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Possible physiological mechanisms for production of hydrogen peroxide by the ichthyotoxic flagellate *Heterosigma akashiwo*

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Abstract. Blooms of the toxic red tide phytoplankton *Heterosigma akashiwo* (Raphidophyceae) are responsible for substantial losses within the aquaculture industry. The toxicological mechanisms of *H.akashiwo* blooms are complex and to date, heavily debated. One putative type of ichthyotoxin includes the production of reactive oxygen species (ROS) that could alter gill structure and function, resulting in asphyxiation. In this study, we investigated the potential of *H.akashiwo* to produce extracellular hydrogen peroxide, and have investigated which cellular processes are responsible for this production. Within all experiments, *H.akashiwo* produced substantial amounts of hydrogen peroxide (up to 7.6 pmol min^{-1} 10^4 $cells^{-1}$), resulting in extracellular concentrations of ~0.5 μmol l^{-1} H_2O_2. Measured rates of hydrogen peroxide production were directly proportional to cell density, but at higher cell densities, accuracy of H_2O_2 detection was reduced. Whereas light intensity did not alter H_2O_2 production, rates of production were stimulated when temperature was elevated. Hydrogen peroxide production was not only dependent on growth phase, but also was regulated by the availability of iron in the medium. Reduction of total iron to 1 nmol l^{-1} enhanced the production of H_2O_2 relative to iron replete conditions (10 μmol l^{-1} iron). From this, we collectively conclude that production of extracellular H_2O_2 by *H.akashiwo* occurs through a metabolic pathway that is not directly linked to photosynthesis.

Introduction

Over the last few decades, marine harmful algal blooms (HABs) have become a global ecological and economical concern. One such algal species is the coastal raphidophyte, *Heterosigma akashiwo*. This alga is the causative agent in massive fish kills that result in millions of dollars of lost fish stocks every year. Specifically, *H.akashiwo* has been involved in yellowtail and red sea bream kills in Japan, Chinook salmon kills in New Zealand and Canada, as well as cultured rainbow trout kills in Canada and the United States (Chang *et al.*, 1990; Black *et al.*, 1991; Honjo, 1993; Yang *et al.*, 1995). In western Canada alone, between 3 and 10 million Canadian dollars are lost every year from the salmon farming industry directly as a result of *H.akashiwo* (Black *et al.*, 1991; Yang *et al*., 1995). More drastically, over a 16 year period in Seto Inland Sea, Japan, in excess of 2 billion yen has been lost from fish culture operations due to *H.akashiwo*. *Heterosigma akashiwo* blooms have now been observed in Denmark, Bermuda and Chile (Honjo, 1993).

The toxicological mechanism(s) of *H.akashiwo* remains controversial and unresolved. Investigators have suggested that specific raphidophytes, particularly *Heterosigma* and *Chattonella* spp., may produce neurotoxins or cardiotoxins that can lead to respiratory and/or cardiac paralysis (Endo *et al.*, 1992; Khan *et al.*, 1996, 1997). As well, others believe that production of extracellular reactive oxygen species (ROS), such as superoxide ($\cdot O_2^-$), hydrogen peroxide (H_2O_2) and

hydroxy radicals (·OH) may alter gill structure and function, resulting in asphyxiation. In studies by Oda *et al.* (Oda *et al.*, 1992, 1997) and other investigators (Tanaka *et al.*, 1994; Yang *et al.*, 1995; Kawano *et al.*, 1996), it has been shown that marine raphidophytes produce extraordinary amounts of ROS, up to 2 nmol 10^4 cells^{-1} min^{-1}. Thus, production of ROS from a raphidophyte bloom has been speculated to be the toxicological agent responsible for fish kills. However, information to support this hypothesis is still largely unproven and being debated.

In this paper, we determined physiological variables that control cellular production of H_2O_2. Hydrogen peroxide is the most stable of the aforementioned ROS, and is also the intermediate between superoxide and hydroxy radicals (Cooper *et al.*, 1989). We employed a unique technique of determining extracellular rates of H_2O_2 production, and tested the effects of cell density, iron limitation and the presence of organically-bound iron on this production. We also report the effects of temperature, light intensity and altered respiratory metabolism on instantaneous rates of H_2O_2 production in an attempt to decipher the controls and sources of this cellular production.

Method

Cultures

Heterosigma sp. (isolate 764) was obtained from the North East Pacific Culture Collection (NEPCC), University of British Columbia, Canada. This isolate originated from waters of Clayoquot Sound, B.C., Canada, and has recently been identified as *H.akashiwo* via ribosomal polymerase chain reaction (rtPCR) for the D_1/D_2 region and ITS regions by comparison with GenBank data for other *H.akashiwo* isolates (unpublished data). This uni-algal, non-axenic culture (25 ml in 125 ml, or 50 ml in 250 ml Erlenmeyer flasks) was grown without rotation at 18°C under a continuous light flux of 65–80 μmol photons m^{-2} s^{-1}. Modified Erd–Schreiber medium was used to maintain all stock and experimental cultures. Sea water obtained from Woods Hole, MA, USA was filtered through Whatman GF/C filters, passed twice through a Chelex-100 resin column, adjusted to pH 8.3, re-filtered and then pasteurized by heating twice to 73°C on consecutive days. Sterile, enriched (final concentrations) sea water was prepared by additions of $NaNO_3$ (2.4×10^{-3} mol l^{-1}), Na_2HPO_4 (2.1×10^{-4} mol l^{-1}), vitamin B_{12} (1.1×10^{-10} mol l^{-1}), Na_2EDTA (2.4×10^{-5} mol l^{-1}), $MnCl_2{\cdot}4H_2O$ (2.9×10^{-6} mol l^{-1}), $ZnCl_2$ (4.4×10^{-7} mol l^{-1}), $CoCl_2{\cdot}6H_2O$ (1.0×10^{-7} mol l^{-1}) and $Na_2MoO_4{\cdot}2H_2O$ (2×10^{-7} mol l^{-1}). Soil water extract (50 ml l^{-1}) was added for maintenance of stock cultures only.

For all experimental cultures, iron was adjusted to the necessary concentration via addition of filter-sterilized $FeCl_3{\cdot}6H_2O$ (final concentrations ranging from 10^{-5} to 10^{-9} mol l^{-1}) and EDTA (10^{-6} mol l^{-1}). Cultures were preconditioned to the appropriate iron concentration prior to the experiment to reduce iron transfer and to ensure that the cells were not satisfying their iron requirements from intracellular iron stores. Preconditioned treatments contained either the same or a lower iron concentration as that of the experiment. Preconditioned cells were inoculated from the exponential growth phase (7–12 days) into the experimental culture media.

Due to the sensitive growth conditions required by *H.akashiwo*, all culture sampling was destructive. This meant that all experimental culture flasks were only sampled from once and then discarded. Every second day, cell enumeration consisted of direct haemocytometer counts or determination of cell density via cellular absorbance measured at 750 nm. Values of absorbance were standardized to cell density by comparison with a standard curve where (absorbance at 750 nm) = $2.65 \times 10^{-3} + 2 \times 10^{-5}$ (cell density) with correlation coefficient (r) = 0.99. Maximal growth rates were determined as given by Guillard (Guillard, 1973) and maximal yields were averaged from three consecutive days.

Hydrogen peroxide determination

Hydrogen peroxide released from cells of *H.akashiwo* was quantified via a fluorometric assay utilizing H_2O_2-dependent oxidation of scopoletin (7-hydroxy-6-methoxy-2H-1-benzopyran-2-one) (Andreae, 1955; Cooper and Zika, 1983). Using a Turner Designs (TD-700) fluorometer (daylight white lamp; Turner #10–045) with excitation filter 300–400 nm (Turner #10–069R) and emission filter 410–600 nm (Turner #10–110R-C), reduction of fluorescence was proportional to the concentration of H_2O_2 present in the suspension. From an experimental culture, 300×10^4 cells were filtered onto a 1 μm polycarbonate filter (Nuclepore, Corning) under low pressure and immediately resuspended in 50 ml artificial seawater medium (ASM) (Harrison *et al.*, 1980). This medium did not contain the nutrient, trace metal or vitamin supplements normally added to ASM. Final cell suspensions were 6×10^4 cells ml^{-1} and incubated at 18°C with a constant light flux of 80 μmol photons m^{-2} s^{-1} unless otherwise noted. At appropriate time intervals, 2.5 ml of the resuspension was removed for H_2O_2 determination. Phosphate buffer (12.5 μl, 0.5 mol l^{-1}, pH 7.0) was added to the 2.5 ml aliquot (2.5×10^{-3} mol l^{-1} final), followed by a 20 μl addition of scopoletin (1.25×10^{-5} mol l^{-1} in 0.2 % ethanol). The aliquot containing the buffer and scopoletin was inverted twice and then its fluorescence set to 100% following stabilization. Once set, 10 μl of a peroxidase solution containing 50 mmol l^{-1} phenol, 1 mg ml^{-1} horseradish peroxidase and 2×10^{-2} mol l^{-1} phosphate buffer were added to catalyze the oxidation of scopoletin to a non-fluorescent compound. Fluorometric values were recorded following another 2 min stabilization period and compared with a standard curve of fluorescence versus H_2O_2 concentration in the absence of cells (a sigmoid concentration–response with r = 0.996). The detection limit for this assay was ~0.5 nmol l^{-1} H_2O_2. Rates of production were determined by the increase in ambient H_2O_2 concentration over the initial 10 or 15 min. Rates were standardized to pmol min^{-1} 10^4 $cells^{-1}$ based on the resuspended cell density and time of observation.

Statistical analysis

All rates of H_2O_2 production and indices of culture growth were statistically compared by one- or two-way analysis of variance (ANOVA) followed by a Tukey multiple comparison test amongst means. Values are expressed as means ± 1 S.E., with $P < 0.05$ being considered significant.

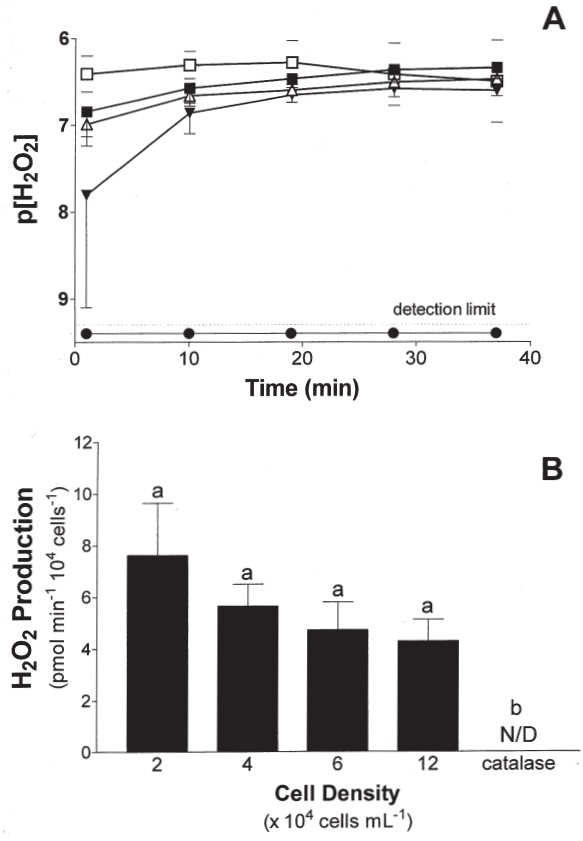

Fig. 1. Effect of cell density on production of hydrogen peroxide. *Heterosigma akashiwo* cells were gently filtered onto a 1 μm polycarbonate filter and resuspended to various densities in artificial seawater medium (ASM). (**A**) Time series measurements of H_2O_2 accumulation (mean ± 1 S.E., $n = 3$) ($p[H_2O_2]$ = -log of H_2O_2 in molarity) are illustrated for different cell density resuspensions: 2×10^4 cells ml^{-1} (-▲-), 4×10^4 cells ml^{-1} (-△-), 6×10^4 cells ml^{-1} (-■-) and 12×10^4 cells ml^{-1} (-□-). An additional treatment included a 6×10^4 cells ml^{-1} (-●-) resuspension where catalase (500 U ml^{-1}) was added to the ASM. (**B**) Calculated rates of hydrogen peroxide production (mean ± 1 S.E., $n = 3$) in the first 10 min following resuspension to various cell densities. This method of H_2O_2 detection is accurate to ~0.5 nmol l^{-1}. Bars labeled with the same lower case letter are not significantly different (i.e. a ≠ b).

Results

Effect of cell density on hydrogen peroxide production

The production of hydrogen peroxide was measured intermittently for various resuspended cell concentrations (Figure 1A). When monitored over time, the initial production of hydrogen peroxide was a transient, catalase-inhibitable, density-dependent response followed by a gradual plateau where the maximum concentration of H_2O_2 attained appeared not to be cell density-dependent. However, according to a 2-way ANOVA, significant differences were observed

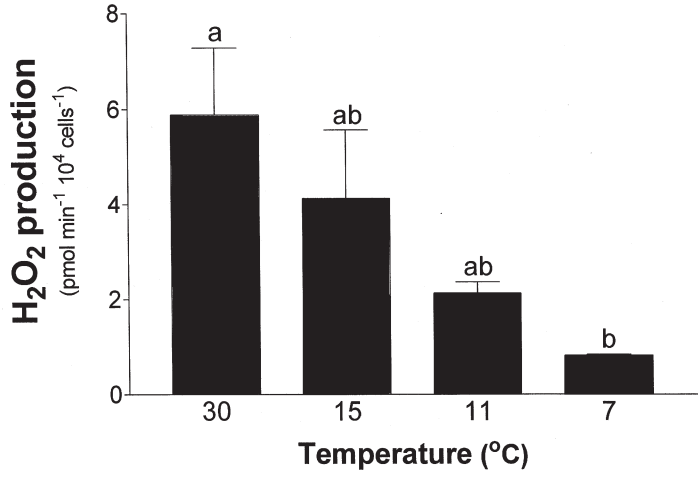

Fig. 2. Effect of temperature on mean rates of hydrogen peroxide production (mean ± 1 S.E., $n = 3$) following cell resuspension for 15 min. Bars labeled with the same lower case letter are not significantly different.

between cell density treatments. When initial rates of hydrogen peroxide production were standardized to a cellular basis (pmol min^{-1} 10^4 $cells^{-1}$ for the initial 10 min period), there was an inverse relationship between H_2O_2 production rates and cell densities (Figure 1B), although no significant differences were evident. Rates of production ranged from approximately 3 to 10 pmol H_2O_2 min^{-1} 10^4 $cells^{-1}$. The maximum hydrogen peroxide concentration obtained for each of the cell density treatments was between 0.5 and 0.1 µmol l^{-1}. To ensure that the bioassay was detecting only H_2O_2 (rather than other reactive oxygen species), catalase was used to enzymatically degrade H_2O_2 into oxygen and water. Measurable rates of hydrogen peroxide production were completely attenuated when the cells were resuspended in the presence of catalase (500 U ml^{-1}) (Figure 1A and 1B).

Effects of temperature, irradiance and respiratory inhibition on production of hydrogen peroxide by H.akashiwo

Temperature has been suspected of being important in the formation of toxic *Heterosigma* blooms (Tomas, 1978; Smayda, 1998). The effect of incubation temperature on instantaneous production of H_2O_2 was assayed. Cells were exposed to the various experimental temperatures only during the 15 min rate-determining incubation period. As temperature was increased from 7 to 30°C, there was a corresponding increase in H_2O_2 production (Figure 2). At 7°C, rates of production were <1 pmol min^{-1} 10^4 $cells^{-1}$, significantly less than the 30°C treatment (~6 pmol min^{-1} 10^4 $cells^{-1}$). This clearly suggests that a metabolic, enzyme-dependent pathway is responsible for H_2O_2 production.

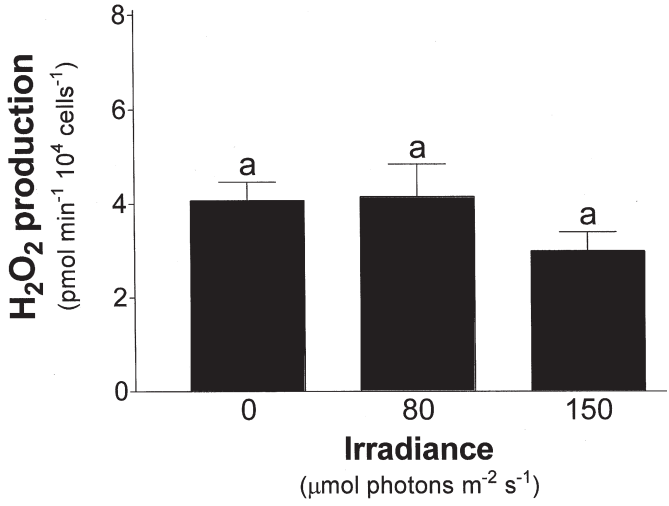

Fig. 3. Effect of irradiance on mean rates of hydrogen peroxide production (mean ± 1 S.E., n = 3) following cell resuspension for 15 min. The dark incubation was pre-treated for 60 min prior to resuspension. Bars labeled with the same lower case letter are not significantly different.

Chloroplasts are the main energy centers for all photoautotrophic organisms. An imbalance in the transfer of photon energy to biochemical energy, or subsequent utilization of this energy can result in the production of ROS such as H_2O_2 (Mehler, 1951). Therefore, the effect of irradiance was investigated on the production of H_2O_2 from *H.akashiwo*. Irradiance was only adjusted for the 15 min incubation period, thus avoiding any acclimatory alterations to the experimental light level. The dark treatment, however, was pre-incubated in total darkness for 60 min prior to the 15 min experimental period. Between each irradiance regime, there were no statistical differences in H_2O_2 production rates, with values ranging from 3.0 to 4.2 pmol min^{-1} 10^4 $cells^{-1}$ (Figure 3). Additionally, the rates of production of H_2O_2 were not altered when cells were treated with 100 μmol l^{-1} DCMU (3-(3,4-dichlorophenyl)-,1-dimethylurea) (unpublished data), an inhibitor of photosynthetic electron transport (Escoubas *et al.*, 1995).

To address the hypothesis that production of hydrogen peroxide is a by-product of a metabolic electron imbalance, the effect of a respiratory inhibitor was investigated. *Heterosigma akashiwo* cells were pre-exposed for 60 min to a range of concentrations of cyanide (KCN), a general inhibitor of many enzymes (including oxidases and peroxidases), prior to the 15 min incubation period. At the end of the cyanide exposure period, cells were intact but mobility was lost. A concentration-dependent response was observed where treatments of 5 mmol l^{-1} and 10 mmol l^{-1} cyanide significantly reduced extracellular production of H_2O_2 by ~2.5× and 3.5×, respectively, relative to the cyanide-free control (Figure 4). Collectively, these data indicate that H_2O_2 production is a temperature-controlled, metabolic process.

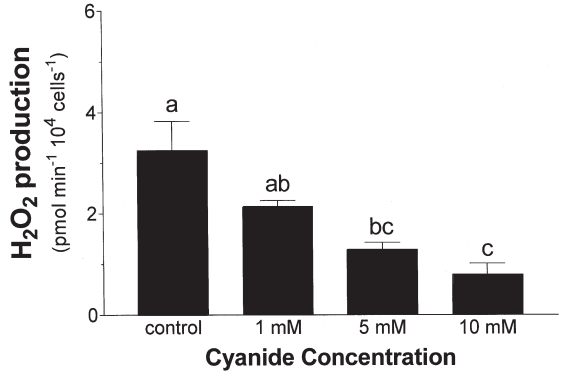

Fig. 4. Effect of cyanide on mean rates of hydrogen peroxide production (mean ± 1 S.E., $n = 3$) following cell resuspension for 15 min. Cyanide, a metabolic inhibitor, was pre-exposed for 60 min. Bars labeled with the same lower case letter are not significantly different.

Effects of desferal and iron limitation on H.akashiwo *growth and production of hydrogen peroxide*

Iron is an essential micronutrient that is believed to have a regulatory role for both growth and toxicity of *Heterosigma* blooms (Taylor and Haigh, 1993; Smayda, 1998). Iron is also important for the photo-redox production of ROS (including H_2O_2) in aquatic systems, commonly known as the Fenton reaction (Cooper and Zika, 1983). Therefore, the effect of iron availability on *H.akashiwo* growth and hydrogen peroxide production was investigated.

The fungal hydroxamate xenosiderophore desferal© (desferrioxamine B) is an organic ligand that is capable of binding iron with high affinity (log k = 31.9) and forming a soluble complex (Morel, 1991). If the desferal/iron complex is available for utilization by a cell culture, then there should be no alterations in culture growth or cellular physiology when iron is organically bound in this form. The presence of desferal was investigated with respect to maximal growth rate (μ_{max}) and maximal yield ($yield_{max}$) of *H.akashiwo* cultures (Figure 5A and 5B). Increasing desferal additions, as shown by the ratio of desferal:ambient iron (where total iron = 10^{-5} mol l^{-1}), resulted in no significant change of either μ_{max} (ranging from 0.64 to 0.74 day^{-1}) or $yield_{max}$ (ranging from 111 to 162 × 10^4 cells ml^{-1}) when the iron concentration was in excess of the desferal concentration (ratio <1). However, when the desferal concentration equalled or exceeded the ambient iron concentration (ratio ≥ 1), both μ_{max} (ranging from 0.1 to 0.001 day^{-1}) and $yield_{max}$ were significantly reduced, particularly for the higher desferal concentration. This suggests that the desferal/iron complex is not a source of iron to *H.akashiwo* and thus, can be used to induce cellular low-iron stress on this alga.

To assess the effect of iron availability on hydrogen peroxide production, three treatments were utilized: an iron-replete treatment (10^{-5} mol l^{-1} Fe^{3+}), a desferal treatment (0.75 × 10^{-5} mol l^{-1} desferal + 10^{-5} mol l^{-1} Fe^{3+}) and an iron-limited treatment (10^{-9} mol l^{-1} Fe^{3+}). For each treatment, production of hydrogen

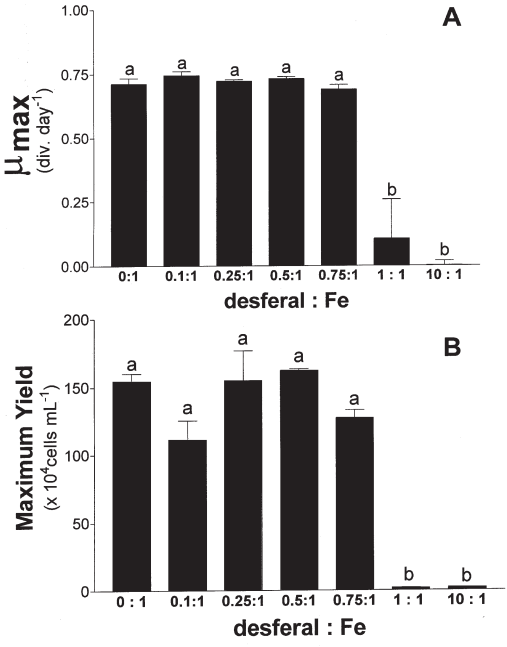

Fig. 5. Effect of desferal on growth rates and yields of *Heterosigma akashiwo*. Illustrated are the effects of desferal additions (0, 0.1, 0.25, 0.5, 0.75, 1, 10 × 10^{-5} mol l^{-1}) on (**A**) maximal growth rate (μ_{max}) and (**B**) maximal yield of batch cultures (mean ± 1 S.E., n = 3) with an initial iron concentration of 10^{-5} mol l^{-1} Fe^{3+}. Desferal additions are expressed as the molar ratio of desferal to iron. Yields were determined using three consecutive maximal points. Bars labeled with the same lower case letter are not significantly different.

peroxide was monitored over the exponential growth stage (days 4 to 10 following inoculation) and the early stationary phase (day 12) (Figure 6). For the iron-replete and iron-limited treatments, H_2O_2 production rates from day to day were variable but not significantly different. However, within the desferal treatment, there was a significant between day difference in H_2O_2 production. Specifically, H_2O_2 production rates on day 8 and day 10 were both different from that on day 6. Between treatments, ANOVA analysis using repeated measures revealed that the iron-limited treatment was significantly different from both the replete and desferal treatments. Collectively, these data suggest that *H.akashiwo* produces H_2O_2 daily and that production rates are significantly elevated when grown in iron-limited conditions (10^{-9} mol l^{-1} Fe^{3+}). Not surprisingly, utilization of desferal did not alter H_2O_2 production relative to the iron-replete treatment. Iron stress was improbable with this particular desferal concentration because neither μ_{max} nor $yield_{max}$ differed from the iron-replete treatment (Figure 5A and 5B).

Discussion

Many isolates of the raphidophyte group (*Chattonella*, *Fibrocapsa* and *Heterosigma* spp.) are commonly known for their harmful and lethal effects to

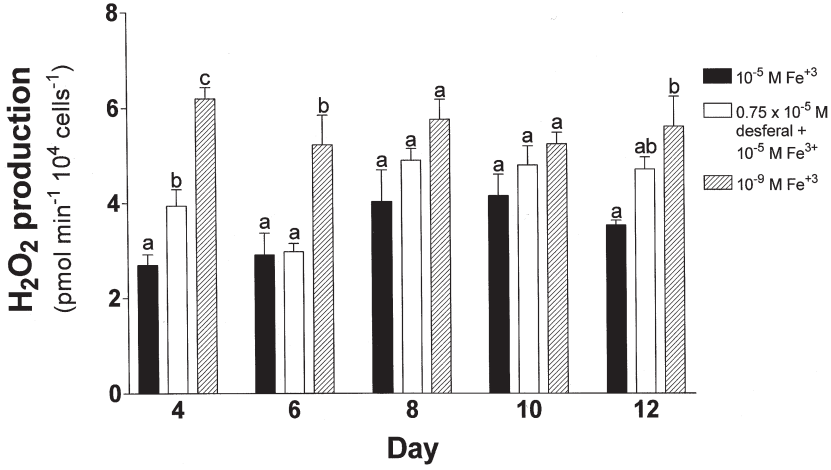

Fig. 6. Effects of desferal and iron limitation on bi-daily hydrogen peroxide production rates by *Heterosigma akashiwo* (mean ± 1 S.E., $n = 3$). The iron-replete treatment contained 10^{-5} mol l^{-1} Fe^{+3}, the desferal treatment contained 0.75×10^{-5} mol l^{-1} desferal + 10^{-5} mol l^{-1} Fe^{+3}, and an iron-limited treatment contained 10^{-9} mol l^{-1} Fe^{+3}. Letters of significance are only calculated between the iron treatments, within a single sampling day. Bars labeled with the same lower case letter are not significantly different.

the fish and shellfish aquaculture industries. Raphidophyte blooms are most prominent and often occur annually in Japan (*Chattonella*, *Fibrocapsa* and *Heterosigma* spp.), New Zealand (*H.akashiwo*), United States (*H.akashiwo*) and Canada (*H.akashiwo*). Relative to other harmful algal species, very little is known about the mechanism(s) by which these algae are toxic to marine invertebrates and fish. The environmental factors that influence toxin production and bloom development are also unresolved. Discussions with salmon aquaculturalists confirm that not all *Heterosigma* blooms are toxic. In coastal British Columbia waters, if a *H.akashiwo* bloom is toxic, it is most toxic during the initial stages of bloom formation, with reduced toxicity once the bloom has fully developed. Thus, experiments that investigate environmental influence on cell physiology are essential for explaining the ecophysiology of toxin production in this genus.

Significant work has been done investigating the role of raphidophyte diel vertical migration in nutrient retrieval (Watanabe *et al.*, 1995; Amano *et al.*, 1998) and the role of sediment cyst-beds as a source of vegetative, surface blooms (Yamochi, 1984; Imai *et al.*, 1993). However, only a few investigators have focused on potential toxin characterization, such as reactive oxygen species (Oda *et al.*, 1997) or various neurotoxins (Khan *et al.*, 1997).

Quantification of H_2O_2

Detection of reactive oxygen species in aquatic environments is not a new technique. The most reliable method involves the use of a fluorometric dye, scopoletin, which shows a stoichiometric loss of fluorescence when it reacts with

H_2O_2 (Andreae, 1955; Perschke and Broda, 1961). This technique has been used to determine H_2O_2 concentrations as a result of photochemical formation in lake studies (Kieber and Helz, 1986; Holm *et al.*, 1987; Cooper *et al.*, 1988), and for quantification of mammalian macrophage production (Nathan and Root, 1977). Recently, this technique has been applied to the determination of algal-produced H_2O_2. Oda *et al.* (Oda *et al.*, 1992) and Yang *et al.* (Yang *et al.*, 1995) have each documented the release of significant amounts of hydrogen peroxide and superoxide from *Chattonella marina* and *H.akashiwo*, respectively, with the implication that ROS are ichthyotoxic. In our experiments, we made a substantial alteration in the set-up of this bioassay as used by Oda *et al.* (Oda *et al.*, 1992) and Yang *et al.* (Yang *et al.*, 1995). During initial experimentation, it appeared that there were interfering effects that may have been caused by extracellular organics (i.e. dissolved organic carbon) (Cooper *et al.*, 1994), or membrane-bound enzymes such as catalase (Miyake *et al.*, 1991), which would each be impacted by cell density. This interference was observed when internal standards of H_2O_2 were injected into cultures. Detection of the internal standard of H_2O_2 was lost, which was proportional to both time and cell density. This observation is supported by some investigators who suggest that there is evidence for algal catalysis of H_2O_2 (Zepp *et al.*, 1987; Cooper *et al.*, 1994). In light of this, we quantified the net change in the concentration of extracellular H_2O_2 after collecting a fixed number of cells with a polycarbonate filter, followed by their immediate resuspension in an organic-free salt solution. This minimized any potential effects by extracellular organics smaller than the pore size of the filter (1 μm).

Production of H_2O_2 by H.akashiwo

For decades, many scientists have believed and subsequently shown that there is a biological component involved in H_2O_2 production in aquatic systems (Van Baalen, 1965; Van Baalen and Marler, 1966). Biologically-produced H_2O_2 is primarily due to the physiology of the community (Palenik *et al.*, 1987; Zepp *et al.*, 1987), leading to the assumption that H_2O_2 is a product of an imbalance in the photosystems. As far as individual species are concerned, it has been observed that at least three raphidophyte genera also contribute to biologically-produced H_2O_2 (Oda *et al.* 1992; Yang *et al.*, 1995). In the present study we were able to quantify this phenomenon for *H.akashiwo.*

Following resuspension of cultured cells, elevations in H_2O_2 over time appear to be density-dependent and completely inhibited by the presence of extracellular catalase. However, when standardized on a per cell basis, there is a non-significant trend that cell density is inversely correlated to H_2O_2 production rates. With this, all subsequent experiments were standardized to 6×10^4 cells ml^{-1}. Interestingly, the maximum concentration of H_2O_2 obtained did not differ between cell density treatments. This may be a function of the balance between the ambient H_2O_2 concentration, and the rates of H_2O_2 degradation and H_2O_2 production. However, density-dependent interference suggests difficulties when comparing data with other reported values that utilize a different protocol. Using the current procedure for *H.akashiwo*, we determined the instantaneous rates of

H_2O_2 production to be ~5 pmol min^{-1} 10^4 $cells^{-1}$. These production rates were consistent with the calculated production rates for other algal isolates, i.e. *Heterosigma carterae* ~20 pmol min^{-1} 10^4 $cells^{-1}$ using 4.5×10^4 cells ml^{-1} (Yang *et al.*, 1995), and *Hymenomonas carterae* ~3.3 pmol min^{-1} 10^4 $cells^{-1}$ using 0.7×10^4 cells ml^{-1} (Palenik *et al.,* 1987), but is considerably lower than the calculated rates for *C.marina* of ~1950 pmol min^{-1} 10^4 $cells^{-1}$ using 2.5×10^4 cells ml^{-1} (Kawano *et al.*, 1996).

Effects of iron availability on H.akashiwo growth and production of H_2O_2

Iron is an essential component for growth and function of marine phytoplankton (Sunda and Huntsman, 1995). *Heterosigma akashiwo* has been shown to require relatively high amounts of iron for bloom initiation and maintenance (Yamochi, 1983, 1987; Taylor and Haigh, 1993). It is also believed that iron availability is important for raphidophyte generation of reactive oxygen species (Tanaka *et al.*, 1992; Kawano *et al.*, 1996).

The production of H_2O_2 may be a physiological mechanism by which these cells obtain iron from either insoluble inorganic iron hydroxides or organically-complexed iron. In some plants, ROS have been implicated in elevating the availability of iron via extracellular ferric reduction (Cakmak *et al.*, 1987). Anderson and Morel have also suggested that marine diatoms take up iron in the ferrous form, a potential product of ROS reacting with ferric hydroxides (Anderson and Morel, 1982). More recently, it has been shown that ROS (particularly superoxide) has the ability to reduce and release ferric iron from the monocatecholate siderophore, aminochelin (Cornish and Page, 1998). This reaction of superoxide may model the effect of ROS on natural iron-binding ligands.

The addition of the siderophore desferal to culture medium had no significant effect on maximal growth rate or maximal yield of *H.akashiwo* until the desferal concentration equaled or exceeded the ambient iron concentration. Upon addition of desferal, the iron becomes complexed with the siderophore, thus chemically lowering the previously free (ferric or ferrous) or EDTA-complexed iron, and the growth of *H.akashiwo* was significantly reduced or in some cases, abolished. This suggests that desferal-complexed iron is not accessible to *H.akashiwo* and can be used to induce iron limitation. *Heterosigma akashiwo* cultures containing high levels of total iron (10^{-5} mol l^{-1}) produced H_2O_2 at rates ranging from 2.3 to 5.1 pmol min^{-1} 10^4 $cells^{-1}$, with little variability due to growth phase. However, when *H.akashiwo* was iron-limited (10^{-9} mol l^{-1} Fe^{3+}), there were significant increases in the daily rate of H_2O_2 production (ranging from 3.9 to 6.9 pmol min^{-1} 10^4 $cells^{-1}$). The ability of desferal to simulate cellular iron limitation by observing an impact on H_2O_2 production was only partially successful. At equimolar concentrations of desferal to iron (ratio of 1:1), growth was insufficient to provide enough cells to determine rates of H_2O_2 production. However, at lesser concentrations of desferal (i.e. ratio of 0.75:1), the cells appeared not to be physiologically affected (maximal growth rates and yields) or significantly altered in their ability to produce H_2O_2 (ranging from 2.7 to 5.6 pmol min^{-1} 10^4 $cells^{-1}$).

Reduced amounts of cellular iron could alter H_2O_2 production in two possible ways: iron stress or limitation could constrain iron-containing enzymes that would normally degrade ROS (i.e. catalase and other iron-containing superoxide dismutases or peroxidases), or iron stress or limitation could be part of an integral negative feedback system where ROS are produced for the purpose of reducing extracellular iron to a more bioavailable form. Thus, in the case of this second postulate, when intracellular iron is sufficient to meet cellular need, less ROS are released from the cells. However, when iron is limiting, more ROS are produced to elevate the extracellular reducing potential surrounding these cells. The latter hypothesis has been shown to be feasible by the work of Cornish and Page (Cornish and Page, 1998). While neither of these postulates has been confirmed, our findings partially conflict with the observations of Kawano *et al.* (Kawano *et al.*, 1996). Their data suggest that increasing desferal concentrations reduced the rates of production of both superoxide and hydrogen peroxide in *C.marina.* However, in parallel experiments, the reduction in ROS production did not correspond to reduced growth, presumptuously attributed to cellular iron limitation. In their studies, when cellular growth did appear to be iron-limited, there was only slight indication that extracellular ROS production was reduced.

Possible mechanisms for production of H_2O_2 from H.akashiwo

The cellular and mechanistic details responsible for H_2O_2 production have not been determined. In the literature, 15°C has been suggested as being a threshold for the formation of 'toxic' *Heterosigma* blooms (Taylor and Haigh, 1993; Smayda, 1998). This temperature threshold may be a function of the temperature required to induce excystment of the dormant cells from the sediments (Yamochi, 1984), rather than a function of cellular toxin production. In our H_2O_2 production assay, we examined the effect of various temperatures on short-term cellular incubation. We observed that H_2O_2 production was directly proportional to the incubation temperature. However, we did not witness a significant critical effect at 15°C relative to the other temperature treatments. This is not to suggest that 15°C is not important for 'toxic' *H.akashiwo* blooms, as toxin production may be linked to cellular growth, which in turn is a function of temperature. However, as instantaneous H_2O_2 production by *H.akashiwo* is directly affected by temperature, it provides evidence that H_2O_2 release is enzyme-dependent, most probably a metabolic process (Palenik *et al.*, 1987; Kawano *et al.*, 1996).

In aquatic systems, H_2O_2 is often produced photochemically where concentrations vary in proportion to the amount of sunlight (Cooper *et al.*, 1988). Hydrogen peroxide is formed via reduction of superoxide, which is produced by an electron transfer to molecular oxygen, catalyzed by the presence of a reduced metal such as ferrous ion (Cooper *et al.*, 1988). However, biological production of H_2O_2, presumably through an imbalanced redox state within the chloroplast, may also play a significant role. We have shown that cellular production of H_2O_2 by *H.akashiwo* is likely a metabolic process and wanted to investigate the potential role of a photosynthetic pathway. Many intracellular processes may result in production of H_2O_2. A primary source of H_2O_2 in photosynthetic organisms

includes oxygen reduction within chloroplasts, specifically as a result of the Mehler reaction (Mehler, 1951). To test this, H_2O_2 production was monitored over a range of irradiance intensities, including a dark treatment, but we observed no significant differences between irradiance treatments. Palenik *et al.* have also shown similar results for *Hymenomonas carterae* (Palenik *et al.*, 1987). Additionally, production of H_2O_2 from *H.carterae* was detectable only in dark treatments. Much of this evidence also lends itself to support a non-photosynthetic, enzyme-dependent process.

Respiratory processes are also significant pathways of energy transfer, leading to the formation of adenosine triphosphate (ATP). Thus, many of these non-photosynthetic, enzyme-dependent processes are potential sources of ROS such as H_2O_2 (Forman and Boveris, 1982). In our experiments, cyanide, an inhibitor of cytochrome oxidase of the respiratory chain (as well as other enzymes such as superoxide dismutase) (Forman and Boveris, 1982), significantly reduced, but did not eliminate, the production of H_2O_2 from *H.akashiwo*. This suggests that H_2O_2 production may at least in part be a function of cyanide-inhibitable oxidase activity, but more investigation is clearly required.

From our experiments, we have shown that not only is *H.akashiwo* producing substantial amounts of H_2O_2, but we have also tried to decipher the cellular processes that are responsible for H_2O_2 production. Our data suggest that production of H_2O_2 by *H.akashiwo* is an enzymatic process that is not photosynthetically derived but is linked to iron availability. Our conclusions are thus in line with Palenik *et al.* (Palenik *et al.*, 1987) who also suggested a respiratory and/or membrane-bound process as the source of ROS formation.

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