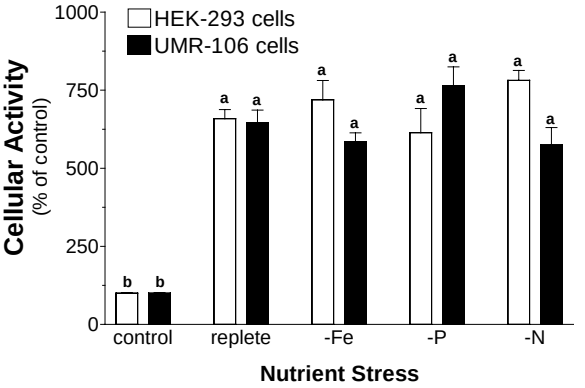


Fig. 4. Effects of exudates collected from nutrient-limited cultures of *H. akashiwo* on cellular activity of HEK-293 and UMR-106 cells. Exudates were collected from batch cultures that were either nutrient replete (replete; 12 μM $Fe^{3+}$, 36 μM $PO_4^{3-}$ and 882 μM $NO_3^-$), iron-limited (–Fe; same as replete except 10 nM $Fe^{3+}$), phosphate-limited (–P; same as replete except 1.8 μM $PO_4^{3-}$) or nitrate-limited (–N; same as replete except 44 μM $NO_3^-$). HEK-293 and UMR-106 cells were exposed to *H. akashiwo* exudates (isolate 764) (1.0 mg ml$^{-1}$) for 24 h. Immediately following removal of exudate treatment, cellular activity was determined by the MTT assay. All data ($n = 4$ wells) are normalized to their respective control and expressed as a percentage. Significant differences within each cell type are shown by letters of significance ($P < 0.05$).

following the initial exposure of the cells to the exudate, or if protracted exposure periods (≥12 h) were necessary, UMR-106 cells were exposed to various concentrations of exudate (0.2, 0.6, 1.0 mg ml$^{-1}$) for two different time regimes (Fig. 3). Exposure of the exudate was for either 1 h (acute exposure) followed by 23 h of medium only (exudate removed), or a 24 h (protracted) exposure period. Acute exposure to each concentration of exudate significantly increased cellular activity up to 3.4-fold of the control values, while the protracted exposure to each concentration of exudate increased cellular activity up to 4.7-fold of the control values. The cellular activities induced by the exudate concentrations of 0.2, 0.6, 1.0 mg ml$^{-1}$ were not significantly different amongst themselves or between exposure periods (acute versus protracted), but they were all significantly different from the controls.

### *3.3. Effect of nutrient-stress on the bioactivity of the H. akashiwo exudates*

*H. akashiwo* (isolate 764) cultures were grown in either nutrient replete (replete), iron-limited (–Fe), phosphate-limited (–P) or nitrate-limited (–N) media. HEK-293 and UMR-106 cells in non-supplemented MEM were exposed for 24 h to 1.0 mg ml$^{-1}$ exudate from each of the cultures of *H. akashiwo* that were grown under the various nutrient conditions. Following exposure to each of the exudates, the cellular activity of both cell lines increased significantly up to 7.8-fold of the normalized, basal cellular activity for both the HEK-293 and UMR-106 cells (Fig. 4). However, for each cell line there were no significant differences in the effect of nutrient limitation on the bioactivity of the exudates relative to the replete treatment.

### *3.4. Collection and testing of exudates from various isolates of H. akashiwo*

Nine isolates of *Heterosigma* spp. (522R, Eddy, 302, 102R, 560R, 764, 630R, 625R, Het PBS) and the non-toxic chlorophyte *T. apiculata* were grown in batch culture and exudates were collected. HEK-293 and UMR-106 cells in non-supplemented MEM were

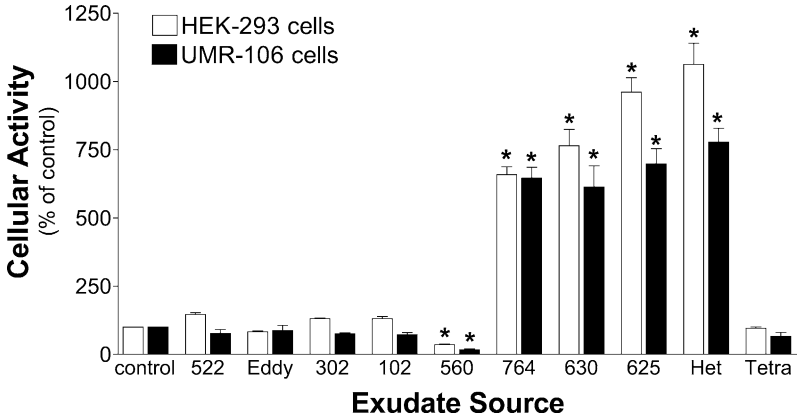


Fig. 5. Effects of exudates from various isolates of *H. akashiwo* on cellular activity of HEK-293 and UMR-106 cells. Cells were exposed to exudate (1.0 mg ml$^{-1}$) collected from cultures of *Heterosigma* spp. (isolates 522R, Eddy, 302, 102R, 560R, 764, 630R, 625R, and Het PBS) and *T. apiculata* 2562 (Tetra) for 24 h. Immediately following removal of exudate treatment, cellular activity was determined by the MTT assay. All data ($n = 6$ cell wells) are normalized to their respective control and expressed as a percentage. Significant differences relative to the control are shown by an asterisk (∗) ($P < 0.05$).

exposed to 1.0 mg ml$^{-1}$ exudate for 24 h prior to testing of cellular activity (Fig. 5). Exudates collected from *Heterosigma* spp. isolates 764, 630R, 625R, and Het PBS significantly increased cellular activity by greater than 6.1-fold relative to the control for both cell lines. However, exudates collected from *Heterosigma* spp. isolates 522R, Eddy, 302, 102R, and *T. apiculata* did not alter the cellular activity of HEK-293 or UMR-106 cells. Unfortunately not further assessed, exudates from *H. akashiwo* isolate 560R depressed cellular activity to less than 45% of control for HEK-293 cells, and less than 25% of control for UMR-106 cells.

## 4. Discussion

In various temperate regions of the globe, blooms of the raphidophyte *H. akashiwo* have been shown to be ecologically and economically very destructive. Economically, aquaculture finfish and shellfish are adversely affected, while ecologically, investigators have illustrated suppressional or lethal effects of *H. akashiwo* on bacteria (Tomas, 1980), fungi (Nagai et al., 1990), phytoplankton (Pratt, 1966) and copepods (Connell et al., 1997). Current research has focused on the ichthyotoxic properties of this alga yet the toxicological details characterizing the ichthyotoxin or other toxic compounds produced by raphidophytes are unclear (Anderson, 1995). In this paper, we have taken the initial steps in identifying a potentially novel extracellular compound that may be responsible for one or more of the ecological phenomena associated with this alga.

Tetrazolium assays have been used to assess cell proliferation, cytotoxicity, activation and survival (Gerlier and Thomasset, 1986; Hussain et al., 1993; Kasugai et al., 1990; Mosmann, 1983). The tetrazolium assay is relatively quick and can be used efficiently to assess the effect of a substantial number of compounds on a variety of cell lines. MTT is a tetrazolium dye that is oxidized by mitochondrial dehydrogenases in living cells. By assessing dehydrogenase activity, one can determine the respiratory status of the cell (cellular activity). Increased dehydrogenase activity can be indicative of either elevated cellular activity or an increase in cell number. Conversely, decreased dehydrogenase activity can indicate either cytotoxicity (reduction in the number of viable cells) or decreased respiratory activity. Recently, the MTT assay has been used to identify and quantify algal toxins such as saxitoxin, brevetoxin, and ciguatoxin (Fairey et al., 1997; Manager et al., 1993), as well as to identify and characterize novel ichthyotoxic compounds, such as that produced by *Pfiesteria piscicida* (Fairey et al., 1999).

Typical exudate concentrations within the original algal cultures at the time of collection were calculated to be in the range of 0.1–0.2 mg ml$^{-1}$. Exposure of two distinct mammalian cell lines to ecologically-relevant concentrations of exudates from cultures of *H. akashiwo* (isolate 764) revealed a unique phenomenon. For instance, exudate concentrations of ≥0.3 mg ml$^{-1}$ with exposure periods of ≥12 h increased cellular activity up to 15-fold. HEK-293 cells appear to be particularly susceptible to stimulation by these organics whereas UMR-106 cells were much more variable throughout the course of experimentation. For instance, stimulation of UMR-106 cells fluctuated from three to eight-fold in response to 1 mg ml$^{-1}$ of the organics. Cell type differences may account for the increased sensitivity in the HEK-293 cells, however, until determination of the mode of action of the bioactive organic(s), this is only speculative. The crude, potentially non-homogenous nature of the organics may have also played a role in the variability of responses within a cell type from experiment to experiment.

Visual examination of HEK-293 cells exposed to *H. akashiwo* exudates revealed two responses: an initial detachment of some of the centrally located cells from the culture plates, and cell lysis following prolonged exposure (>24 h). Subsequent experiments have shown that cell death occurs after 24 h via apoptosis (Twiner et al., 2001). Cell lysis may be the result of metabolic burnout from the drastic increase in respiratory metabolism that was observed. It is also observed that the increase in respiratory metabolism induced by the organic(s) appears to be irreversible. Wash out experiments where the cells were only exposed to the organic(s) for a short period of time (1 h) were equally stimulated relative to the protracted treatments (24 h). Although cellular and organismal toxicity of this compound will also be dependent on its stability, preliminary evidence suggest that the efficacy appears to be relatively high. The ecological significance of stimulating cellular respiratory rates of cells or organisms is unknown with respect to ichthyotoxicity, allelopathy, anti-grazing or otherwise, however enhancement of metabolic activity to the degree observed in the current study will likely have a toxicological impact. It would not be unreasonable to suggest that toxins such as cholera toxin act in a similar manner by disrupting the metabolic rate of a cell. Cholera toxin activates adenylate cyclase resulting in an increase of cAMP of up to 100-fold (Darnell et al., 1986).

Recent evidence suggests that brevetoxin-like compounds are produced by *H. akashiwo* (Khan et al., 1997) and *Chattonella cf. verruculosa* (Bourdelais et al., 2002). However, our investigation could not confirm or negate this suggestion as assessed both experimentally and by methodological design. The exudate collection protocol used in the current study only collected compounds >1000 Da, whereas brevetoxin (PbTx-3) is ∼897 Da (Nakanishi, 1985). As well, voltage-gated sodium channels, the target for brevetoxin, are often specific to neuronal, cardiac and skeletal muscle tissues (Bottein Dechraoui and Ramsdell, 2003; Mandel, 1992). UMR-106 cells are not believed to contain voltage-gated sodium channels, whereas the HEK-293 cells may contain a very small portion of them (Black and Waxman, 1996). Additionally, the observed increases in cellular activity induced by the stimulatory exudates collected from *Heterosigma* cultures (isolate 764) were not altered in the presence of various sodium channel ligands such as brevetoxin, veratridine, or tetrodotoxin (data not shown). Although the data and experimental protocols collectively suggest that the bioactive exudate is not a response consistent with brevetoxin, we have obviously not discerned whether brevetoxin-like compounds are concurrently produced by *Heterosigma* spp. Taken together, this does suggest that a novel compound(s) contained within the exudate is responsible for increasing the cellular activity of the two cell types used in this study.

Since aquaculturalists suggest that not all *H. akashiwo* blooms are toxic it is believed that environmental conditions may trigger alterations in cellular physiology that in turn, govern bloom toxicity. Although eutrophication often initiates a bloom, subsequent nutrient limitation, particularly by phosphate, has been shown to enhance the toxicity of some cultured algae and bacteria (Anderson et al., 1990; Doucette and Trick, 1995; Hallegraeff, 1993). As such, *H. akashiwo* (isolate 764) was grown in nutrient-limited (iron, phosphate, nitrate) batch cultures prior to exudate extraction. However, the bioactivity of the exudates obtained from the various nutrient-stressed cultures was not altered relative to the exudates collected from nutrient replete cultures. This suggests that production of a bioactive

compound by *H. akashiwo* is not regulated by iron, phosphate or nitrate limitation. Future analysis may indicate that another essential nutrient may be important in regulation of cellular production of the bioactive compound(s). Moreover, production of the bioactive exudate may not be constitutive during all phases of growth, as exudates were collected only during late exponential or early stationary growth.

Although stimulatory exudates from *H. akashiwo* (isolate 764) were used for most of the current investigation, a survey of exudates collected from eight other *Heterosigma* spp. was done to determine if these stimulatory bioactive compounds were commonly produced by geographically distinct isolates. Four of the assessed isolates (764, 630R, 625R, and Het PBS) produced the stimulatory bioactive compound, whereas four other *H. akashiwo* isolates (522R, Eddy, 302, and 102R) and exudates from *T. apiculata* had no significant effect on cellular activity of the mammalian cells. Although there is no geographical correlation with respect to isolates inducing a bioactive response, the variability of responses observed between isolates may be the first step towards discerning potentially toxic *H. akashiwo* isolates from non-toxic isolates. As a control for common metabolic waste products, exudates collected from non-toxic cultures of *T. apiculata* were also assessed; they did not alter cellular activity. Additionally, *H. akashiwo* 560R induced an inhibitory response, significantly depressing cellular activity. Although not investigated further, the inhibitory exudates from this isolate may have important properties relevant to the ecology and/or toxicology of this alga. Currently, the identities of the stimulatory and inhibitory bioactive compounds revealed in this study are unknown.

Using a cell line approach and determining metabolic activity via the tetrazolium assay, we have investigated potential mechanisms by which *Heterosigma* spp. may cause ecological and economical damage. In the pursuit of identifying potential organic toxins, exposure of two distinct mammalian cell lines to *Heterosigma* spp. exudates caused a dramatic irreversible increase in respiratory activity that was organism- and isolate-specific but not affected by nutrient limitation of the alga. Bioactive compounds produced by raphidophytes have long been hypothesized. Allelopathic and self-stimulatory extracellular agents have been thought to be responsible for algal species succession involving *H. akashiwo* and *Skeletonema costatum* (Itakura et al., 1996). A classical study by Pratt (1966) documented the successional dynamics of dominating phytoplankton species. It was shown that *H. akashiwo* exudates act in a self-stimulatory fashion, while growth of the diatom *S. costatum* was inhibited. The allelopathic compound in this study was believed to be a high molecular weight tannin. Moreover, Honjo (1990, 1993) has suggested that secretion or exfoliation of polysaccharide–protein complexes, proposed to be from the glycocalyx of this wall-less alga, may be the source of these bioactive allelopathic compounds.

Tetrazolium assays serve as efficient methods for identifying, isolating and purifying bioactive compounds from potentially toxic phytoplankton such as *H. akashiwo*. Steps outlined in this study have identified the production of bioactive compounds by specific isolates of cultured *H. akashiwo* and assessed the effect of nutrient limitation on the production of these compounds. The diverse nature of the responses in cellular activity induced by the various *Heterosigma* spp. exudates suggests that there is either more than one bioactive organic produced by the various isolates of *H. akashiwo*, or the organics obtained from *H. akashiwo* isolate 560R are either more efficacious or more concentrated resulting in enhanced rates of cellular mortality. In the former scenario suggesting greater than one bioactive compound, a stimulatory organic may be functioning as an allelopathic compound, whereas the inhibitory compound could be ichthyotoxic in nature. Regardless, the use of these cell lines in conjunction with the MTT assay may be applied in future assays to decipher toxic *Heterosigma* blooms from non-toxic *Heterosigma* blooms. However, subsequent experiments will have to determine the identity of the bioactive compounds involved, determine the precise mechanism of action of these compounds, and assess their relevance to fish kills, algal succession and other ecological aspects.

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