Vol. 23: 101–108, 1995 | DISEASES OF AQUATIC ORGANISMS Dis aquat Org | Published October 26

Oxygen-radical-mediated effects of the toxic phytoplankter *Heterosigma carterae* on juvenile rainbow trout *Oncorhynchus mykiss*

C. Z. Yang*, L. J. Albright, A. N. Yousif

Department of Biological Sciences, Simon Fraser University, Burnaby, British Columbia, Canada V5A 1S6

ABSTRACT: *Heterosigma carterae* (formerly *Heterosigma akashiwo*) is a flagellated marine phytoplankter which can be toxic to many finfish, including salmonids. We have determined that, when toxic to juvenile rainbow trout *Oncorhynchus mykiss*, *H. carterae* produces high concentrations of superoxide and hydroxyl radicals as well as hydrogen peroxide. If superoxide dismutase (dismutates superoxide radical to hydrogen peroxide) and/or catalase (catalyzes the formation of water from hydrogen peroxide) are added to a toxic culture of *H. carterae*, it becomes markedly less toxic to juvenile rainbow trout. We propose that the toxicity of *H. carterae* to the salmonid *O. mykiss* is due to the formation of toxic concentrations of superoxide and hydroxyl radicals and hydrogen peroxide.

KEY WORDS: *Heterosigma carterae* · Toxic phytoplankter · Salmon · Superoxide radicals · Hydroxyl radicals · Hydrogen peroxide · Superoxide dismutase · Catalase

INTRODUCTION

Toxic phytoplankters are a major problem in the culture of marine finfishes since they can cause mass mortalities. *Heterosigma carterae* [formerly *Heterosigma akashiwo* (Taylor, 1992)] is such a toxic phytoplankter. This organism has caused kills of cultured finfish in Japan, New Zealand and the west coast of North America (Gowen 1987, Boustead et al. 1989, Chang 1990). For example, losses of farmed salmon to *H. carterae* in coastal marine waters of Washington State (USA) from 1986 to 1990 have been estimated to be in excess of US $11 million. Since 1986, major farmed salmon kills due to *H. carterae* have also been recorded in coastal British Columbia (Canada). In 1989, for example, losses in the salmon farming industry in British Columbia's Agamemnon Channel alone were C $4.2 million (Black et al. 1991).

The mode of action by which *Heterosigma carterae* kills finfish has been difficult to determine. Chang (1990) noted that moribund chinook salmon in the presence of toxic *H. carterae* had severe acute exudative and degenerative changes to the gill tissue. He suggested that edematous gill lamellae were caused by local impairment of osmoregulation, which in turn induced death due to oxygen deficiency.

However, the observations of Oda et al. (1992a, b) have been a useful guide in determining the toxic mode of action of *Heterosigma carterae*. These authors have shown that the toxic phytoplankter *Chattonella marina* produces superoxide and hydroxyl radicals as well as hydrogen peroxide. They also found that superoxide dismutase (SOD) and/or catalase additions to a *C. marina* culture, in which bacteria were suspended, could protect these bacteria against the toxic action of this phytoplankter.

Since *Heterosigma carterae* is a raphidophycean as is *Chattonella marina* and therefore taxonomically closely related to *C. marina*, we hypothesized that the toxic mode of action of the former may be similar to this latter one. This study was therefore undertaken to examine the generation of reactive oxygen intermediates of superoxide and hydroxyl radicals as well as hydrogen peroxide by *H. carterae* and to study the effects of these oxygen species on viability of juvenile rainbow trout *Oncorhynchus mykiss*.

*E-mail: chunzhen@sfu.ca

MATERIALS AND METHODS

Culture. The strain of *Heterosigma carterae* was obtained from Dr I. Whyte (Pacific Biological Station, Canada Dept of Fisheries and Oceans, Nanaimo, BC). This culture was initially isolated by Dr I. Whyte and Mr E. Black (British Columbia Ministry of Agriculture, Fisheries and Food) from an intense and toxic bloom of *H. carterae* in San Mateo Bay, Vancouver Island, British Columbia. The culture was unialgal, but not axenic.

Culture technique. *Heterosigma carterae* was cultured in 7 l boiling flasks at 20°C using 6 l of Harrison's medium (Harrison et al. 1980) at an initial pH of 8.2. Each culture was aerated and illuminated using 1000 lx of continuous illumination. The cell concentrations in each culture were determined using the Utermöhl technique (Utermöhl 1958). Under these conditions, a toxic culture of *H. carterae* was obtained within 7 d following the inoculation of 3 l of fresh medium with 3 l of a 7 d old culture.

Effects of different pre-treatments on production of superoxide radical. The effects of different pre-treatments on release of superoxide radicals by *Heterosigma carterae* were determined spectrophotometrically on the basis of their reduction of cytochrome *c* (Shimada et al. 1989). Following incubation of *H. carterae* in Harrison's medium for 6 d, the reducing ability of these cells was measured as follows. Firstly, 3 ml of the culture was transferred into each of 7 quartz cuvettes, each of which contained 100 µg ml^{-1} of cytochrome *c* (type III from horse heart; Sigma). Reduction of cytochrome *c* was measured as an increase in absorbance at 550 to 540 nm. This was the unshaken culture. Secondly, 7 cuvettes, each of which contained 3 ml of the culture and 100 µg ml^{-1} of cytochrome *c*, were vigorously shaken 20 times by hand to give absorbances following shaking. Thirdly, 7 cuvettes, each of which contained 3 ml of the culture and 100 µg ml^{-1} of cytochrome *c*, were sonicated for 7 s at the maximum power setting using a sonicator (Fisher, model 300) to give absorbances following cell destruction. After addition of cytochrome *c*, the absorbances of the material in all cuvettes were immediately read in the spectrophotometer against a blank containing 100 µg ml^{-1} of cytochrome *c* in Harrison's medium. The concentration of *H. carterae* used in this experiment was 4.1×10^4 cells ml^{-1}.

SOD-inhibition of cytochrome *c* reduction using different pre-treatments. The experimental protocol was identical to that described above, except that SOD (100 U ml^{-1}; Sigma) was added to an additional set of reaction mixtures. To determine the amount of superoxide produced under different pre-treatment conditions, SOD was added before the administration of cytochrome *c*.

Effects of different concentrations of *Heterosigma carterae* on production of superoxide radical. Following the addition of 100 µg ml^{-1} of cytochrome *c* to various concentrations (1.25×10^4, 1.94×10^4, 2.58×10^4, 3.23×10^4 and 3.88×10^4 cells ml^{-1}) of *H. carterae* suspension in Harrison's medium, each reaction mixture was incubated at 20°C for 30 min in the presence or absence of 100 U SOD ml^{-1}, and the absorbance determined (see above). The absorbance reading was converted to nmol cytochrome *c* reduced by using a molar absorption coefficient of 19.1×10^3 $nmol^{-1}$.

Effects of different incubation times on production of superoxide radical. Following the addition of 100 µg ml^{-1} of cytochrome *c* to various concentrations (0.5×10^4, 2×10^4, 2.5×10^4, 3.3×10^4 and 4×10^4 cells ml^{-1}) of *Heterosigma carterae* suspension in Harrison's medium, each reaction mixture was incubated at 20°C for 0.5, 2, 4, 8, 12 and 24 h in the presence or absence of 100 U SOD ml^{-1} and the absorbance determined.

Detection of SOD activity in Harrison's medium. The oxidation of epinephrine to adrenochrome by xanthine oxidase is mediated by superoxide radicals. This reaction is inhibited by SOD. Reactions were performed at 20°C in 3 ml of Harrison's medium. Each cuvette contained 2×10^{-4} M epinephrine (Sigma), 1×10^{-4} M xanthine (Sigma) and 1×10^{-8} M xanthine oxidase (Sigma). SOD concentrations used to inhibit the reaction were 32 and 133 ng ml^{-1}.

Determination of hydrogen peroxide concentrations. Release of H_2O_2 from *Heterosigma carterae* was measured quantitatively by the horseradish-peroxidase-dependent oxidation of scopoletin (Nathan & Root 1977). Briefly, after addition of 1 µg scopoletin and 20 U ml^{-1} horseradish peroxidase to various concentrations of *H. carterae* suspension in Harrison's medium, each reaction mixture was incubated at 20°C in the presence or absence of 500 U ml^{-1} catalase (Sigma). Decrease in fluorescence intensity was measured using a fluorescence spectrophotometer at an excitation wavelength of 350 nm and an emission wavelength of 460 nm and was calibrated for concentration of H_2O_2 by using a standard curve of diluted H_2O_2 in cell-free Harrison's medium. Under these assay conditions, loss of fluorescence was proportional to the concentration of H_2O_2. Release of H_2O_2 was expressed as nmol per 30 min per 10^4 cells.

Determination of hydroxyl radicals. The generation of hydroxyl radicals in *Heterosigma carterae* suspension was measured by electron spin resonance (ESR) trapping. Janzen et al. (1978) reported that hydroxyl radical reacts with a spin trap, 5,5-dimethyl-1-pyrroline-N-oxide (DMPO, Sigma), to yield a DMPO-OH signal (aN = aH = 1.48 mT). A mixture of algal suspension (4.3×10^4 cells ml^{-1}) and diethylenetriaminepen-

Table 1. Reduction of cytochrome *c* by *Heterosigma carterae* exposed to various treatments, and inhibitory effects of SOD on reducing ability. Means ± SEM (n = 7). OD: optical density

Culture treatment	OD (4.1×10^4 cells $ml^{-1})^{-1}$ $(2\ min)^{-1}$	nM of reduced cytochrome *c*	% inhibitory effects of SOD
Unshaken	0.0294 ± 0.0013	1.593 ± 0.068	35
Shaken	0.0481 ± 0.0013	2.518 ± 0.068	55
Sonicated	0.0773 ± 0.0027	4.047 ± 0.141	64

taacetic acid (DTPA, 500 µM) was illuminated for 10 min using 1000 lx illumination. DMPO was then added to a concentration of 90 mM. The mixture was immediately transferred to the ESR quartz flat cell (inner size, 60 × 10 × 0.31 mm), and the cell was placed in a Bruker X-Band ECS106 ESR spectrometer. ESR spectra were recorded at room temperature under the following conditions: modulation frequency, 100 kHz; modulation amplitude, 0.498 G; scanning field, 334 ± 10 mT; receiver gain, 10^6; time constant, 0.65 s; sweep time, 167 s; microwave power, 76.9 mW; and microwave frequency, 9.67 GHz.

Bioassay for protection of yearling rainbow trout from toxic *Heterosigma carterae* cells by catalase or catalase plus SOD. Domestic rainbow trout of 5 to 10 g weight were taken to the Simon Fraser University aquatic facility and cultured in fresh water at 12°C for 1 mo prior to use, such that they became acclimated.

Expt 1: Five fish were placed in each of five 2 l Erlenmeyer flasks. Flask 1 contained 1.5 l of aerated Harrison's medium; flask 2 contained 1.5 l of aerated toxic *Heterosigma carterae* culture in Harrison's medium; flask 3 contained 1.5 l of aerated toxic *Heterosigma carterae* culture in Harrison's medium to which 1000 U ml^{-1} of catalase was added. Flask 4 contained 1.5 l of aerated sea water (salinity 26‰) and flask 5 contained 1.5 l of aerated 30-min-sonicated toxic *H. carterae* culture in Harrison's medium. The incubation temperature of all flasks was 19°C. The experiment was stopped after 6 h. This experiment was repeated 3 times. The data from all 3 experiments are presented as means ± SEM.

Expt 2: Five fish were placed in each of five 2 l Erlenmeyer flasks. The contents of flasks 1 to 3 were as described above. Flask 4 contained 1.5 l of aerated toxic *Heterosigma carterae* culture in Harrison's medium into which 500 U ml^{-1} of catalase and 100 U ml^{-1} of SOD were added and flask 5 contained 1.5 l of aerated sea water. The incubation temperature of all flasks was 19°C. The experiment was stopped after 7 h.

RESULTS

Superoxide. Unshaken, shaken and sonicated cultures of *Heterosigma carterae* reduced cytochrome *c* (Table 1) as a result of $^{\cdot}O_2^-$ release. However, the lowest rate of release of $^{\cdot}O_2^-$ occurred with the unshaken culture. When *H. carterae* was shaken, the culture showed approximately twice the $^{\cdot}O_2^-$ release rate as compared to the unshaken one ($p < 0.001$). The sonicated culture showed approximately 3 times the rate of $^{\cdot}O_2^-$ release as compared to the unshaken one ($p < 0.001$) (Table 1).

Rates of SOD-inhibitable cytochrome *c* reduction under different pre-treatment situations. To ascertain that the reduction of cytochrome *c* was mainly due to superoxide radicals, SOD was added to the culture. Table 1 shows that approximately 35, 55 and 64% of the cytochrome *c* reduction caused by the unshaken, shaken and sonicated cultures, respectively, was due to superoxide when 100 U ml^{-1} SOD was added.

Effects of different concentrations of *Heterosigma carterae* culture on production of superoxide radical: The effect of cell density on the rate of $^{\cdot}O_2^-$ release is shown in Fig. 1. As expected, a higher cell density resulted in greater $^{\cdot}O_2^-$ release. The reduction of cytochrome *c* at a concentration of 3.88×10^4 ml^{-1} *H. carterae* cells was 6.02 nmol; this decreased to 3.0 nmol by addition of SOD. Thus, $^{\cdot}O_2^-$-dependent reduction of cytochrome *c* was estimated as approxi-

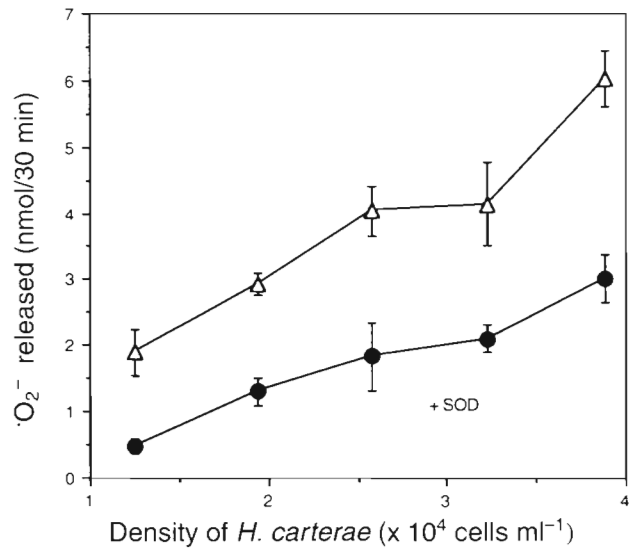

Fig. 1. Effects of different concentrations of *Heterosigma carterae* on the production of $^{\cdot}O_2^-$, on the basis of reduction of cytochrome *c*, in the presence (●) or absence (△) of SOD

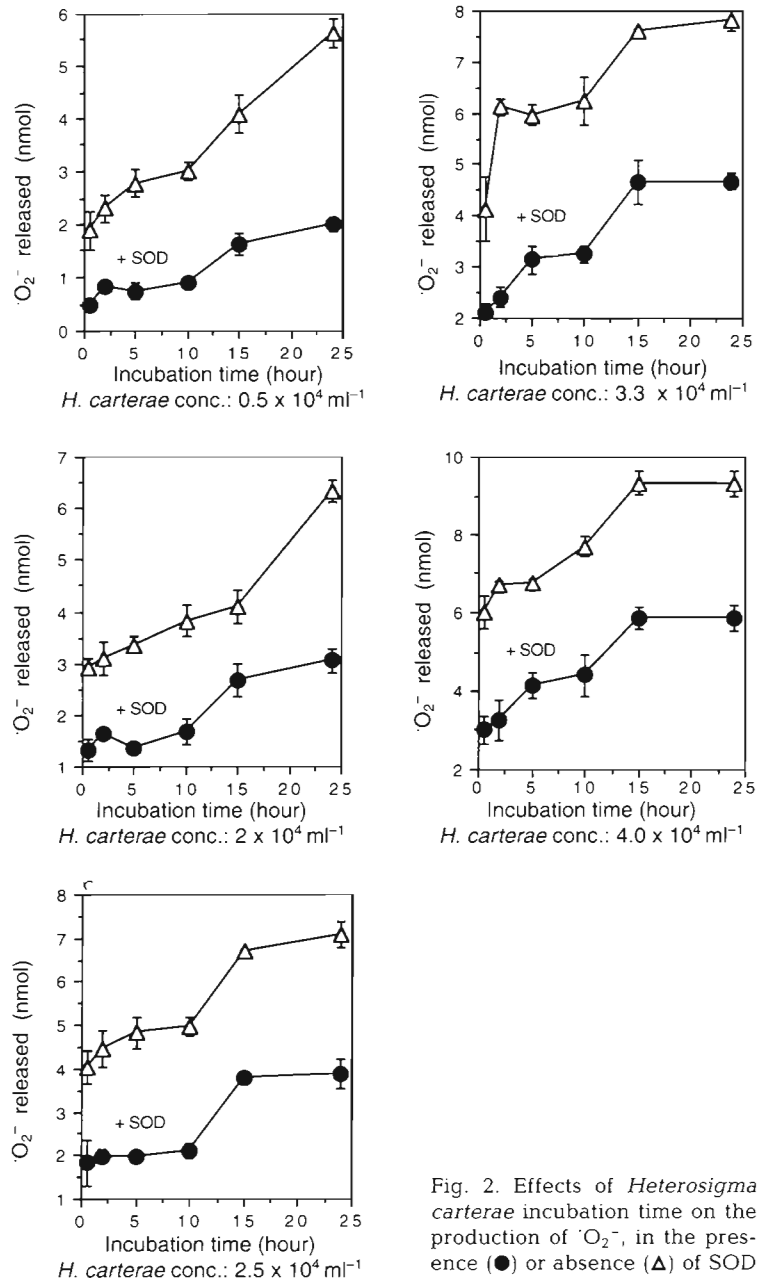

Fig. 2. Effects of *Heterosigma carterae* incubation time on the production of O_2^-, in the presence (●) or absence (△) of SOD

mately 3 nmol per 3.88×10^4 ml^{-1} *H. carterae* cells for 30 min (Fig. 1).

Effects of incubation time on production of superoxide radical. The effect of incubation time on the release of O_2^- by *Heterosigma carterae* is shown in Fig. 2. The results indicate that the amount of O_2^- released by *H. carterae* was dependent on incubation time; a longer incubation time resulted in greater O_2^- release. The reduction of cytochrome *c* in release of O_2^- in this experiment was inhibited approximately 50% by addition of 100 U ml^{-1} SOD.

SOD activity in Harrison's medium. Fig. 3 shows that the formation of adrenochrome was inhibited by several concentrations of SOD. Based upon these observations Harrison's medium does not appear to inhibit SOD activity.

Hydrogen peroxide release by *Heterosigma carterae*. As shown in Fig. 4, H_2O_2 is readily released by *H. carterae*. The amount of H_2O_2 released is dependent on the cell density; a higher cell density resulted in greater H_2O_2 release. Significant increases in H_2O_2 production were observed at *H. carterae* cell concentrations greater than 2×10^4 ml^{-1}. Hydrogen peroxide production was almost entirely eliminated by the addition of 500 U ml^{-1} catalase to the culture (Fig. 4).

Determination of hydroxyl radical. Fig. 5 shows that DMPO-OH adducts (hydroxyl radical) with a 4-lined, 1:2:2:1 ratal signal (aN = aH = 1.49 mT) were produced in the suspension of intact *Heterosigma carterae*.

Mortality. ***Expt 1:*** The mean cumulative mortality of the juvenile rainbow trout was 90% within 3 h upon exposure to the toxic *Heterosigma carterae* culture. However, the mean cumulative mortality of the fish was only 10% when exposed to the same culture in which catalase was added immediately prior to the addition of fish. The mean cumulative mortality of the fish in Harrison's medium was also 10%. No fish died in either the seawater or sonicated culture (Fig. 6).

Expt 2: All of the juvenile rainbow trout died within 30 min upon exposure to a toxic culture of *Heterosigma carterae* (Fig. 7). However, only 20% of the fish died when exposed to the culture in which 1000 U ml^{-1} catalase was added immediately prior to addition of fish. The mortalities of the fish exposed to the culture containing 100 U ml^{-1} and 500 U ml^{-1} catalase were 40%. The mortality of the fish in Harrison's medium was 20%. No fish died in seawater (Fig. 7).

DISCUSSION

Free radicals are quite unstable and highly reactive. They can attack and degrade organic molecules as diverse as lipids, proteins, and nucleic acids and cause lipid peroxidation which results in altered

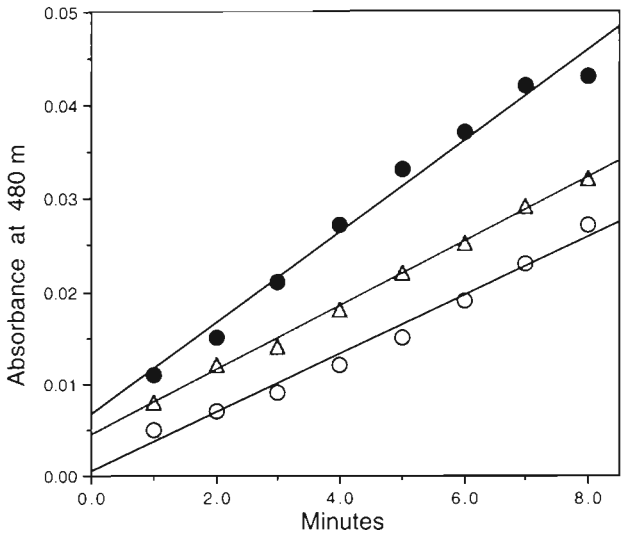

Fig. 3. Detection of SOD activity in Harrison's medium. SOD concentrations used to inhibit the reaction were 32 ng ml^{-1} (Δ), 133 ng ml^{-1} (O) and zero (●)

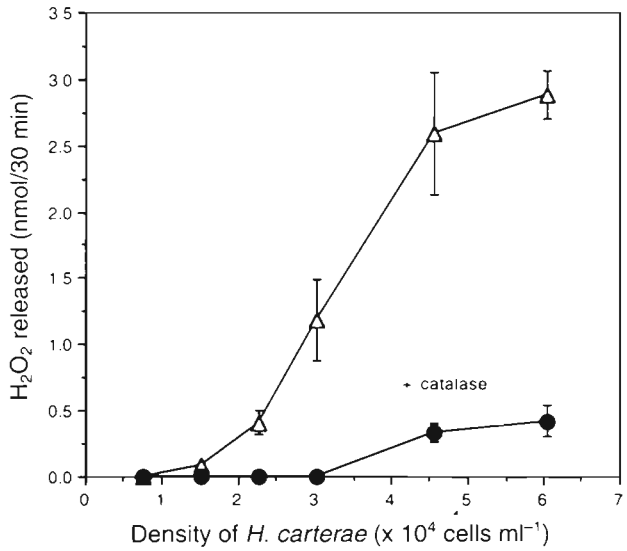

Fig. 4. Detection of H_2O_2 released by *Heterosigma carterae* in the presence (●) or absence (Δ) of catalase

membrane fluidity, permeability, and even cell lysis, inactivation of calcium adenosine triphosphatase (ATPase) in the cell plasma membrane and crosslinking or scission of deoxyribonucleic acid (DNA) strands (Schiller et al. 1993). Superoxide is such a radical species and therefore can directly damage biological materials, for instance, by reacting with enzymes (Fridovich 1983). Other direct consequences may include damage to bronchiolar or alveolar cells, endothelial cells, or fibroblasts of humans (Nakashima et al. 1987).

The superoxide radical can be converted in biological systems to a variety of potentially more damaging radicals, for instance, the hydroxyl radical. Our results (Table 1, Figs. 1 & 2) show that toxic cultures of *Heterosigma carterae* are able to produce superoxide radicals without any *H. carterae* cell destruction. The amount of $^{\cdot}O_2^-$ released by ca 10^4 cells of *H. carterae* is approximately the same as that released by 10^6 mouse peritoneal macrophage cells upon contact with the surface-active agent phorbol myristate acetate (PMA), which can disrupt the plasma membrane of microorganisms and mammalian cells (Slater 1972, Johnston et al. 1978). Activated macrophages produce $^{\cdot}O_2^-$ and H_2O_2, which play a key role in killing bacteria (Babior 1978). Thus, the amount of $^{\cdot}O_2^-$ released by 10^4 *H. carterae* cells is probably sufficient to damage primary and secondary lamellae cells of finfish.

Oda et al. (1992b) have reported that the toxic phytoplankter *Chattonella marina* produces superoxide radicals. They found that the reduction of cytochrome *c* by *C. marina* reached a plateau within 10 min. However, we found that the reduction of cytochrome *c* by *Heterosigma carterae* continued for a 24 h period. This may be because we used a greater concentration of cytochrome *c*.

Our results (Table 1) show that when disturbed, the culture of *Heterosigma carterae* increased its release of $^{\cdot}O_2^-$. This probably mimics the situation when water containing cells of *H. carterae* passes over the secondary lamellae of finfish gills. The turbulence associated with the respiratory current will likely disturb the cells, increasing the amount of $^{\cdot}O_2^-$ release. Previous studies also support the hypothesis that the respiratory epithe-

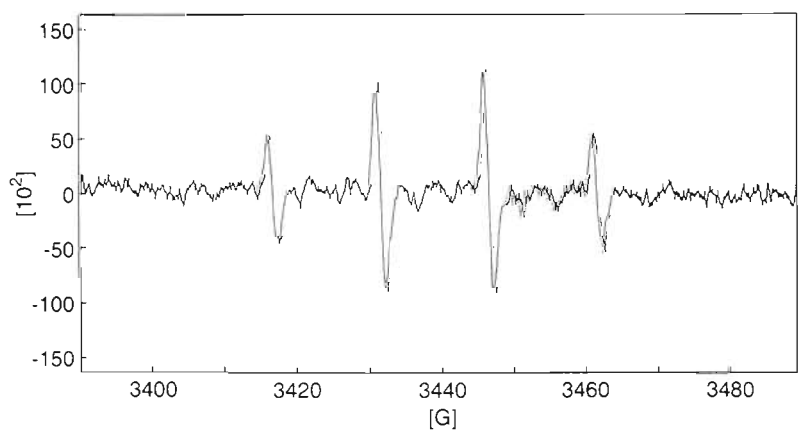

Fig. 5. ESR signal of hydroxyl radical ($^{\cdot}OH$) produced by *Heterosigma carterae*

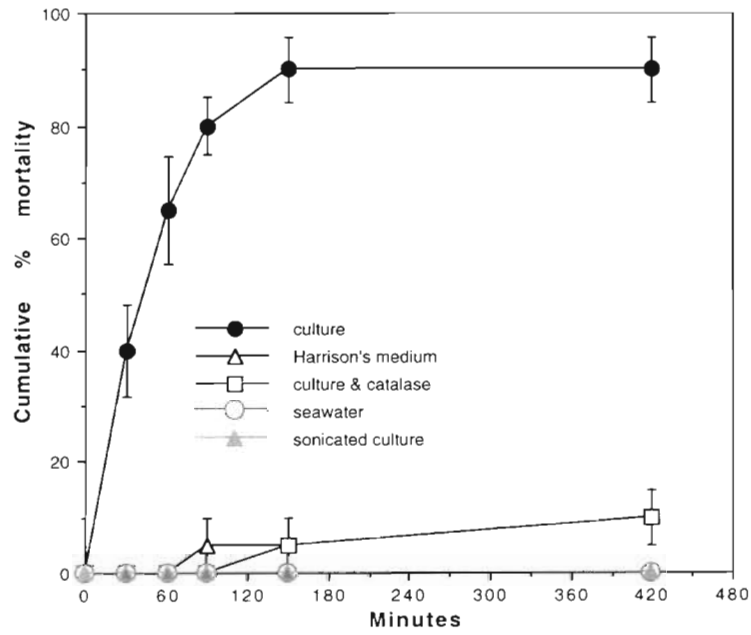

Fig. 6. Cumulative mortalities of rainbow trout *Oncorhynchus mykiss* exposed to *Heterosigma carterae* culture, Harrison's medium, *H. carterae* culture plus catalase, seawater, and sonicated culture

lial cells of finfish are the target sites of *H. carterae* toxicity (Chang 1990).

Hydrogen peroxide is not a free radical, but a toxic reactive oxygen metabolite (Schiller et al. 1993). Our results (Fig. 5) show that toxic cultures of *Heterosigma carterae* produce hydrogen peroxide. The amount of H_2O_2 released by 10^4 cells of *H. carterae* is similar to that of PMA-induced H_2O_2 release by 10^6 peritoneal macrophages from mice, which can kill bacteria (Nathan & Root 1977). Oda et al. (1992b) reported that H_2O_2 was generated by *Chattonella marina* at a rate of 0.062 nmol min^{-1} 10^{-4} cells. Other authors (Lai et al. 1993) have determined the selectivity of H_2O_2 toxicity. Their results showed that 90% of human lymphocyte cells are killed at 200 µM H_2O_2. Dean et al. (1986) reported that a rapid depolarization of the cells occurred within 5 min of treatment by iron and hydrogen peroxide and cells became greatly permeable to macromolecules after 35 min of treatment. They suggested that the derangement of membrane potential, reflecting abnormalities in ionic homeostasis, can be a sufficient factor inducing later lysis of the target cells. Pellmar (1986) reported that once depolarization has commenced under the attack of iron and H_2O_2, it cannot be stopped by adding iron chelators and catalase. However, if the iron chelator and catalase are added prior to the addition of iron and H_2O_2, all the subsequent events can be prevented. H_2O_2 readily crosses cell membranes. Therefore, H_2O_2 may diffuse into epithelial gill cells, damaging them. Hydrogen peroxide production was almost entirely eliminated by the addition of 500 U ml^{-1} catalase (Fig. 4).

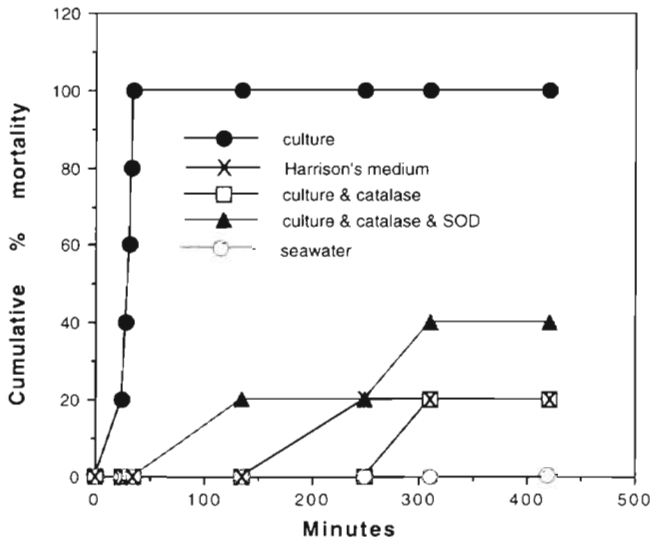

Fig. 7 The cumulative mortalities of rainbow trout *Oncorhynchus mykiss* exposed to *Heterosigma carterae* culture, Harrison's medium, *H. carterae* culture plus catalase, *H. carterae* culture plus catalase and SOD, and seawater

Our results show that the rates of production of $^{\cdot}O_2^-$ and H_2O_2 by *Heterosigma carterae* depend on the cell concentrations of *H. carterae*. As shown in Figs. 1 & 4, production of $^{\cdot}O_2^-$ and H_2O_2 by less than 10^4 cells is barely detectable, but a higher cell density results in greater $^{\cdot}O_2^-$ and H_2O_2 release. This may explain why fish kills induced by *H. carterae* under laboratory conditions occur only when the concentration of *H. carterae* cells exceeds 10^4 ml^{-1} cells.

The addition of specific stimulants, reported as essential for the release of $^{\cdot}O_2^-$ by phagocytic cells (Romeo et al. 1973, Nathan & Root 1977), was not required in the case of *Heterosigma carterae*. However, we cannot exclude the possibility that the presence of unknown stimulants in the culture medium triggered the release of $^{\cdot}O_2^-$ from *H. carterae*. A physical stimulation, such as agitated pretreatment as shown above, did trigger more release of $^{\cdot}O_2^-$ from *H. carterae*. Although $^{\cdot}O_2^-$ and H_2O_2 are toxic entities, much of their apparent toxicity is likely due to their interaction to produce the much more reactive hydroxyl radical through the iron- or copper-catalyzed Haber-Weiss-Fenton reaction, as follows (Klebanoff 1982, Halliwell & Gutteridge 1984);

$$^{\cdot}O_2^- + Fe^{3+} \rightarrow O_2 + Fe^{2+}$$
$$H_2O_2 + Fe^{2+} \rightarrow OH^- + {}^{\cdot}OH + Fe^{3+}$$

$$\text{Net:} \quad {}^{\cdot}O_2^- + H_2O_2 \rightarrow O_2 + {}^{\cdot}OH + OH^-$$

Coastal seawaters contains sufficient Fe or Cu to allow this reaction to occur. Our results (Fig. 6) show that *Heterosigma carterae* produces hydroxyl radical. This radical, once generated, can react with molecules in its immediate surroundings and destroy various important biomolecules (Simpson et al. 1988).

Catalase accelerates the formation of water from hydrogen peroxide (Forman & Fisher 1981), thus preventing the secondary generation of the hydroxyl radical by superoxide anion. SOD catalyzes the dismutation of the superoxide anion free radical to hydrogen peroxide (Bannister et al. 1987). And, as part of the Haber-Weiss-Fenton reaction, iron (II) becomes oxidised to iron (III). Superoxide radicals are thought to play a crucial role in reducing these salts so that the reaction can continue (Halliwell & Gutteridge 1985). This catalysis prevents the accumulation of superoxide anions, which, if available, can react with hydrogen peroxide to form the hydroxyl radical via the Haber-Weiss-Fenton reactions.

Our results (Figs. 6 & 7) show that a toxic culture of *Heterosigma carterae* caused high mortalities of juvenile rainbow trout under laboratory conditions and that this mortality was markedly decreased by the addition of catalase alone or catalase plus SOD to the culture immediately prior to the addition of test fish. Since the fish exposed to the toxic culture containing catalase showed the same mortality as those in Harrison's medium, the mortality in each tank was likely due to the effect of Harrison's medium, i.e. catalase could protect the fish. Our results also indicate that catalase is more efficient than SOD at protecting the fish, a result which we would expect based upon their reaction products. These results further confirm that toxicity of *H. carterae* for salmon is due to oxygen radicals and reactive oxygen species since SOD enzyme is a specific scavenger for $^{\cdot}O_2^-$.

The reactive oxygen intermediates generated by *Heterosigma carterae* probably first attack the membranes of the epithelial cells of the secondary lamellae and then cause lipid peroxidation damage and inactivation of ATPase of epithelial cell plasma membrane, which leads to the formation of edema in gill lamellae (Schiller et al. 1993). Chang (1990) observed the ultrastructural change in the apical surface of chloride cells caused by *H. carterae*, which represents changes in the ion transport function in gill filaments. This supports our suggestion that the formation of edema is due to degeneration by reactive oxygen radicals. Dean (1987) suggested that damage to plasma membrane transport proteins (pumps, carriers and channels) is an important mechanism by which free radicals perturb ionic homeostasis. Marinov (1985) has proposed that sodium channel operation involves radical intermediates; this implies that exogenous radicals might interfere with ion transport not only by damaging proteins, but also by interaction directly with transport intermediates.

In summary, *Heterosigma carterae* can produce reactive oxygen intermediates such as superoxide and hydroxyl radicals and hydrogen peroxide, which are toxic to juvenile rainbow trout, and by extension to other salmonids, if present in high enough concentrations, and the mortality of salmonids due to these toxins can be prevented by catalase and SOD. These results suggest that the main toxic effects of *H. carterae* on salmonids are likely due to superoxide and hydroxyl radicals and hydrogen peroxide.

Acknowledgements. We thank Drs Paul Percival and Frank Orfino for their generous help in operating the ESR spectrometer and interpreting ESR data. We thank Mr Frank Pouw for aid in cell culture. We also thank Dr J. N. C. Whyte for providing the toxic culture of *Heterosigma carterae*. We are grateful for useful discussions with Dr T Honjo and A. Ishimatsu when this research was initiated. The research was supported by a grant from the Science Council of British Columbia.

LITERATURE CITED

Babior BM (1978) Oxygen-dependent microbial killing by phagocytes. New Engl J Med 298:721–725

Bannister JV, Bannister WH, Rotilio G (1987) Aspects of the structure, function, and applications of superoxide dismutase. CRC crit Rev Biochem 22:111–180

Black EA, Whyte JNC, Bagshaw JW, Ginther NG (1991) The effects of *Heterosigma akashiwo* on juvenile *Oncorhynchus tshawytscha* and its implications for fish culture. J appl Ichthyol 7:168–175

Boustead N, Chang H, Pridmore R, Todd P (1989) Big Glory Bay algal bloom identified. Freshwat Catch 39:3–4

Chang FH (1990) First record of a *Heterosigma* (Raphidophyceae) bloom with associated mortality of cage-reared salmon in Big Glory Bay, New Zealand. NZ J mar Freshwat Research 24:461–469

Dean RT (1987) Free radicals, membrane damage and cell-mediated cytolysis. Br J Cancer 55 (Suppl VIII):39–45

Dean RT, Thomas SM, Vince G, Wolff SP (1986) Oxidation induced proteolysis and its possible restriction by some secondary protein modifications. Biomed Biochim Acta 45:11

Forman HJ, Fisher AB (1981) Antioxidant defenses. In: Gilbert DL (ed) Oxygen and living processes: an interdisciplinary approach. Springer-Verlag. New York, p 235–249

Fridovich I (1983) Superoxide radical: an endogenous toxicant. A Rev Pharmacol Toxicol 23:239–257

Gowen R (1987) Toxic phytoplankton in Scottish waters, the ecology of phytoplankton in Scottish coastal waters. Rapp P-v Reun Cons int Explor Mer 187:89–93

Halliwell B, Gutteridge JMC (1984) Oxygen toxicity, oxygen radicals, transition metals and disease. Biochem J 219: 1–14

Halliwell B, Gutteridge JMC (1985) Free radicals in biology and medicine. Clarendon press, Oxford, p 139–187

Harrison PJ, Waters RE, Taylor FJR (1980) A broad spectrum artificial medium for coastal and open ocean phytoplankton. J Phycol 16:28–35

Janzen EG, Nutter DE, Davis ER, Blackburn BJ, Poyer JL, McCay PB (1978) On spin trapping hydroxyl and hydroperoxyl radicals. Can J Chem 56:2237–2242

Johnston RB, Godzik CA, Cohn ZA (1978) Increased superoxide anion production by immunologically activated and chemically elicited macrophages. J exp Med 148:115–127

Klebanoff SJ (1982) Oxygen-dependent cytotoxic mechanisms of phagocytes. In: Gallin JI, Fauci AS (eds) Advances in host defense mechanisms. Raven Press, New York, p 111–162

Lai M, Griffiths H, Pall H, Williams A, Lunec J (1993) An investigation into the role of reactive oxygen species in the mechanism of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine toxicity using neuronal cell lines. Biochem Pharmacol 45:927–933

Marinov BS (1985) Na^+ channel antagonists act as electron donors while agonists act as electron acceptors in reactions with dye free radicals. FEBS Lett 191:159

Nakashima H, Ando M, Sugimoto M, Suga M, Soda K, Araki S (1987) Receptor-mediated O_2^- release by alveolar macrophages and peripheral blood monocytes from smokers and nonsmokers. Am Rev respir Dis 136:310–315

Nathan CF, Root RK (1977) Hydrogen peroxide release from mouse peritoneal macrophages. J exp Med 146: 1648–1662

Oda T, Akaike T, Sato K, Ishimatsu A, Takeshita S, Muramatsu T, Maeda H (1992a) Hydroxyl radical generation by red tide algae. Arch Biochem Biophy 294:38–43

Oda T, Ishimatsu A, Shimada M, Takeshita S, Muramatsu T (1992b) Oxygen-radical-mediated toxic effects of the red tide flagellate *Chattonella marina* on *Vibrio alginolyticus*. Mar Biol 112:505–509

Pellmar T (1986) Electrophysiological correlates of peroxide damage in guinea pig hippocampus *in vitro*. Brain Res 364:377

Romeo D, Zabucchi G, Rossi F (1973) Reversible metabolic stimulation of polymorphonuclear leukocytes and macrophages by concanavalin A. Nature New Biol 243:111–112

Schiller HJ, Reilly PM, Bulkley GB (1993) Antioxidant therapy. Crit Care Med 21(2):S92–S102

Shimada M, Shimono R, Murakami TH, Yoshimatsu S, Ono C (1989) Red tide, *Chattonella antiqua* reduced cytochrome *c* from horse heart. In: Okaichi T, Anderson DM, Nemoto T (eds) Red tides: biology, environmental science, and toxicology. Elsevier, New York, p 443–446

Simpson JA, Cheeseman KH, Smith SE, Dean RT (1988) Free-radical generation by copper ions and hydrogen peroxide. Stimulation by Hepes buffer. Biochem J 254:519–523

Slater TF (1972) Free radical mechanisms in tissue injury. Pion Ltd, London

Taylor FJR (1992) The taxonomy of harmful marine phytoplankton. G Bot Ital 126:209–219

Utermöhl H (1958) Zur Vervollkommnung der quantitativen Phytoplankton-Methodik. Mitt int Ver theor angew Limnol 9:1–38

Editorial responsibility: Managing Editor

Manuscript first received: December 15, 1994
Revised version accepted: May 17, 1995