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# Antioxidant responses and lipid peroxidation in gills and erythrocytes of fish (*Rhabdosargus sarba*) upon exposure to *Chattonella marina* and hydrogen peroxide: Implications on the cause of fish kills

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## Abstract

*Chattonella marina*, a red tide or harmful algal bloom species, has caused mass fish kills and serious economic loss worldwide, and yet its toxic actions remain highly controversial. Previous studies have shown that this species is able to produce reactive oxygen species (ROS), and therefore postulated that ROS are the causative agents of fish kills. The present study investigates antioxidant responses and lipid peroxidation in gills and erythrocytes of fish (*Rhabdosargus sarba*) upon exposure to *C. marina*, compared with responses exposed to equivalent and higher levels of ROS exposure. Even though *C. marina* can produce a high level of ROS, gills and erythrocytes of sea bream exposed to *C. marina* for 1 to 6 h showed neither significant induction of antioxidant enzymes nor lipid peroxidation. Antioxidant responses and oxidative damage did not occur as fish mortality began to occur, yet could be induced upon exposure to artificially supplied ROS levels an order of magnitude higher. The result of this study implies that ROS produced by *C. marina* is not the principal cause of fish kills.

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**Keywords:** Antioxidant responses; Harmful algal blooms; Lipid peroxidation; Reactive oxygen species

## 1. Introduction

During the past several decades, Harmful Algal Blooms (HABs) have increased in their frequency of occurrence, geographical distribution, number of causative species, intensity and damages (Anderson, 1989; Smayda, 1990; Hallegraeff, 1993; Sournia, 1995). Blooms of *Chattonella* spp., particularly *Chattonella marina*, have been reported worldwide including in

North and South America, Europe, Japan, China, Australia, South East Asia and Russia and have caused massive fish kills and economic losses in many of these countries (Okaichi, 1989; Hallegraeff et al., 1998; Marshall and Hallegraeff, 1999; Backe-Hansen et al., 2000; Bourdelais et al., 2002).

The precise toxic mechanism of *Chattonella* spp. remains highly controversial. Several hypotheses have been proposed, including gill damage and hence impairment of oxygen transfer (Matsusato and Kobayashi, 1974; Sakai et al., 1986; Endo et al., 1988; Ishimatsu et al., 1990, 1991, 1996a,b; Tsuchiyama et al., 1992; Oda et al., 1995, 1997, 1998), production of

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neurotoxins (Onoue and Nozawa, 1989; Endo et al., 1992; Khan et al., 1996b, 1997) and polyunsaturated fatty acids (PUFAs) (Nichols et al., 1987; Suzuki and Matsuyama, 1995), as well as generation of reactive oxygen species (ROS) by *Chattonella* spp. (Shimada et al., 1991, 1993; Oda et al., 1992a,b, 1994, 1995, 1997; Tanaka et al., 1992, 1994; Kim et al., 1999a). No conclusive scientific evidence is, however, available to prove any of these inferences.

All raphidophytes are known to produce ROS (Tanaka et al., 1992, 1994; Tanaka and Muto, 1992; Shimada et al., 1993; Oda et al., 1992a,b, 1994, 1995, 1997; Kim et al., 1999b), including superoxide ( $\cdot\text{O}_2^-$ ), hydrogen peroxide ( $\text{H}_2\text{O}_2$ ) and hydroxyl radicals ( $\text{OH}\cdot$ ). The highest ROS production rate was reported for *Chattonella* spp. (Oda et al., 1997), especially during exponential growth phase (Oda et al., 1992b, 1994, 1995; Ishimatsu et al., 1996a; Kawano et al., 1996). As a result, ROS are generally believed to be a plausible ichthyotoxic agent (Yang et al., 1995; Oda et al., 1997; Twiner and Trick, 2000). The fact that raphidophytes can produce ROS *per se* however, does not necessarily mean that ROS is the cause of fish kills, since the levels of ROS during *Chattonella* blooms and fish kills are unknown, and it has not been demonstrated that the levels of ROS produced are sufficiently high to kill fish. Shimada et al. (1993) observed that mucus produced by gill lamellae of young yellowtail fish induced the release of mucocysts from the large protrusions located on the glycocalyx of *Chattonella antiqua*. The glycocalyx, which generates  $\cdot\text{O}_2^-$ , may be discharged when flagellate cells are inhaled into the mouth and touch the gill surface. Furthermore, the glycocalyx may stick to the gill surface, resulting in continuous  $\cdot\text{O}_2^-$  generation and hence severely damage gill tissue (Kim et al., 2001). There is, however, no direct evidence to demonstrate whether the level of ROS induced by fish mucus is sufficient to cause oxidative stress, gill damage or fish mortality. As such, the toxic mechanism of *Chattonella* spp. remains unknown.

High levels of  $\text{H}_2\text{O}_2$  were produced in the exponential growth phase of *Cochlodinium polykrikoides* (~6000 cells/ml) under laboratory conditions (Kim et al., 1999a,b) and concomitant lipid peroxidation of fish gill tissue was observed. This lends further support to the hypothesis that damage was mediated through radical-dependent oxidation. Likewise, Twiner and Trick (2000) suggested that ROS production by *Heterosigma akashiwo* could alter gill structure and function, resulting in asphyxiation. Recently, Tang et al. (2005) reported significant induction of chloride cells in the gill, concomitant with significant reduction of osmol-

ality in the blood, were found in fish exposed to *C. marina*, while similar changes were not observed when fish was exposed to 50  $\mu\text{M}$   $\text{H}_2\text{O}_2$ , thus offering cytological and physiological evidence to refute the postulation that hydrogen peroxide is the principal cause of fish kills associated with *C. marina*.

In the course of evolution, aerobic organisms have evolved a suite of enzymatic and non-enzymatic proteins to prevent damage of lipid, protein and DNA caused by endogenous free radicals produced during their metabolism (Di Giulio, 1991; Ahmad, 1995). Should these antioxidant responses be overwhelmed by ROS, lipid peroxidation (LPO) and membrane damage will occur (Nakano et al., 1999). Indeed, the induction of these antioxidant responses and LPO have been commonly employed as biomarkers of oxidative stress (Di Giulio, 1991; Livingstone, 1993; Lackner, 1998; Ahmad et al., 2000) both under field and laboratory conditions.

The present study examines antioxidant responses and lipid peroxidation in gills and erythrocytes of fish (*Rhabdosargus sarba*) upon exposure to various levels of *C. marina* in comparison with equivalent and higher levels of artificially supplied  $\text{H}_2\text{O}_2$  exposure. The objective is to study the antioxidant responses of fish upon exposure to blooming concentration of *C. marina* and equivalent concentration of  $\text{H}_2\text{O}_2$ , with a view to decipher whether ROS produced by *C. marina* is the principal cause of fish kills.

## 2. Materials and methods

### 2.1. Culture of *C. marina* and *Dunaliella tertiolecta*

*C. marina* (Subrahmanyam) Hara et Chihara (NIES-3) kindly provided by the National Institute for Environmental Studies (NIES), Japan was cultured in K-medium (temperature:  $22 \pm 1$  °C, salinity:  $30 \pm 1$ ‰, pH:  $7.5 \pm 0.5$ , no aeration, photoperiod: 12 h light/12 h dark, provided by a 5000 lx cool-white fluorescent lamp). Culture was then transferred to an autoclaved transparent polypropylene bag (24" × 36") (Cole Parmer) mounted inside a 30 L Perspex tank and incubated until *C. marina* cells reached their maximum cell density ( $2\text{--}8 \times 10^3$  cells/ml) in exponential growth phase. These cells were then used for the exposure experiments.

In order to eliminate the possibility that observed effects were due to physical damage of fish gills caused by high concentrations of algae, the same biomass of a non-toxic algae which does not produce ROS, *D. tertiolecta* (American Type Culture Collection No. 30929), was cultured in K-medium under the same condition and used as an algal control in all of the exposure experiments.

## 2.2. Test species

Preliminary  $LT_{50}$  experiments on common aquaculture fish species in Hong Kong showed that the goldlined seabream (*R. sarba*) was most sensitive to both *C. marina* and  $H_2O_2$ . This species was, therefore, selected as a model fish species in the present study. Juvenile goldlined seabream (body weight  $180 \pm 20$  g) were obtained from a local fish farm and acclimatized in clean, running seawater (temperature:  $22 \pm 1$  °C, dissolved oxygen:  $5.5 \pm 0.5$  mg/L, salinity:  $30 \pm 1$ ‰, pH:  $7.5 \pm 0.5$ ) for at least 7 days prior to experiments. During the acclimation period, fish were fed to satiation with frozen shrimp daily. Previous studies have shown that the gill is the major site attacked by ROS produced by *C. marina* (Hishida et al., 1997, 1998; Nakamura et al., 1998). In contrast, fish erythrocytes are well protected by high enzymatic and non-enzymatic radical scavenging activities (Matkovics et al., 1977; Wdziejczak et al., 1982; Filho and Boveris, 1993). Thus, antioxidant responses in fish gills and erythrocytes were studied in our experiments to detect oxidative stress that might be caused by ROS.

## 2.3. Algal exposure experiment

Results of our preliminary experiments showed that  $2 \times 10^3$  cells/ml of *C. marina* produced a significant high level of  $H_2O_2$ , and the highest ROS production was found at cell densities of  $8 \times 10^3$  cells/ml. In light of the above, *C. marina* cell densities of  $2 \times 10^3$  cells/ml and  $8 \times 10^3$  cells/ml were used to represent “low concentration” and “high concentration”, respectively in our experiments. In each exposure experiment, an equivalent biomass of *D. tertiolecta* was used in the algal control and a seawater control was also set up in parallel. For each level of treatment, the algal control and seawater control each consisted of 6 replicate tanks, and each tank contained three fish. Stable physical conditions (temperature:  $22 \pm 1$  °C, dissolved oxygen:  $5.5 \pm 0.5$  mg/L, salinity:  $30 \pm 1$ ‰, pH:  $7.5 \pm 0.5$ , photoperiod: 12 h light/12 h dark, provided by a 5000 lx cool-white fluorescent lamp) were maintained throughout the experiment and only gentle aeration was provided during the exposure period.

Results of  $LT_{50}$  experiments showed that the  $LT_{50}$  of *R. sarba* exposed to low concentration and high concentrations of *C. marina* were 6 h and 3 h, respectively. Thus, for the low concentration treatment, fish ( $n=9$ ) were sampled at 3 h (i.e. at which time 10% mortality was observable), and another 9 fish were sampled at 6 h (at which time 50% mortality occurred).

Likewise, 9 fish each were sampled from the high concentration treatment at 1 h (i.e. when 10% mortality was observable), and 9 fish were again sampled from the high concentration treatment at 3 h (when 50% fish mortality occurred). During each of the above samplings, the same number of fish was sampled from the algal and seawater controls.

Sampled fish were anesthetized in 0.1 g/L tricaine methanesulfonate (MS-222) (Sigma). The tail was quickly cut with a scalpel blade and blood collected within 30 s from the caudal vessel using a heparinized capillary tube (Marienfeld) kept on ice. The first and second right gill arches were dissected out within 1–2 min and washed in ice-cold 0.9% sodium chloride solution (NaCl) and subsequently ice-cold SEI buffer (150 mM sucrose, 10 mM EDTA, 50 mM Imidazole, pH 7.3) to remove excessive blood, body fluid and algal cells. The first gill arch was used to determine antioxidant enzyme activities and the second for the  $Na^+, K^+$ -ATPase. Gill filaments were carefully trimmed from the gill arches, cut into pieces of <1 cm and put into an Eppendorf tube. Tissue was either homogenized immediately or frozen in liquid nitrogen and stored at  $-80$  °C until analysis. Blood samples were collected from individual fish using heparinized capillary tubes (Marienfeld), and centrifuged at  $3000 \times g$  for 10 min at 4 °C. The red cells were resuspended and diluted to a final volume of 1 ml with ice-cold 0.9% NaCl, centrifuged at 4 °C at  $3000 \times g$  for 10 min at 4 °C and subsequently double rinsed in ice-cold 0.9% NaCl. Packed erythrocytes were either used for preparation of hemolysates immediately, or stored in liquid nitrogen at  $-80$  °C until analysis.

Algal density and ROS levels were measured before exposure and also at 20 min intervals during the entire experimental period. Two aliquots of algae and water were collected from the surface of each treatment tank and transferred into an ice-cold Eppendorf tube. Cells were counted using a hemocytometer and total ROS levels quantified using the method of Buxser et al. (1999) and expressed as  $\mu M H_2O_2$ .

## 2.4. $H_2O_2$ exposure experiment

ROS levels produced by cultures of  $2 \times 10^3$  and  $8 \times 10^3$  cells/ml of *C. marina* were measured at  $9.69 \pm 0.19$   $\mu M$  and  $18.95 \pm 1.01$   $\mu M$  respectively. Experiments were therefore designed to study antioxidant responses and lipid peroxidation in fish exposed to an equivalent level of ROS. If the observed effects are principally attributable to ROS, similar antioxidant responses and lipid peroxidation induction in gills and erythrocytes

should occur. Although  $\cdot\text{OH}$  is highly reactive and damaging when produced within the cell,  $\cdot\text{OH}$  has an extremely short life ( $<1$  ms) and is not membrane permeable. Thus, it is unlikely that extra-cellular  $\cdot\text{OH}$  produced by algae can reach and affect fish/mussel to a significant extent, especially it has been demonstrated that *C. marina* does not attach to the gills of affected fish (Tang and Au, 2004).  $\text{H}_2\text{O}_2$  is the intermediate between superoxide ( $\cdot\text{O}_2^-$ ) and hydroxyl radicals ( $\cdot\text{OH}$ ), and is the most stable form of ROS, with a half-life of several hours in seawater.  $\text{H}_2\text{O}_2$  is membrane permeable, and reacts readily with oxidizable metals (e.g.  $\text{Fe}^{2+}$  in fish blood) or organic matters and produce  $\cdot\text{OH}$  (by the Fenton reaction) when in contact with tissues (Cooper and Zepp, 1990; Mao et al., 2002).

Since antioxidant responses and lipid peroxidation were not observable at these two algal concentrations, higher concentrations of  $\text{H}_2\text{O}_2$  (0.5 mM, 50-fold higher than ROS levels in the high concentration of *C. marina*) were used in this experiment to investigate whether antioxidant responses and LPO would occur at higher ROS levels. The experimental set up for the  $\text{H}_2\text{O}_2$  exposure experiment was similar to that described in the algal exposure experiment. The  $\text{H}_2\text{O}_2$  treatment consisted of 6 replicate tanks and each tank contained 3 fish. A seawater control was also set up in parallel. Nine fish each were sampled at 3 h, and another nine fish sampled at 6 h. The same number of fish was also removed from the seawater control at each of the above samplings.

ROS levels at each of the treatment and control were quantified before exposure and at 10 min intervals

during the exposure period and expressed as  $\mu\text{M}$   $\text{H}_2\text{O}_2$ , as described in the algal exposure experiments.

## 2.5. Measurements of antioxidant responses and lipid peroxidation

Subcellular fractions of gill filaments from the upper part of the first gill arch were homogenized using an electrical homogenizer (Ultra-Turrax T8 IKA Labor-technik) in 1:15 tissue weight (g): buffer volume (ml) (0.05 M phosphate buffer, pH 7.4 containing 0.15 M KCl) at 4 °C. The homogenate was centrifuged at  $3000\times g$  for 15 min at 4 °C to remove unbroken cells and intact nuclei, and the supernatant further centrifuged at  $10,000\times g$  for 20 min to obtain the post-mitochondrial supernatants (PMS). The cytosolic fraction was isolated from the  $10,000\times g$  supernatant by further centrifuging at  $100,000\times g$  for 60 min. The cytosolic supernatant was used for superoxide dismutase (SOD), glutathione peroxidase (GPx), glutathione reductase (GR) and lipid peroxidation (LPO) analyses. Cytosolic supernatants were separated into two aliquots: one was used for SOD and LPO determinations and the other for selenium containing glutathione peroxidase (Se-GPx) and GR determinations. Aliquots were sampled only once for each assay and were not frozen again for re-analysis to prevent or minimize contamination. The PMS fraction was used for Catalase (CAT) assay. All supernatants were stored at  $-80$  °C for subsequent analysis or kept on ice for immediate analysis. The lower part of same filaments were stored at  $-80$  °C and only homogenized immediately before reduced

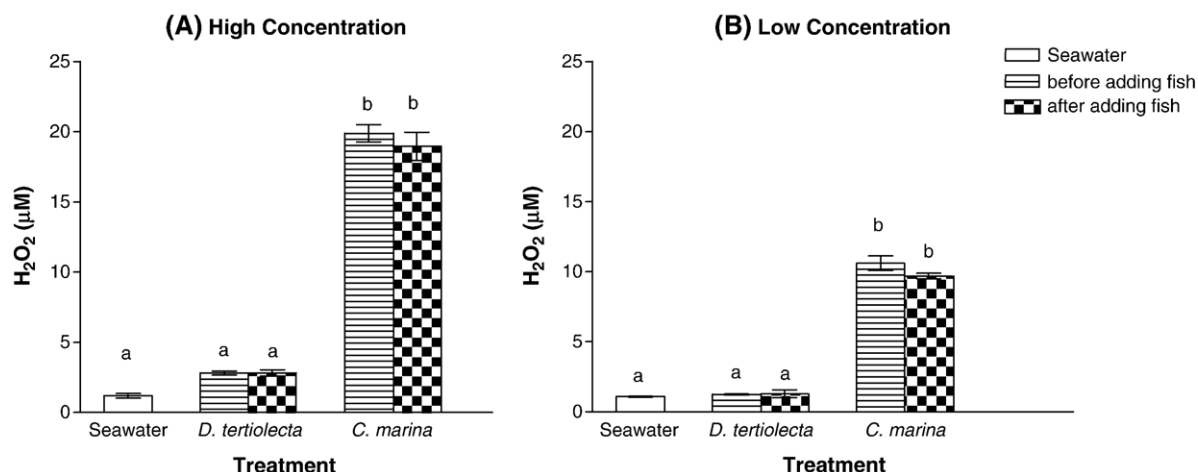
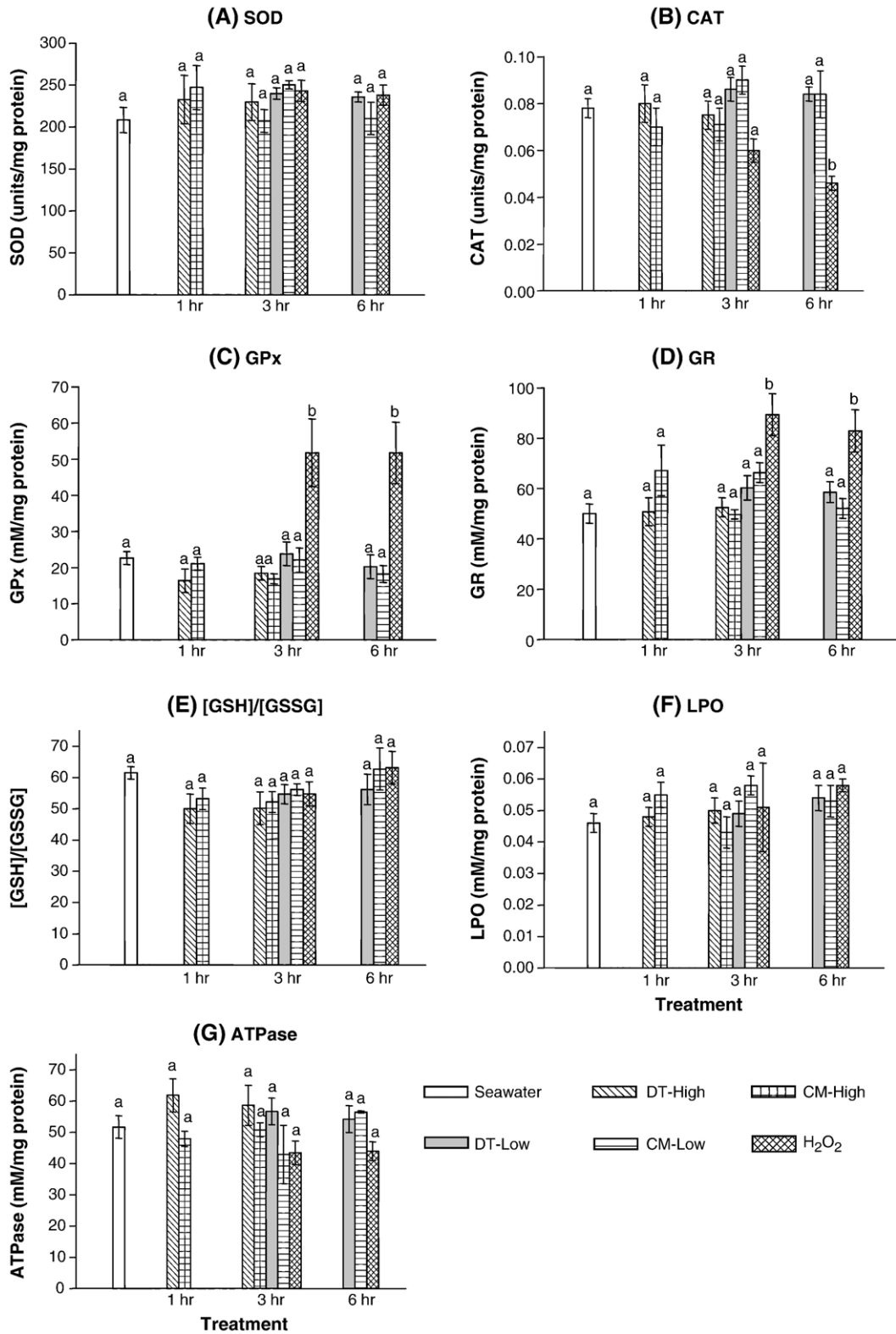


Fig. 1.  $\text{H}_2\text{O}_2$  levels ( $\mu\text{M}$ ) in (A) high (8000 cells/ml) and (B) low (2000 cells/ml) concentrations of *C. marina*, *D. tertiolecta* and seawater control before and after adding fish. Data are expressed as mean  $\pm$  S.E.M. ( $n=4$ ). Treatments with the same letter are not significantly different from one another in the Tukey test ( $p \geq 0.001$ ).





glutathione (GSH) and oxidized glutathione (GSSG) analyses.

Packed erythrocytes were suspended in an approximately equal volume of homogenized buffer (0.05 M phosphate buffer, pH 7.4 containing 0.15 M KCl). 50  $\mu$ l of the suspension was then added to 450  $\mu$ l homogenized buffer to further dilute this to a 1:20 ratio. Packed erythrocytes were sonicated for 5 min on ice to ensure complete cell disruption. The hemolysate was centrifuged at 3000 $\times$ g for 20 min at 4 °C to remove broken cells and debris and stored at –80 °C prior to analysis or kept on ice for immediate analysis.

Enzyme activities were measured under conditions of saturating substrate at 25 °C in polystyrene microwell plates (Nunc) (128 $\times$ 86 mm), using a 96-well microplate reader (Spectra Max 340PC) in accordance with the methodology describe below. Protein concentrations of tissue were determined according to the method of Bradford (1976). The hemoglobin (Hb) content of hemolysates was determined by the method of Drabkin and Austin (1935). All biochemical reagents, enzymes and chemicals were purchased from Sigma Chemical Co. SOD and CAT were determined following Marklund and Marklund (1974) and Cohen et al. (1996) respectively. GPx and GR activities were determined by NADPH consumption (measured at 340 nm) and based on the methods of Carlberg and Mannervik (1985) respectively. GSH and GSSG were analyzed using the fluorescence probe *o*-phthalaldehyde (OPA) (Senft et al., 2000). Fluorescence was measured on a black microplate (FluoroNunc) at 365 nm excitation and 430 nm emission. Na<sup>+</sup>,K<sup>+</sup>-ATPase was analyzed using the method of McCormick (1993). Lipid peroxidation was measured based on the detection of malondialdehyde (MDA, a byproduct of lipid peroxidation), using the thiobarbituric acid (TBA) test (Draper and Hadley, 1990). Results of all the above biochemical determinations were expressed as mM/mg protein (for gill tissue) or mM/g Hb (for erythrocytes).

## 2.6. Statistical analysis

ROS levels in the H<sub>2</sub>O<sub>2</sub> and two levels of *C. marina* treatments, algal control and seawater control were compared using one-way ANOVA. Two-way ANOVA

was used to investigate the effects of low and high algal/H<sub>2</sub>O<sub>2</sub> concentrations, duration of exposure, and their interactions on different enzyme activities and lipid peroxidation. If significant differences were revealed by the ANOVA test, a Tukey test was used to further elucidate which treatments and time intervals were significantly different. Significance level ( $\alpha$ ) was set at 0.05 in all tests.

## 3. Results

### 3.1. ROS measurement in *C. marina* mass culture

The mean concentrations of H<sub>2</sub>O<sub>2</sub> in low and high concentrations of *C. marina* were 9.69 $\pm$ 0.2  $\mu$ M and 18.95 $\pm$ 1  $\mu$ M respectively, and were some 10 to 20 times higher than that in the *D. tertiolecta* and seawater controls (Fig. 1,  $P < 0.001$ ).

### 3.2. Antioxidant enzymes and lipid peroxidation

Throughout the exposure periods, SOD, CAT, GPx, GR activities and [GSH]/[GSSG], in both fish gills (Fig. 2) and erythrocytes (Fig. 3), as well as Na<sup>+</sup>,K<sup>+</sup>-ATPase in the gills (Fig. 2) showed no significant difference between high and low concentrations of *C. marina*, *D. tertiolecta* and seawater control ( $P \geq 0.05$ ). LPO levels in gills and erythrocytes also showed no significant differences between high and low concentrations of *C. marina*, *D. tertiolecta* and seawater control ( $P \geq 0.05$ ) (Figs. 2F and 3F).

### 3.3. H<sub>2</sub>O<sub>2</sub> exposure experiments

Compared with seawater control and *C. marina* treatments, no significant change in SOD activities was found in fish gills upon treatments with H<sub>2</sub>O<sub>2</sub> ( $P \geq 0.05$ ) (Fig. 2A). CAT activities were significantly lower upon exposure to 0.5 mM H<sub>2</sub>O<sub>2</sub> for 6 h ( $P < 0.05$ ) (Fig. 2B). GPx, GR as well as [GSH]/[GSSG] activities in fish gills after treatment with 0.5 mM H<sub>2</sub>O<sub>2</sub> for 3 and 6 h were significantly elevated compared with those treated with low and high concentrations of *C. marina* and seawater control ( $P < 0.05$ ) (Fig. 2C and D).

No significant change in LPO levels in the gills were found between high and low concentrations of *C.*

Fig. 2. (A) SOD activities, (B) CAT activities, (C) GPx activities, (D) GR activities, (E) [GSH]/[GSSG], (F) LPO levels and (G) ATPase activities in the gill of goldlined seabream (*Rhabdosargus sarba*) after exposure to high (8000 cells/ml) and low (2000 cells/ml) concentrations of *C. marina*, *D. tertiolecta*, H<sub>2</sub>O<sub>2</sub> and seawater control from 1 to 6 h. Data are expressed as mean $\pm$ S.E.M. ( $n=9$ ). Treatments with the same letter are not significantly different from one another in the Tukey test ( $p \geq 0.05$ ).

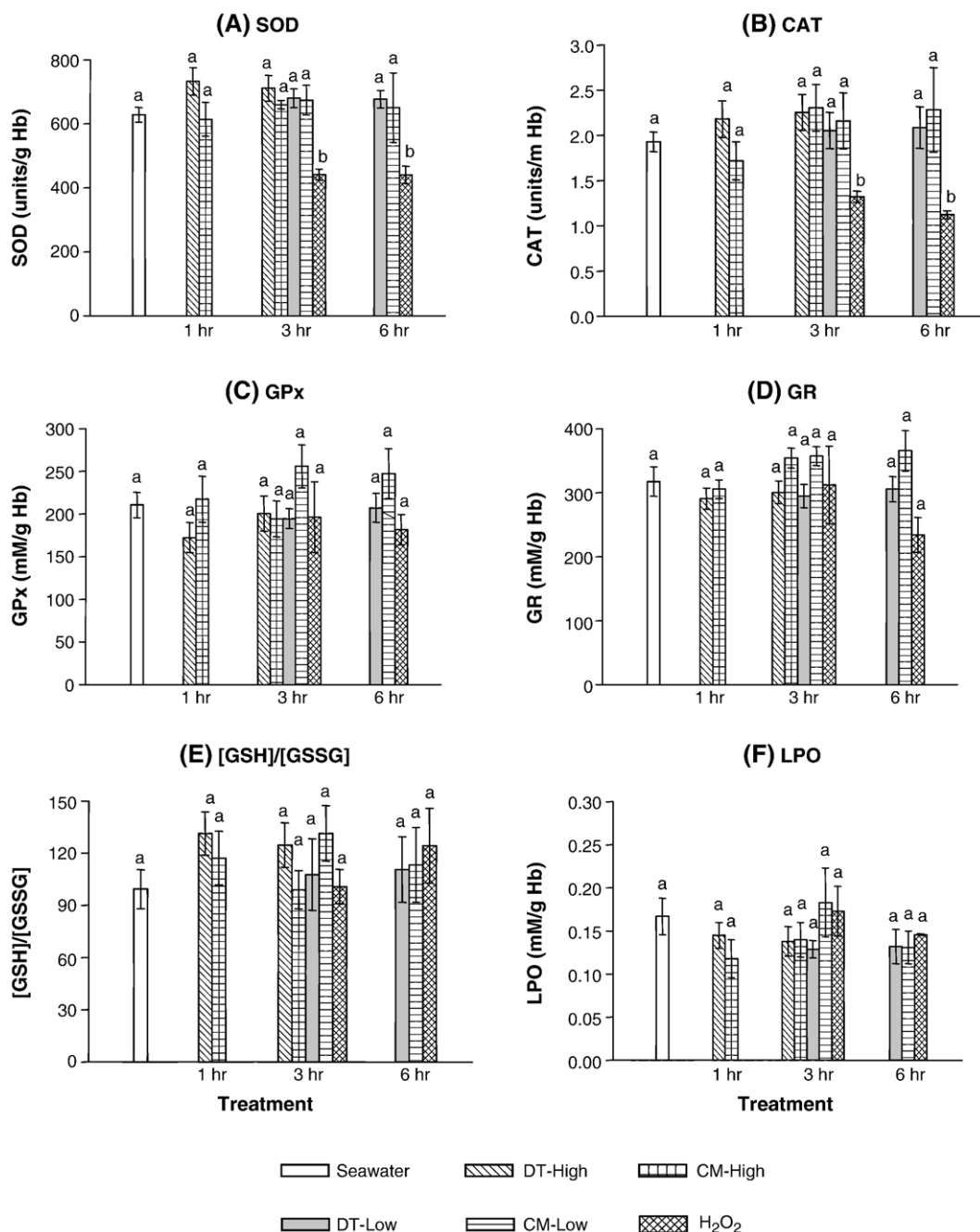


Fig. 3. (A) SOD activities, (B) CAT activities, (C) GPx activities, (D) GR activities, (E) [GSH]/[GSSG] and (F) LPO levels in the erythrocytes of goldlined seabream (*Rhabdosargus sarba*) after exposure to high (8000 cells/ml) and low (2000 cells/ml) concentrations of *C. marina*, *D. tertiolecta*, H<sub>2</sub>O<sub>2</sub> and seawater control from 1 to 6 h. Data are expressed as mean ± S.E.M. ( $n=9$ ). Treatments with the same letter are not significantly different from one another in the Tukey test ( $p \geq 0.05$ ).

*marina*, high and low concentrations of H<sub>2</sub>O<sub>2</sub>, and seawater control ( $P \geq 0.05$ ) (Fig. 2F). Likewise, no difference was found in Na<sup>+</sup>,K<sup>+</sup>-ATPase activities between *C. marina* treatments, the H<sub>2</sub>O<sub>2</sub> treatments and seawater control ( $P \geq 0.05$ ) (Fig. 2G).

SOD and CAT activities in erythrocytes upon exposure to H<sub>2</sub>O<sub>2</sub> for 3 and 6 h were significantly lower than those exposed to low and high concentrations of *C. marina* and the seawater control ( $P < 0.05$ ) (Fig. 3A and B). No significant change in GPx, GR

activities and [GSH]/[GSSG] were found in erythrocytes in fish exposed to low and high concentrations of *C. marina*, the H<sub>2</sub>O<sub>2</sub> treatment and the seawater control ( $P \geq 0.05$ ) (Fig. 3C and E). Throughout the exposure period, no significant changes in erythrocyte LPO levels between both concentrations of *C. marina*, the H<sub>2</sub>O<sub>2</sub> treatment and the seawater control could be found ( $P \geq 0.05$ ) (Fig. 3F).

#### 4. Discussion

Several studies postulated that ROS produced by *C. marina* were the principal ichthyotoxic agent (Shimada et al., 1991, 1993; Oda et al., 1992a,b, 1994, 1995, 1997; Tanaka et al., 1992, 1994; Tanaka and Muto, 1992; Kim et al., 2000). In these studies however, ROS were only measured using relative scales, to demonstrate that exponential growth phase *C. marina* produced higher levels of ROS than stationary phase cells (Oda et al., 1995; Kawano et al., 1996), or that ROS produced by *C. marina* in the absence of SOD or CAT were higher than in the presence of these inhibitors (Oda et al., 1994, 1995, 1997). No evidence was ever presented to show that ROS production by this species was sufficiently high to cause oxidative damage or to kill fish. For the first time, here we demonstrate that H<sub>2</sub>O<sub>2</sub> levels produced by sub-bloom ( $2130 \pm 167$  cells/ml) and bloom concentrations ( $8220 \times 259$  cells/ml) of exponential phase *C. marina* were  $\sim 10$   $\mu$ M and  $\sim 20$   $\mu$ M, respectively. These levels were some 10–20 times higher than those of non-toxic algal (*D. tertiolecta*) and seawater controls.

Fifty percent of fish mortality began to occur after 6 h exposure to low *C. marina* cell densities and after 2 h at the high concentration, and yet no significant changes of antioxidant enzymes were found in gills or erythrocytes. Three possible reasons may account for the lack of antioxidant response: (1) our exposure period was too short (1–6 h), whereas the induction of antioxidant enzymes (i.e., synthesis of relevant mRNA and protein) may take more than 6 h; (2) Despite *C. marina* producing ROS, the levels were insufficient to induce oxidative stress in fish; or (3) antioxidant responses of goldlined seabream (*R. sarba*) are not sensitive to ROS insult.

The first possibility can be eliminated for two reasons. First, a number of studies provided ample evidence to demonstrate that antioxidant responses in a variety of fish occur within hours. For example, Gabryelak and Klekot (1985) observed a transitory increase in erythrocytes CAT activity in crucian carp (*Carassius carassius*) after exposure to 10 ppm paraquat

(a herbicide) for 4 h. Similarly, gill SOD activity in carp (*Cyprinus carpio* L.) increased following exposure to 10 ppm paraquat for 8 h (Vig and Nemcsok, 1989). Likewise, Li et al. (2003) showed that gill SOD activities in carp increased after 0.5 h, whereas CAT and GPx activities increased after 6 h upon exposure to 10  $\mu$ g/L mycrocystin-LR toxin. Ritola et al. (2000) also showed activities of hepatic GPx of Arctic charr (*Salvelinus alpinus*) increased 30 min after exposure to 0.34 mg/l of ozone.

Second, significant oxidative damage and lipid peroxidation should theoretically occur if antioxidant defenses were overwhelmed by ROS production (Kappus, 1987; Halliwell and Gutteridge, 1989; Winston and Di Giulio, 1991). For example, increases of erythrocytes SOD, CAT and GPx activities in *C. carassius* L. were thought to be caused by H<sub>2</sub>O<sub>2</sub> produced by 10 ppm paraquat exposure for 4 h. This exposure also led to an increase in LPO level (Gabryelak and Klekot, 1985). These authors postulated that antioxidant enzyme activities were insufficient to protect against the ROS. Thus, even though the antioxidant enzymes did not respond within the short exposure times used in our experiment, the effects of ROS, if significant, should also have been manifested as oxidative damage such as lipid peroxidation. The lack of antioxidant enzyme responses and lipid peroxidation in fish upon exposure to *C. marina* suggest that ROS produced by the algae was not sufficient to elicit antioxidant responses or oxidative damage, let alone cause fish kills.

In order to test possibilities 2 and 3 (see above), a second set of experiments was carried out. Fish were exposed to a level of H<sub>2</sub>O<sub>2</sub> 50 times higher than that produced by *C. marina*, to cover possible variations in ROS production under laboratory and field conditions. Significant increases in gill GPx, GR and [GSH]/[GSSG] occurred readily after 3 and 6 h upon H<sub>2</sub>O<sub>2</sub> treatment. The fact that antioxidant enzymes in fish gills were enhanced by H<sub>2</sub>O<sub>2</sub> treatment further confirmed that antioxidant responses in goldlined seabream (*R. sarba*) are responsive to ROS within 3 to 6 h. The concentrations needed to elicit a response are, however, at least an order of magnitude ( $> 50$  times) higher than those produced by “bloom” concentrations of *C. marina*. Notwithstanding, oxidative stress (formation of LPO) was still not observed in the gills and erythrocytes despite the much higher levels of H<sub>2</sub>O<sub>2</sub> than were produced by *C. marina*. Results of our studies, therefore, provide unequivocal biochemical evidence to reject the hypothesis that ROS produced by *C. marina* are the principal ichthyotoxic agents



involved in fish kills, as postulated by previous workers (Oda et al., 1992a,b, 1994, 1995, 1997).

In fact, previous studies only demonstrated that ROS can be produced by *C. marina* and did not provide any evidence to show the causal relationship between fish mortality and ROS. Oda et al. (1992b) found that  $O_2^-$  (1–6 nmol) and  $H_2O_2$  (0.4–2 nmol) levels increased with increasing density of *C. marina* (from  $0.5 \times 10^4$  to  $2.5 \times 10^4$  cells/ml), and the proliferation of the marine bacteria, *Vibrio alginolyticus* was strongly inhibited in a plankton/bacteria co-culture after 7 h incubation. Based on these results, it was concluded that ROS produced by *C. marina* was responsible for fish-kills. However, the level of ROS sufficient to kill bacteria would almost certainly be lower than that required to kill fish.

Ishimatsu et al. (1990, 1997) showed that plasma ions (i.e.  $Cl^-$ ,  $Na^+$ ,  $Mg^{2+}$ ) in yellowtails increased after exposure to *C. marina* (4000 cells/ml) for 1 h. These results suggested that osmoregulatory impairment might be a possible reason leading to fish kills. In our study,  $Na^+$ ,  $K^+$ -ATPase activities in the gill remained unchanged after exposure to *C. marina*.  $Na^+$ ,  $K^+$ -ATPase in the gill chloride cells continuously excretes NaCl and plays an important role in the active transport of  $Na^+$  and  $K^+$  across the cell membrane. It is the primary driving force for membrane-associated transport channels such as  $Na^+/Ca^{2+}$  and  $Na^+/H^+$  antiporters (Skou, 1965) and energy-requiring processes (Racker et al., 1983). The fact that  $Na^+$ ,  $K^+$ -ATPase activities remained unchanged in the *C. marina* studies suggests that osmoregulatory impairment is unlikely. In contrast, a decrease in  $PaO_2$  and blood osmolality, upon 3 h exposure to *C. marina* (2000 cells/ml) has been observed and this was accompanied by proliferation of chloride cells (Tang and Au, 2004). Their results appear to suggest that *C. marina* may impair osmoregulation in fish, but the precise mechanisms remain unknown.

The present study focused on antioxidant responses and lipid peroxidation of goldlined seabream (*R. sarba*) upon exposure to *C. marina*. The biochemical evidence presented herein indicates that ROS is unlikely to be the principal toxic mechanism of *C. marina* in fish kills. Several possibilities regarding ichthyotoxicity of *C. marina* have been postulated and remain unresolved. *C. marina* may: (1) cause gill damage and, therefore, impairment of oxygen transfer; (2) produce brevetoxin-like toxins, and (3) produce toxic PUFAs.

Suffocation due to clogging of gills by algal cells is generally supposed to be the direct cause of fish death killed by *C. marina*. Physical clogging may lead to

over-production of mucus or edema, which were observed in the gills of fish killed by exposure to *Chattonella* in both laboratory (Shimada et al., 1983; Ishimatsu et al., 1996b; Hishida et al., 1997; Nakamura et al., 1998) and field (Tiffany et al., 2001) conditions. Physical clogging leads to the impairment of oxygen transfer and hence fish kills. Unfortunately, the study of Tiffany et al. (2001) on tilapia did not have any control, nor was the cell density reported. Excessive mucus observed in their study may also be due to other factors (e.g., toxins) rather than physical clogging by *C. marina*. In our study, we did not find any mucus or edema in fish exposed to *C. marina* or to  $H_2O_2$  treatments.

Several studies have suggested that brevetoxin-like compounds are produced by *C. marina* (Onoue et al., 1990; Ahmad, 1995; Khan et al., 1995, 1996a; Haque and Onoue, 2002). The toxin profiles reported were, however, inconsistent between studies. Importantly, the 24-h  $LC_{50}$  of toxin fractions extracted from *Chattonella* to juvenile red sea bream was 4.5 mg/L (1.4 mg of toxin was extracted from  $10^9$  cells) (Onoue et al., 1990), while the 24-h  $LD_{50}$  of brevetoxin PbTx-2 produced by *Karenia brevis* (= *Gymnodinium breve*) at  $10^9$  cells/L was only 2–6  $\mu$ g/L (Baden, 1983; Stuart and Baden, 1988). It is, therefore, clear that the toxicity of brevetoxin-like compounds extracted from high and environmentally unrealistic concentrations of *C. marina* ( $10^9$  cells/ml) is still some 2000 to 6000 times lower than that produced by *G. breve*. Neither laboratory nor field data yet support that sufficiently high levels of brevetoxins can be produced during *Chattonella* blooms to cause gill damage or fish mortality.

Suzuki and Matsuyama (1995) reported the production of 20:5, 18:4 and 16:0 PUFA by *C. antiqua* ( $4.8 \times 10^5$  cells/ml). These PUFA were, however, not characterized and their toxicity not tested. Rather, these PUFA were only suspected to be toxic, based on the fact that 18:5n3 PUFA produced by *Karenia mikimotoi* (= *Gymnodinium cf. mikimotoi*) is toxic. The ichthyotoxicity of PUFA produced by *K. cf. mikimotoi* was assumed to inhibit ATPase activities and distort chloride cells and thus impair osmoregulation, but whether the concentration of PUFA produced by *C. marina* is sufficiently high to cause such damage and kill fish remains unknown. Correlations between toxic PUFA production and fish mortality during *Chattonella*-exposure or blooms have also not been demonstrated. The available scientific information to support the notion that PUFA is ichthyotoxic or the principal toxic agent leading to fish kills is limited.

## 5. Conclusions

Gill and erythrocytes of sea bream exposed to bloom and sub-bloom concentrations of *C. marina* for 1 to 6 h showed no significant induction of antioxidant enzyme activities or scavengers. Lipid peroxidation also did not occur. The fact that: (a) both antioxidant responses and oxidative damage did not occur even though fish died upon exposure to *C. marina*, and (b) certain antioxidant responses could be induced when fish were exposed to ROS levels an order of magnitude higher, therefore, provides for the first time unequivocal experimental evidence demonstrating that ROS levels produced by *C. marina* are not sufficient to cause oxidative stress or oxidative damage of fish, let alone mortality. Our analysis also showed that the evidence supporting other explanations for fish mortality, such as, gill damage (and therefore impairment of oxygen transfer), production of brevetoxin-like toxins and toxic PUFAs, is inconclusive thus far. Further studies are, therefore, required to decipher the ichthyotoxicity of *C. marina*.

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