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Toxic effects of *Heterosigma akashiwo* do not appear to be mediated by hydrogen peroxide

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Abstract

The ichthyotoxic red tide organism *Heterosigma akashiwo* (Raphidophyceae) has been associated with fish kill events within the aquaculture industry for many years. The precise toxicological mechanism involved in these fish kills is unclear; however, much research attention has focused on the production of reactive oxygen species (ROS) by these toxic algae. In this study, we investigated the production of hydrogen peroxide (H_2O_2) by isolates of *H. akashiwo* and the nontoxic chlorophyte *Tetraselmis apiculata.* Subsequently, we tested those concentrations of H_2O_2 on vertebrate cell lines and the invertebrate *Artemia salina* (brine shrimp) to investigate mortality. Net production rates for the *H. akashiwo* isolates ranged from 0.46 to 7.89 pmol H_2O_2 min^{-1} $(10^4$ cells$)^{-1}$ while obtaining maximum concentrations between 0.14 and 0.91 μM H_2O_2. Conversely, *T. apiculata* produced only 0.03 pmol H_2O_2 min^{-1} $(10^4$ cells$)^{-1}$ with a maximum level on 0.04 μM. However, toxic effects on UMR-106 and HEK-293 cells were only induced by acute and protracted exposure to concentrations of $H_2O_2 \geq 0.1$ mM. Additionally, significant mortality of *A. salina* in the presence or absence of ferric and ferrous iron was induced by H_2O_2 levels ≥ 1 mM. Iron is a redox metal that reduces H_2O_2 to hydroxy radicals. These data collectively indicate that production of H_2O_2 by multiple isolates of *H. akashiwo* is orders of magnitude less than that required for mortality of either the vertebrate cell lines or the invertebrate *A. salina.* Other nonichthyotoxic roles for extracellular ROS are proposed.

Harmful algal blooms (HAB) of the coastal raphidophyte *Heterosigma akashiwo* (Hada) Hara et Chihara have been increasing in prevalence over the last few decades. The devastating effects of this noxious alga on fish and shellfish have become of the utmost concern for many aquaculturists around the world (Black et al. 1991). *H. akashiwo* outbreaks have occurred in Canada (Taylor and Haigh 1993), Japan (Honjo 1993), New Zealand (Chang et al. 1993), the United States (Smayda 1998), and China (Tseng et al. 1993), as well as in Europe, Chile, and Bermuda (Honjo 1993). In western Canada alone, losses by the salmon farming industry in excess of Can$3 million yr^{-1} are attributed to toxic *H. akashiwo* blooms (Black et al. 1991; Yang et al. 1995). Similarly, *H. akashiwo* blooms in Puget Sound, Washington, have resulted in an estimated US$4 to 5 million annual loss to fish aquaculturists (Horner et al. 1991).

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Until recent years, the toxicological mechanism by which *H. akashiwo* and other closely related raphidophytes, such as *Chattonella* spp. and *Fibrocapsa* spp., kill fish has remained a mystery. However, much information has been compiled regarding the putative toxin that may be involved in this alga's ichthyotoxic action. Amidst controversy and debate, three hypotheses have arisen. The first is the involvement of mucus or other lectin-like polysaccharides that may cause asphyxiation by covering fish gills. However, it is not known if this mucus is a defense response by the fish or an exfoliated substance from the algal surface (Nakamura et al. 1998; Oda et al. 1998; Smayda 1998). The second hypothesis involves the production of an organic toxin. A putative neurotoxin has been isolated from waters containing bloom concentrations of raphidophyte cells, both in situ and in vitro (Khan et al. 1996, 1997). These neurotoxinlike compounds are believed to be brevetoxins (Khan et al. 1997) and may lead to cardiac disorders and/or gill damage (Endo et al. 1992). The third hypothesis involves excessive production of reactive oxygen species (ROS) such as superoxide ($O_2^{\cdot -}$), hydrogen peroxide (H_2O_2), and hydroxy radicals ($OH^{\cdot}$) by raphidophyte species and there is a suggestion that these ROS are the ichthyotoxic agents (Yang et al. 1995; Oda et al. 1997; Twiner and Trick 2000). If concentrations of ROS are high enough, it is probable that fish gill tissue will be damaged, reducing oxygen uptake leading to asphyxiation.

The purpose of this study was to examine the hypothesis

Table 1. Summary of algal strains used and their origin and production of H_2O_2 following resuspension to 6×10^4 cells ml^{-1}. Data are shown as means (½ range); $n = 2$.

Organism	Isolation location	Net H_2O_3 production (pmol min^{-1} [10^4 cells]$^{-1}$)	Max. H_2O_2 (μM)
Heterosigma akashiwo 764*	Clayoquot Sound, Canada	7.89 (0.93)	0.906 (0.214)
H. akashiwo 560R*	Long Island Sound, U.S.A.	1.23 (0.22)	0.130 (0.024)
H. akashiwo 102R*	English Bay, Canada	0.85 (0.38)	0.195 (0.024)
H. akashiwo 625R*	Genoa Bay, Canada	0.46 (0.20)	0.139 (0.003)
Tetraselmis apiculata 2562†	Lincolnshire, England	0.03 (0.01)	0.044 (0.004)

* North East Pacific Culture Collection.
† University of Texas Culture Collection.

that ROS are responsible for *Heterosigma*-induced toxicity in aquatic systems. We characterized the generation of ROS by determining the net rates of production of H_2O_2, as well as the maximum concentration produced by four isolates of *H. akashiwo.* We then compared the toxicities of these concentrations of H_2O_2 on vertebrate cell lines and the marine invertebrate *Artemia salina* (brine shrimp).

Materials and methods

Algal cultures—H. akashiwo cultures (strains 560R, 102R, 625R) were obtained from the North East Pacific Culture Collection (NEPCC), Vancouver, Canada. Isolate 764, also obtained from NEPCC, was a known *Heterosigma* sp. and has subsequently been identified as *H. akashiwo* via sequencing of the large subunit ribosomal RNA gene (D_1/D_2) and the internal transcribed spacer (ITS) regions of the ribosomal RNA gene (unpubl. data). Isolates 764, 102R, and 625R were all originally obtained from blooms off the British Columbia (Canada) coast, whereas isolate 560R originated in the Long Island Sound, U.S.A. (Table 1), and has long been used as a typical lab isolate. The toxicities of the isolates are unknown. For a nontoxic control, the chlorophyte *Tetraselmis apiculata* 2562 was obtained from the University of Texas Culture Collection, Austin, Texas. This alga was chosen because of its similar growth rate relative to the *H. akashiwo* isolates and the lack of any evidence suggesting that this alga is toxic. Each nonaxenic stock algal culture was maintained in f/2 medium (–Si) (Guillard and Ryther 1962) before transfer to artificial seawater medium (ASM) (Harrison et al. 1980) supplemented with f/2 nutrients, metals, and vitamins. Each isolate was grown in 50-ml batch cultures (in 250-ml erlenmeyer flasks) until late exponential/early stationary phase (10–12 d following inoculation). Cells were grown without rotation at 18°C under a continuous light flux of 65–80 μmol photons m^{-2} s^{-1}.

Hydrogen peroxide determination—Hydrogen peroxide release from *H. akashiwo* was quantified via a fluorometric assay utilizing H_2O_2-dependent oxidation of scopoletin (7-hydroxy-6-methoxy-2*H*-1-benzopyran-2-one) (Andreae 1955). In short, as previously described by Twiner and Trick (2000), 300×10^4 exponentially growing cells were filtered onto a 1-μm pore diameter polycarbonate filter (Nuclepore®, Corning) under low pressure and immediately were resuspended in 50 ml of nominally H_2O_2-free, nonsupplemented ASM salt solution (Harrison et al. 1980). At 0 and 15 min, 2.5 ml of the resuspension was removed for H_2O_2 determination. Phosphate buffer (12.5 μl, 0.5 M, pH 7.0) was added to the 2.5-ml aliquot, followed by 20 μl scopoletin solution (1.25×10^{-5} M in 0.2% ethanol). Once the fluorescence reading of the reaction mixture had stabilized (15 to 60 s), 10 μl of a peroxidase solution containing 50 mM phenol, 1 mg ml^{-1} horseradish peroxidase, and 2×10^{-2} M phosphate buffer was added to catalyze the oxidation of scopoletin to a nonfluorescent derivative. Fluorescence intensity was recorded following another 2-min stabilization period and compared to a standard curve of fluorescence versus H_2O_2 concentration in the absence of cells. In the absence of cells, these standard H_2O_2 aliquots remained detectable and stable for >60 min. Net rates of production (pmol H_2O_2 min^{-1} [10^4 cells]$^{-1}$) were determined from the increases in ambient H_2O_2 concentration over the initial 15 min, whereas final steady-state concentrations of H_2O_2 were recorded at 45 min.

Cell lines and cytotoxicity assay—Vertebrate 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 a ratio of 1 : 7 every 2 to 3 d by washing with Dulbecco's phosphate-buffered saline followed by release with a trypsin/EDTA solution. Cultures were maintained in 5% : 95% humidified CO_2 : air at 37°C. For viability assays, cells were seeded in a volume of 100 μl in supplemented MEM at a density of $1–2 \times 10^4$ cells $well^{-1}$ in sterile, 96-well culture plates. After 12–24 h to allow for attachment, the medium was aspirated and replaced with fresh, nonsupplemented MEM (100 μl). To this, hydrogen peroxide (Caledon Laboratories Ltd., No. 4060-1) was added for either 1 or 24 h. Following exposure, medium was replaced with fresh nonsupplemented MEM (100 μl) prior to viability testing either immediately or at 24 or 48 h. Cell viability was colorimetrically determined by the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) assay, which measures the activity of mitochondrial succinate dehydrogenase (Mosmann 1983). Briefly, 10 μl of MTT so-

lution (5 mg ml^{-1} in PBS; 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 succinate dehydrogenase to form insoluble, blue crystals. The overlying medium (and noncleaved dye) was aspirated and replaced with 100 μl of an acid/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) with KC4 software using a test wavelength of 562 nm and a reference wavelength of 690 nm. Cell viability was determined from the ratio of 562 and 690 nm. All data were expressed as a percentage of the control when no hydrogen peroxide was present.

Artemia salina *growth and toxicity—Artemia salina* eggs were hydrated in a small aquarium containing nonsupplemented ASM salt solution. Eggs were sprinkled into a darkened end of the aquarium that was separated from an aerated, lightened area by a perforated polycarbonate divider. With a hatching time of ~48 h, *A. salina* nauplii (1-d-old) that had accumulated in the lightened area of the aquarium were transferred into 96 well plates with five live nauplii per well, containing a final volume of 300 μl ASM. A total of 30 shrimp per treatment were tested. *A. salina* were exposed to H_2O_2 (10^{-7}–1 M) with or without 10 μM ferric or ferrous iron for 24 h. Individuals were considered dead if they were lying immobile in the well. Controls in which H_2O_2 was omitted were adjusted for volume with and without added iron.

Statistical analysis—Data reporting production of H_2O_2 are expressed as means ± ½ range ($n = 2$). All other values are expressed as means ± SE ($n \geq 5$) and were compared statistically by one- or two-way analysis of variance (ANOVA) followed by Tukey multiple comparison tests ($P \leq 0.05$ being considered significant). Raw data from the cell line experiments were analyzed prior to percent transformation, and *A. salina* data were analyzed by using probit transformation of percent mortality plotted versus log[H_2O_2] to determine 50% mortality (LC_{50} ± SE) at 24 h (Sam 1993).

Results

Production of hydrogen peroxide by H. akashiwo—Cells from cultures of *H. akashiwo* were suspended in nominally H_2O_2-free medium and monitored for changes in ambient H_2O_2 concentrations. Net production rates were determined by assaying duplicate flasks within the late exponential phase. It was during this phase that production rates were maximal as determined by Twiner and Trick (2000). Production rates differed between species and *H. akashiwo* isolates (Table 1). For individual *H. akashiwo* isolates, rates of H_2O_2 production were between 0.46 and 7.89 pmol min^{-1} $(10^4$ cells$)^{-1}$, which were considerably higher than the rate of production for *T. apiculata* (0.03 pmol min^{-1} [10^4 cells$]^{-1}$). During this assay, the concentration of ambient H_2O_2 reaches steady state after ~30 min (Twiner and Trick 2000). Therefore, maximal H_2O_2 yields were determined for each isolate 45 min following resuspension of 6×10^4 cells ml^{-1}. Maximal yields of H_2O_2 among the different *H. akashiwo* isolates ranged from 0.14 to 0.90 μM, whereas *T. apiculata* was in steady state at 0.04 μM H_2O_2 (Table 1).

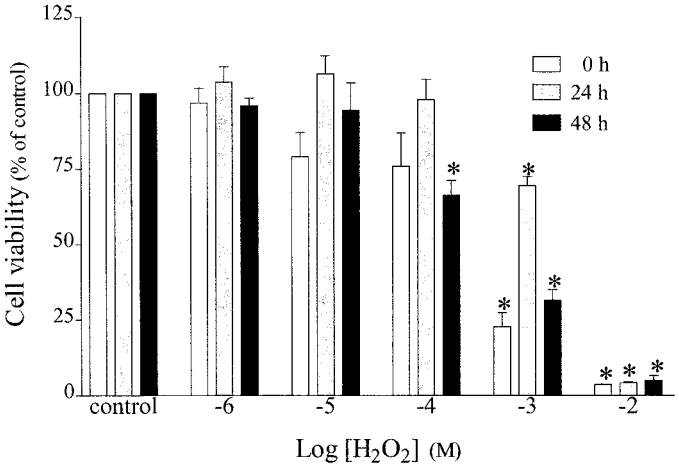

Fig. 1. Effects of an acute exposure to hydrogen peroxide on cell viability. UMR-106 cells were exposed to the indicated concentrations of H_2O_2 for 1 h. Medium was then replaced, and cells were allowed to recover for various time periods (0, 24, 48 h). Cells were assayed for viability following the respective recovery period by the MTT assay. Data are expressed as a percentage of control (no H_2O_2) and are illustrated as means ± SE ($n = 5$ cell wells). Significant differences ($P \leq 0.05$) relative to the control are indicated by asterisks.

Effects of hydrogen peroxide on cell viability—Cultured vertebrate cells in nonsupplemented MEM were exposed to H_2O_2 (10^{-6} to 10^{-2} M initial concentrations) for 1 or 24 h. To assay for cell viability and recovery of cellular function following treatments, succinate dehydrogenase activity was measured using the MTT assay at 0, 24, and 48 h following the exposure period. Following an acute 1-h exposure to H_2O_2, UMR viability was significantly reduced for H_2O_2 treatments $\geq 10^{-3}$ M, regardless of the length of the recovery period. Even for cells allowed to recover for up to 48 h following acute exposure to H_2O_2, viability remained significantly suppressed below control values (Fig. 1). In addition, cellular activity was significantly suppressed for the 10^{-4} M H_2O_2 treatment with the 48-h recovery period, but not at 0 or 24 h. Hydrogen peroxide $< 10^{-4}$ M had no significant effect on cell viability. An almost identical cytotoxic response was observed when HEK-293 cells were exposed acutely to H_2O_2 for 1 h with a 24-h recovery period (data not shown).

To assess the effects of a protracted exposure period, H_2O_2 (10^{-6}–10^{-2} M) was added to UMR-106 and HEK-293 cells for 24 h (Fig. 2). Exposure to 10^{-3} and 10^{-2} M H_2O_2 significantly reduced cell viability to <10% of control for both cell types. In contrast, $H_2O_2 \leq 10^{-4}$ M had no significant effect on cell viability. Comparisons of acute and protracted exposure indicate that cell viability is reduced to a greater extent with the longer, protracted exposure period. This is clearly seen in the 10^{-3} M treatments, where the acute treatment with no recovery time reduced cell viability to 22% of control values, whereas the comparable protracted treatment reduced viability to 5.5% of control values (Figs. 1, 2).

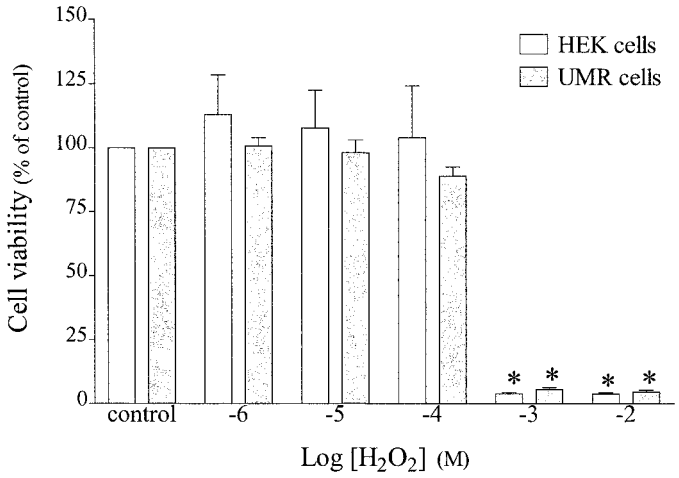

Fig. 2. Effects of a protracted exposure to hydrogen peroxide on cell viability. UMR-106 and HEK-293 cells were exposed to the indicated concentrations of H_2O_2 for 24 h. Cells were assayed for viability immediately following removal of H_2O_2 using the MTT assay. Data are expressed as a percentage of control (no H_2O_2) and illustrated as means ± SE (n = 5 cell wells). Significant differences ($P \leq 0.05$) relative to the control are indicated by asterisks.

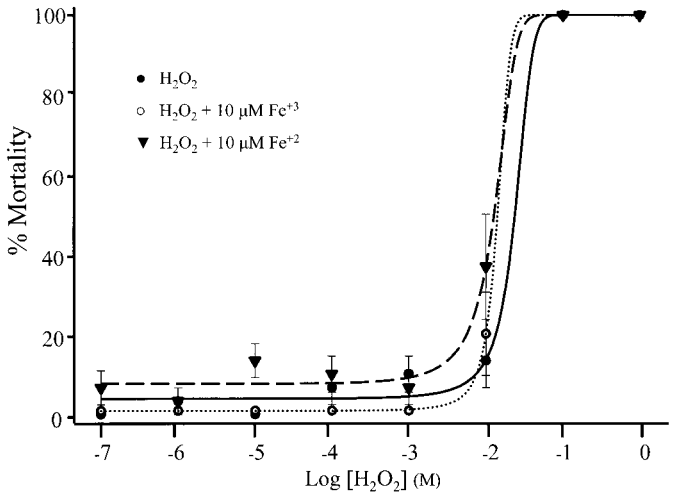

Fig. 3. Effects of hydrogen peroxide and iron on *Artemia salina* survival. *A. salina* nauplii (1 d old) were exposed to either H_2O_2 alone or to H_2O_2 in the presence of 10 μM ferric or ferrous iron for 24 h. Data are plotted as percent mortality (mean ± SE; n = 6 replicates of five shrimp) and were analyzed by probit transformation and a sigmoidal regression to obtain LC_{50} values for each treatment. Appropriate controls for the iron treatments omitted H_2O_2 but included the iron addition (not shown).

Effects of hydrogen peroxide on Artemia salina—Nauplii of the marine zooplankter *A. salina* were exposed to H_2O_2 (10^{-7}–1 M initial concentrations) for 24 h. Survivorship of *A. salina* nauplii was relatively consistent at H_2O_2 concentrations of 10^{-3} M or less, where a minimum of 26 of the 30 nauplii survived for each H_2O_2 treatment (<14% mortality) (Fig. 3). In the absence of H_2O_2, no mortality was observed (data not shown). However, at concentrations of 10^{-1} and 1 M H_2O_2, there was 100% mortality. The LC_{50} value for H_2O_2 was determined to be 2.6×10^{-2} M ($\pm 0.2 \times 10^{-2}$ M).

Iron is a redox metal that can catalyze the formation of more reactive intermediates such as hydroxy radicals ($OH^{\cdot}$) from H_2O_2. This process is commonly referred to as the Fenton reaction (Walling 1975). *A. salina* nauplii were exposed to hydrogen peroxide (10^{-7}–1 M initial concentrations) in the presence of 10 μM ferric ($FeCl_3$) or ferrous ($FeSO_4$) iron. There was almost complete survival of the nauplii at H_2O_2 concentrations $<10^{-3}$ M (Fig. 3). In treatments where nauplii were exposed to 10^{-2} M H_2O_2 in the presence of Fe^{+3} and Fe^{+2}, 20 and 37% mortality was observed, respectively. In the absence of iron, 13% mortality was observed. Greater concentrations of H_2O_2 induced 100% nauplii mortality in each iron treatment (Fig. 3). Nauplii LC_{50} values were calculated as 1.5×10^{-2} M ($\pm 0.2 \times 10^{-2}$ M) and 1.3×10^{-2} M ($\pm 0.2 \times 10^{-2}$ M) for the ferric and ferrous iron treatments, respectively; both were significantly different relative to the LC_{50} value for H_2O_2 alone.

Collectively, the addition of iron to H_2O_2 significantly enhanced the toxicity of H_2O_2 to *A. salina*; however, the initial form of iron (ferric or ferrous) did not significantly influence toxicity. Elevations in H_2O_2 toxicity by iron are presumably by the catalytic reduction of H_2O_2 into $OH^{\cdot}$ radicals. *A. salina* metanauplii (4-d-old) were also tested yielding similar results as the nauplii with respect to toxic levels of H_2O_2 in the presence and absence of iron (data not shown). However, LC_{50} values could not be quantified because of exceptionally high basal levels of mortality in the 4-d-old shrimp (>55%).

Discussion

Each year, blooms of the raphidophyte *H. akashiwo* result in millions of dollars of lost aquaculture stocks. Locations that are particularly susceptible to blooms of *H. akashiwo* include coastal areas of the United States (Horner et al. 1991), New Zealand (Chang et al. 1993), Canada (Taylor and Haigh 1993), and Japan (Honjo 1990). Although much new information has been attained within the last 15 yr, very little concrete evidence has been gathered regarding the toxicological mechanism by which this alga and other raphidophytes affect fish and shellfish.

Reactive oxygen species as ichthyotoxins—Many ichthyotoxic HAB raphidophytes produce considerably more ROS than do nonichthyotoxic reference strains. Reports of HAB species that produce significant levels of ROS include *H. akashiwo* (Twiner and Trick 2000), *Chattonella* spp. (Tanaka et al. 1992), *Olisthodiscus luteus* (Kim et al. 1999*b*), as well as the coccolithophorid *Hymenomonas carterae* (Palenik et al. 1987) and the dinoflagellate *Cochlodinium polykrikoides* (Kim et al. 1999*a*). In this study, we have shown that four geographically distinct *H. akashiwo* isolates have different levels of H_2O_2 production. However, it is evident that production of H_2O_2 by the *H. akashiwo* isolates is much greater than in that of nontoxic *T. apiculata*. Because of this type of experimental evidence, ROS have been suggested as the causative agents responsible for ichthyotoxicity. However, our current study shows that the concentrations of H_2O_2 that induce significant cell toxicity or mortality of the marine invertebrate *A. salina* is two to three orders of magnitude higher than the

amount of H_2O_2 produced by isolates of *H. akashiwo.* Other studies assessing the toxicity of H_2O_2 toward fish support our findings. Tests of hydrogen peroxide on rainbow trout (*Oncorhynchus mykiss*) fry and fingerlings revealed that NOEC (no observable effect concentrations) for exposure times of 60 and 90 min were in excess of 6.9×10^{-4} M (Gaikowski et al. 1999). Additionally, studies using *O. mykiss* determined the threshold for H_2O_2 toxicity (mortalities $> 20\%$) to fry and fingerlings to be 1.2×10^{-2} M for 30 min, 8.2×10^{-3} M for 60 min, and 5.0×10^{-3} M for 120 min (Arndt and Wagner 1997).

Cytotoxic effects of H_2O_2 on the cell lines indicate that irreversible cell damage by H_2O_2 was not observed at concentrations less than 10^{-4} M. In fact, the concentration required to kill $>90\%$ of the cells was 10^{-3} M or greater for both the acute (1 h) and protracted (24 h) exposure treatments. Thus, we conclude that concentrations of H_2O_2 greater than 10^{-4} M are required to induce cell toxicity. Experimental evidence using a neuronal CG4 cell line supports these findings (Bhat and Zhang 1999). In both cases, toxicity appears to be mediated by an initial, probably necrotic, mechanism that is irreversible.

In the study by Lush and Hallegraeff (1996), it was shown that *A. salina* were sensitive to both whole cultures and cell-free cultures (spent medium) of *H. akashiwo.* Experiments investigating *A. salina* nauplii mortality to 24-h treatments of H_2O_2 indicated that 50% mortality was induced by 2.6×10^{-2} M H_2O_2. The addition of a saturating amount of iron (both ferric or ferrous) to the nauplii reduced the LC_{50} value (i.e., enhance mortality), suggesting that the presence of iron does influence ROS toxicity. Iron is a redox catalyst for the production of hydroxy radicals ($OH^{\cdot}$) from H_2O_2 (Walling 1975). Additionally, *A. salina* metanauplii appeared to be more sensitive to H_2O_2 than nauplii—abnormally high mortality (up to 77%) was observed for the metanauplii controls (no H_2O_2). Nonetheless, H_2O_2 in the presence or absence of iron does not induce significant *A. salina* mortality in the range that is produced by isolates of *H. akashiwo* (up to 10^{-6} M). A comparison of H_2O_2-induced toxicity of the cell lines versus *A. salina* suggests that marine invertebrates are not as sensitive to H_2O_2 as vertebrate cells.

Collectively, there is a large discrepancy between the level of H_2O_2 that *H. akashiwo* isolates produce and the amount required to cause cell toxicity, *A. salina* mortality, or fish mortality. Although net rates of H_2O_2 production appear to be very different between *H. akashiwo* isolates, maximal concentrations of H_2O_2 produced by *H. akashiwo* are only in the submicromolar range. This suggests that algal-produced H_2O_2 is still more than two orders of magnitude less than that required to induce significant cellular toxicity and three to four orders of magnitude less than required to induce *A. salina* mortality and to affect *O. mykiss* fry and fingerlings (Arndt and Wagner 1997). However, it should be noted that many of these toxicological assays involve an initial inoculation of H_2O_2 and that H_2O_2 is susceptible to decay or degradation. During a natural *H. akashiwo* bloom event, H_2O_2 would likely be produced on a sustained basis over prolonged periods of time. However, given the extreme differences observed in concentrations of algal-produced H_2O_2 and the levels required to induce any significant biological effect, a nonichthyotoxic role of ROS production needs to be considered.

Alternative roles for extracellular reactive oxygen species—Reactive oxygen species are released from cells for a variety of purposes, one of which is related to the iron status of the cells. For bacterial cells that supply their iron via a siderophore-mediated system, it has been shown that ferric iron bound to the monocatecholate siderophore aminochelin can be reduced to ferrous iron by superoxide, resulting in the release of the iron ion (Cornish and Page 1998). Alternatively, in nonsiderophore systems, one method of iron uptake is the reduction of free ferric iron or organically bound ferric iron mediated by ferric chelate reductases (Schmidt 1999). It has been suggested that superoxide may be the electron-rich intermediate that acts in conjunction with these reductases to maintain the high degree of reducing power (Cakmak et al. 1987). Recently, it has been shown that H_2O_2 production by *H. akashiwo* is inversely proportional to iron availability (Twiner and Trick 2000), suggesting a relationship between iron and ROS.

As observed in mammalian macrophages, the oxidative burst of ROS can be a defense system targeted toward pathogens (Nathan and Root 1977). Release of ROS by algae could be a metabolically ''low-cost'' chemical defense system (Collen et al. 1995). ROS contain no carbon, nitrogen, or phosphate, which are typical components of organic toxins; thus, ROS are produced with a minimal nutritional burden to the cells. The only requirement is oxygen and a source of electrons. These electrons could be supplied by a photosynthetic/respiratory imbalance or by reduced activity of ROS detoxification enzymes such as peroxidases, superoxide dismutases, and catalases.

Alternatively, ROS production may not be independent of neurotoxin production and potency. It has recently been observed that brevetoxinlike compounds are produced by raphidophytes such as *H. akashiwo* (Khan et al. 1997). Many different brevetoxin subtypes exist, where some are considerably more potent than others (Baden et al. 1988) and some of these differences in potency can be attributed to the degree of oxidation of the polyether compound. Thus, in line with the belief that not all *H. akashiwo* blooms are constitutively toxic, ROS production could be a mechanism of controlling brevetoxin toxicity (D. Baden pers. comm.).

We have compared the amount of H_2O_2 produced by isolates of *H. akashiwo* to the amount of H_2O_2 that is required to elicit toxicologically relevant effects. Our models for toxicity included vertebrate cell lines and the marine invertebrate *A. salina.* Our data clearly show that under the assay conditions used here, *H. akashiwo* does not produce enough ROS to induce significant mortality, even in the presence of large quantities of ferrous iron. Further research should be focused on isolating the mechanism of ROS production and the role of extracellular ROS in algae. Much knowledge could be obtained by measuring ambient ROS and iron concentrations in situ during a *H. akashiwo* bloom.

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