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

Stephanie P.S. Woo [1], Wenhua Liu [1], Doris W.T. Au[1], Donald M. Anderson[2] and Rudolf S.S. Wu[1]*

[1] Centre for Coastal Pollution and Conservation,

City University of Hong Kong,

Tat Chee Avenue, Kowloon,

Hong Kong SAR, China

[2] Biology Department,

Woods Hole Oceanographic Institution,

Woods Hole, MA 02543,

United States of America

* Tel: (852) 2788 7401; Fax: (852) 2788 7406; Email: bhrswu@cityu.edu.hk

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 (*Rhabdosarga 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.

Keywords: Harmful Algal blooms, Reactive oxygen species, Antioxidant responses, Lipid peroxidation

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 *C. 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 & 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 & Kobayashi, 1974; Sakai et al., 1986; Endo et al., 1988; Ishimatsu et al., 1990, 1991, 1996a, 1996b; Tsuchiyama et al., 1992; Oda et al., 1995, 1997, 1998), production of neurotoxins (Onoue & Nozawa, 1989; Endo et al., 1992; Khan et al., 1996 b; 1997) and polyunsaturated fatty acids (PUFAs) (Nichols et al., 1987; Suzuki & Matsuyama, 1995), as well as generation of reactive oxygen species (ROS) by *Chattonella* spp. (Shimada et al., 1991, 1993; Oda et al., 1992a, 1992b, 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 & Muto, 1992; Shimada et al., 1993; Oda et al., 1992a, 1992b, 1994, 1995, 1997; Kim et al., 1999b), including superoxide ($O_2^{·-}$), hydrogen peroxide (H_2O_2) and hydroxyl radicals ($OH^·$). 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 & 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 *C. antiqua*. The glycocalyx, which generates $O_2^{·-}$, 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 $O_2^{·-}$ 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 H_2O_2 were produced in the exponential growth phase of *C. polykrikoides* (~ 6,000 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 though radical-dependent oxidation. Likewise, Twiner & 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 osmolality in the blood, were found in fish exposed to *C. marina*, while similar changes were not observed when fish was exposed to 500 μM H_2O_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 (*Rhabdosarga sarba*) upon exposure to various levels of *C. marina* in comparison with equivalent and higher levels of artificially supplied H_2O_2 exposure. The objective is to study the antioxidant responses of fish upon exposure to blooming concentration of *C. marina* and equivalent concentration of H_2O_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 Chattonella marina and *Dunaliella tertiolecta*

Chattonella marina (Subrahmanyan) Hara et Chihara (NIES-3) kindly provided by the National Institute for Environmental Studies (NIES), Japan was cultured in

K-medium (temperature: 22 ± 1 °C, salinity: 30 ± 1 ‰, pH: 7.5 ± 0.5, no aeration, photoperiod: 12 h light/12 h dark, provided by a 5000 lux cool-white fluorescent lamp). Culture was then transferred to an autoclaved transparent polypropylene bag (24” x 36”) (Cole Parmer) mounted inside a 30 L Perspex tank and incubated until *C. marina* cells reached their maximum cell density (2 - 8 x 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, *Dunaliella 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 (*Rhabdosargus 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 ± 20 g) were obtained from a local fish farm and acclimatized in clean, running seawater

(temperature: 22 ± 1 °C, dissolved oxygen: 5.5 ± 0.5 mg/L, salinity: 30 ± 1 %o, pH: 7.5 ± 0.5) for at least 7 d 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 *Chattonella 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; Wdzieczak et al., 1982; Filho et al., 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 x 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 x 10^3 cells/ml. In light of the above, *C. marina* cell densities of 2 x 10^3 cells/ml and 8 x 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 ± 1 °C, dissolved oxygen: 5.5 ± 0.5 mg/L, salinity: 30 ± 1 ‰, pH: 7.5 ± 0.5, photoperiod: 12 h light/12 h dark, provided by a 5000 lux 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 *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 *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 µM H_2O_2.

2.4. H_2O_2 Exposure Experiment

ROS levels produced by cultures of 2 x 10^3 and 8 x 10^3 cells/ml of *C. marina* were measured at 9.69 ± 0.19 μM and 18.95 ± 1.01 μM 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 •OH is highly reactive and damaging when produced within the cell, •OH has an extremely short life (< 1 m-sec.) and is not membrane permeable. Thus, it is unlikely that extra-cellular •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). H_2O_2 is the intermediate between superoxide ($\bullet O_2^-$) and hydroxyl radicals (•OH), and is the most stable form of ROS, with a half-life of several hours in seawater. H_2O_2 is membrane permeable, and reacts readily with oxidizable metals (e.g. Fe^{2+} in fish blood) or organic matters and produce •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 H_2O_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 H_2O_2 exposure experiment was similar to that described in the algal exposure experiment. The H_2O_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 μM H_2O_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 Labortechnik) 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 x ***g*** for 15 min at 4 °C to remove unbroken cells and intact nuclei, and the supernatant further

centrifuged at 10,000 ***g*** for 20 min to obtain the post-mitochondrial supernatants (PMS). The cytosolic fraction was isolated from the 10,000 x ***g*** supernatant by further centrifuging at 100,000 x ***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 2 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 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 µl of the suspension was then added to 450 µl 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 x ***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 x 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 *et al.* (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^+,K^+-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 & 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_2O_2 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_2O_2 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 (α) was set at 0.05 in all tests.

3. Results

3.1. ROS measurement in C. marina mass culture

The mean concentrations of H_2O_2 in low and high concentrations of *C. marina* were 9.69 ± 0.2 µM and 18.95 ± 1 µM 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^+,K^+-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 ($P \geq 0.05$) (Fig. 2F and Fig. 3F).

3.3. H_2O_2 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_2O_2 ($P \geq 0.05$) (Fig. 2A). CAT activities were significantly lower upon exposure to 0.5 mM H_2O_2 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_2O_2 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-D).

No significant change in LPO levels in the gills were found between high and low concentrations of *C. marina,*, high and low concentrations of $H_2O_{2,}$ and seawater

control ($P \geq 0.05$) (Fig. 2F). Likewise, no difference was found in Na^+,K^+-ATPase activities between *C. marina* treatments, the H_2O_2 treatments and seawater control ($P \geq 0.05$) (Fig. 2G).

SOD and CAT activities in erythrocytes upon exposure to H_2O_2 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-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_2O_2 treatment and the seawater control ($P \geq 0.05$) (Figs. 3C – E). Throughout the exposure period, no significant changes in erythrocyte LPO levels between both concentrations of *C. marina*, the H_2O_2 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, 1992b, 1994, 1995, 1997; Tanaka et al,. 1992, 1994; Tanaka & 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_2O_2 levels produced by sub-bloom (2,130 ± 167 cells/ml) and bloom concentrations (8,220 ± 259 cells/ml) of exponential phase *C. marina* were ~ 10 μM and ~ 20 μM, 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 (*Rhabdosargus 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 & Klekot (1985) observed a transitory increase in erythrocytes CAT activity in crucian carp (*Carassius carrassius*) 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 & 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 µg/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 & Gutteridge, 1989; Winston & Di Giulio, 1991). For example, increases of erythrocytes SOD, CAT and GPx activities in *Carassius carassius* L. were thought to be caused by H_2O_2 produced by 10 ppm paraquat exposure for 4 h. This exposure also led to an increase in LPO level (Gabryelak & 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 were carried out. Fish were exposed to a level of H_2O_2 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_2O_2 treatment. The fact that antioxidant enzymes in fish gills were enhanced by H_2O_2 treatment further confirmed that antioxidant responses in goldlined seabream (*Rhabdosargus 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_2O_2 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, 1992b, 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 casual relationship between fish mortality and ROS. Oda *et al.* (1992b) found that $O_2^{\cdot -}$ (1 - 6 nmol) and H_2O_2 (0.4 - 2 nmol) levels increased with increasing density of *C. marina* (from 0.5 x 10^4 to 2.5 x 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* (4,000 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* (2,000 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 (*Rhabdosargus 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 un-resolved. *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; Ahmed, 1995; Khan et al., 1995, 1996a; Haque & Onoue, 2002). The toxin profiles reported were, however, inconsistent between studies. Importantly, the 24-hour 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-hour LD_{50} of brevetoxin PbTx-2 produced by *Karenia brevis* (=*Gymnodinium breve)* at 10^9 cells/L was only 2 - 6 µg/L (Baden, 1983; Stuart & 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 2,000 to 6,000 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 & Matsuyama (1995) reported the production of 20:5, 18:4 and 16:0 PUFA by *C. antiqua* (4.8 x 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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Figure Legends

Figure 1 H_2O_2 levels (μM) in (A) high (8,000 cells/ml) and (B) low (2,000 cells/ml) concentrations of *C. marina, D. tertiolecta* and seawater control before and after adding fish. Data are expressed as mean ± S.E.M. (n = 4). Treatments with the same letter are not significantly different from one another in the Tukey test ($p \geq 0.001$).

Figure 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 (8,000 cells/ml) and low (2,000 cells/ml) concentrations of *C. marina*, *D. tertiolecta,* H_2O_2 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 ($p \geq 0.05$).

Figure 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 (8,000 cells/ml) and low (2,000 cells/ml) concentrations of *C. marina*, *D. tertiolecta*, H_2O_2 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 ($p \geq 0.05$).

Figure 1

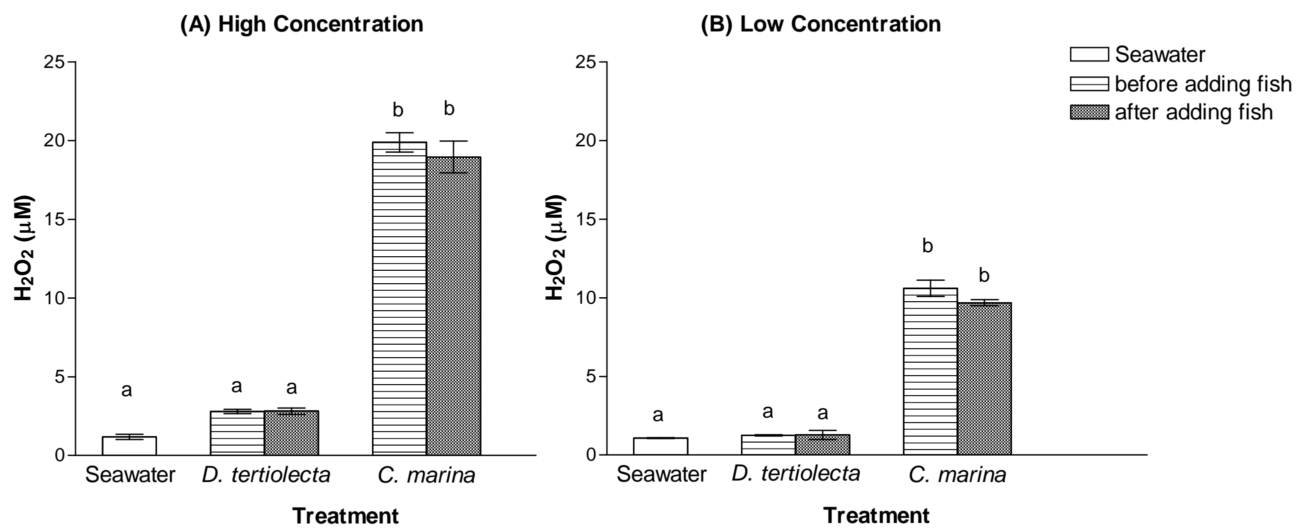

Figure 2

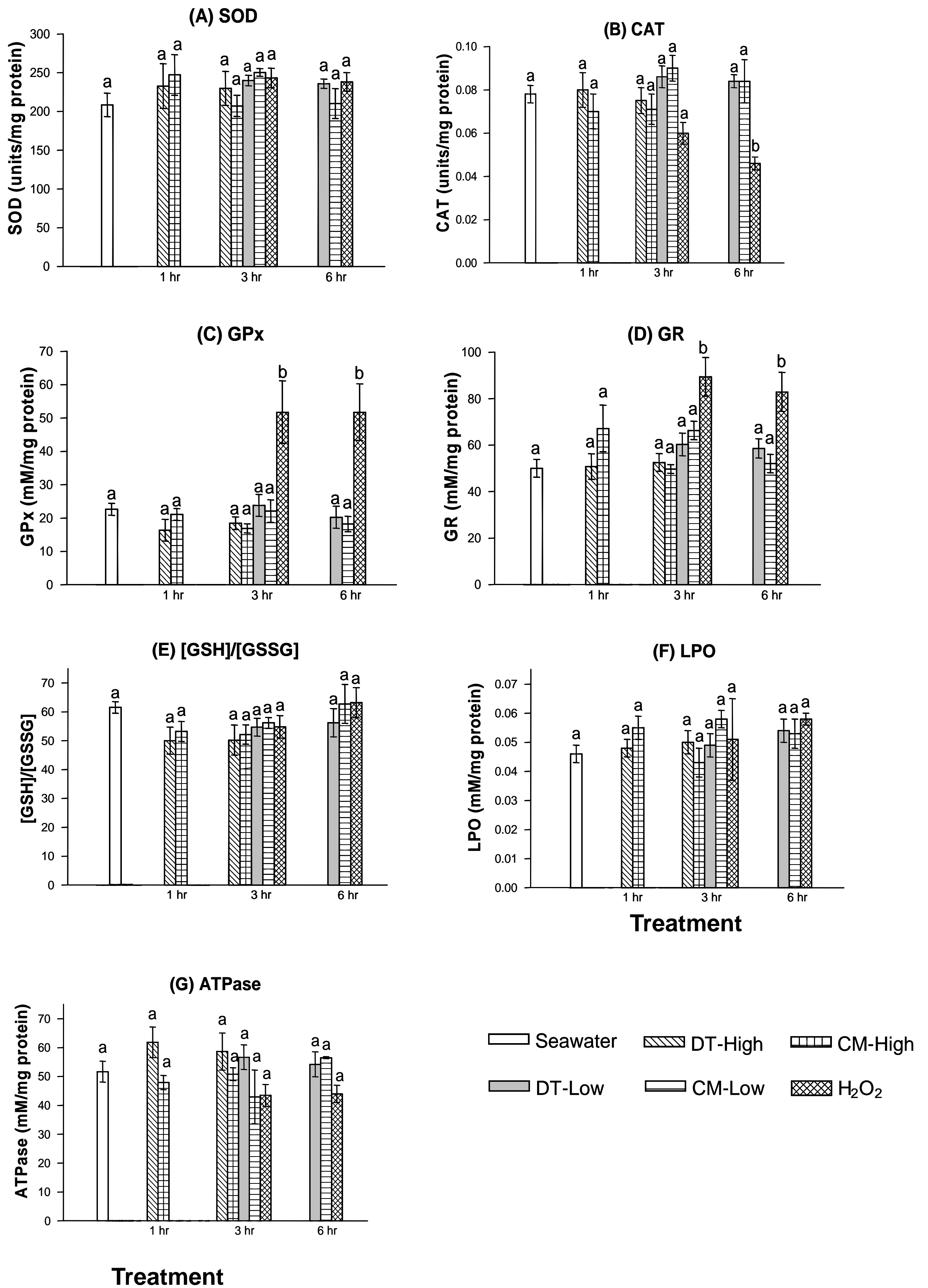

Figure 3