

Effects of *Cochlodinium polykrikoides* on Oxidative Status and Immune Parameters in the Marine Medaka *Oryzias javanicus*

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In this study, the marine medaka *Oryzias javanicus* was exposed to two concentrations of non-toxin-producing red tide dinoflagellate *C. polykrikoides* (1,000 and 2,000 cells ml⁻¹) for 96 h, and the time-course biochemical responses of antioxidant and immunity parameters were analyzed in the liver tissue. Significant ichthyotoxicity with increasing cell concentrations of *C. polykrikoides* and exposure period was observed for 96 h. Opercular respiratory rate was lowered in marine medaka exposed to 2,000 cells ml⁻¹ of *C. polykrikoides*. Intracellular malondialdehyde (MDA) content significantly elevated in response to both cell concentrations. In the case of glutathione (GSH) content, the levels were significantly elevated by 1,000 cells ml⁻¹ of *C. polykrikoides*, but the contents significantly depleted upon exposure to 2,000 cells ml⁻¹ of *C. polykrikoides*. Similarly, enzymatic activities of catalase (CAT) and superoxide dismutase (SOD) were increased by 1,000 cells ml⁻¹ of *C. polykrikoides*, whereas their activities were lowered by 2,000 cells ml⁻¹ of *C. polykrikoides*. Analysis of the two immunity parameters, alternative complement pathway and lysozyme, showed significantly lowered activities in 2,000 cells ml⁻¹ of *C. polykrikoides*-exposed liver tissue. These biochemical effects of *C. polykrikoides* on marine medaka would be helpful for understanding its acute effects in marine fish.

Keywords: *Cochlodinium polykrikoides*, Red tide, Marine medaka, Oxidative stress, Immunity

Introduction

Harmful algal blooms (HABs) are known as to be occurred through excessive growth of several microscopic algae or algae-like bacteria in aquatic ecosystems, although their blooms are natural phenomena (Landsberg, 2002). HABs are strongly associated with direct damage and/or their toxins to aquaculture and fisheries and are responsible for mass mortality of fish and shellfish (Hallegraeff, 2003). Detrimental effects of HABs on the economically important aquaculture and fisheries have been highlighted worldwide (Whyte et al., 2001; Kudela and Gobler, 2012). Dinoflagellates are abundant marine planktons with high diversity and are also crucial food sources in aquatic tropic levels (Shumway, 1990). HABs induced by dinoflagellates are often called red tides in marine ecosystem. In particular, several species of dinoflagellates can produce ichthyotoxic compounds even at low cell densities

and induce numerous harmful effects through bioaccumulation in different trophic levels including marine mammals and human (Landsberg, 2002; Hallegraeff, 2003; Mindy et al., 2010). Several physical and physiological damages were reported in fish species exposed to dinoflagellate. For example, direct physical contact of *C. polykrikoides* with gill tissues and epithelial hyperplasia was observed in fish (Gobler et al., 2008). Detrimental effects such as gill clogging, anoxia, convulsions, swelling and necrosis of the lamellar epithelium, osmoregulatory disruption, and/or excess mucus secretion have been observed in numerous fish species exposed to dinoflagellates (Kim et al., 1999; Chen and Chou, 2001; Cembella et al., 2002; Landsberg, 2002; Band-Schmidt et al., 2003; Shin et al., 2019).

The unarmored, chain-forming mixotrophic dinoflagellate *Cochlodinium polykrikoides* is one of the most HAB-forming species with a wide range of distribution in the world (Whyte et

al., 2001; Gárate-Lizárraga et al., 2004; Anton et al., 2008; Curtiss et al., 2008; Kudela et al., 2008; Tomas and Smayda, 2008). The genus *Cochlodinium* is known to be well adapted to environmental challenges, such as daily or seasonal fluctuations in temperature, salinity, irradiation, and nutrient (Kim et al., 2004; Gobler et al., 2008; Kudela et al., 2008; Mulholland et al., 2009). Apart from the toxins produced from *Cochlodinium* species, the mechanism of ichthyotoxicity of *C. polykrikoides* has been consistently substantiated. *C. polykrikoides* can cause gill injury, hyperventilation, and respiratory impairment through direct attachment on the gill tissues of fish (Shin et al., 2019). Several non-toxin-producing *Cochlodinium* species are known to generate reactive oxygen species (ROS) such as superoxide anions and hydrogen peroxide that would be responsible for direct or indirect ichthyotoxicity (Kim et al., 1999; Marshall et al., 2002; Kim et al., 2004; Liu et al., 2007). In addition, a cocktail of highly reactive ROS-like compounds, mucus-like substances or biologically active metabolites released from *Cochlodinium* species were suggested as additives to ichthyotoxicity (Kim et al., 2002; Tang and Gobler, 2009).

In general, fish undergo immunological fluctuations in response to environmental stressors. However, studies on HAB-triggered immunity response are scarce. Several immunity parameters such as measurement of enzymatic activity of complement and lysozyme have been used to reflect the health status and homeostasis of fish. The complement system has a crucial role in innate immunity with a wide range of immune recognition capabilities. Measurement of lysozyme activity has been consistently used for monitoring the potential impact of exogenous stress on innate immunity. To minimize the detrimental effects of *Cochlodinium* blooms and subsequent economic losses, it is crucial to understand the precise ichthyotoxicity mechanism, but there is limited knowledge on the actual effects of *C. polykrikoides* on fish liver, particularly the modulations of the antioxidant defense system and immunity.

In this study, we analyzed acute effects of *C. polykrikoides* on the liver tissue of the marine model fish, *Oryzias javanicus*. Although potential effects of *C. polykrikoides* have been reported in marine fish (e.g., red seabream), there is no information on the effects in small marine model fish. To estimate potential detrimental effects, biochemical assays for antioxidant defense system and innate immunity were performed with 1,000 and 2,000 cells ml⁻¹ of *C. polykrikoides* in this study. Intracellular contents of malondialdehyde (MDA) and glutathione (GSH) were monitored to confirm evidence for *C. polykrikoides*-mediated modulation in redox homeostasis. In addition, GSH-mediated antioxidant responses,

as well as two immunity parameters, alternative complement pathway (ACH₅₀) and lysozyme, were analyzed in the liver tissue after exposure to 1,000 and 2,000 cells ml⁻¹ of *C. polykrikoides*. Our findings can help to understand *C. polykrikoides* toxicity in marine fish by providing biochemical responses for the threats of HABs.

Material and Methods

1. Experimental animals

Animal handling and experimental procedures were approved by the Animal Welfare Ethical Committee and the Animal Experimental Ethics Committee of the Incheon National University (Incheon, South Korea). The marine medaka *Oryzias javanicus* used in this study were maintained in artificial seawater (TetraMarine Salt Pro, Cincinnati, OH, USA; 30 ± 0.5 practical salinity unit; 6.01 ± 0.76 mg O₂ l⁻¹) at 20 ± 1°C under a 14/10 h L/D cycle. They were fed frozen mosquito larvae and an artificial diet twice a day until satiation.

A laboratory culture of *C. polykrikoides* was established in our previous study (Shin et al., 2019). It was grown at 20°C in GSe medium dissolved with filtered seawater with a 14/10 h L/D cycle under a 120 μmol photons m⁻² s⁻¹ light source. Subculture was performed with an inoculation of approximately 50~60 cells ml⁻¹ into 10 l GSe medium before experiment. Cell number of *C. polykrikoides* was counted in a Leica DMLA microscope (Leica Microsystems, Wetzlar, Germany) equipped with UV epifluorescence and an AxioCam HRc camera (Zeiss, Göttingen, Germany) using a Sedgwick-Rafter counting chamber (VWR, Langenfeld, Germany). For exposure, *C. polykrikoides* was harvested between the exponential and early stationary phases to adjust final concentrations equivalent to approximately 10,000 *C. polykrikoides* cells ml⁻¹ before treatment.

2. Analysis of acute effects of *C. polykrikoides*

During the survival rate measurement, fish were not pooled and one fish was reared on one experimental tank. Additional three-day acclimation was conducted in the experimental tanks to diminish potential stress on marine medaka. To measure sublethal ranges, 30 marine medaka were individually exposed to control (GSe medium) 1,000, 2,000, 3,000, 4,000, and 5,000 cells ml⁻¹ of *C. polykrikoides* for 96 h. The fish were not fed during exposure. No marine medaka died for 96 h in the control group. Survival

rates were monitored at 0, 3, 6, 12, 24, 48, and 96 h in the control and exposed marine medaka. The opercular respiratory rate of 1,000 and 2,000 cells ml⁻¹-exposed marine medaka was monitored in a transparent chamber. Ten marine medaka in each concentration were moved into the chamber and the breath beats for each marine medaka were recorded for 5 min.

3. *C. polykrikoides* exposure and sample preparation

Sixty-three marine medaka per each concentration were distributed into three groups and exposed to each concentration of *C. polykrikoides*. Three marine medaka per each concentration were collected at 0, 3, 6, 12, 24, 48, and 96 h. To sample liver tissue, individuals were anesthetized by immersion in a solution of tricaine methanesulfonate (Sigma-Aldrich, Inc. St. Louis, MO, USA). Each marine medaka was dissected to obtain liver tissues. Three liver samples from each subgroup were pooled for analysis, and the three pooled specimens were analyzed in triplicate.

4. Biochemical analysis

Pooled liver tissues were homogenized in a cold buffer (20 mM Tris, 150 mM NaCl, 10 mM β-mercaptoethanol, 20 μM leupeptin, 2 μM aprotinin, and 100 μM benzamidine) and centrifuged for 30 min at 30,000 × *g* at 4°C. After heat-denaturation of the supernatants for 15 min at 75°C, thiobarbituric acid reactives (TBARs) were measured at 535 nm with a Thermo Varioskan Flash spectrophotometer (Thermo Fisher Scientific, Tewksbury, MA, USA) using malonaldehyde bis (MDA; tetramethoxypropan, Sigma-Aldrich, Inc.) as standard. The total content of lipid peroxidation compounds was calculated as nM of MDA per gram of liver tissue. Total soluble protein was determined with the Bradford method.

Intracellular GSH content in the pooled liver tissues was analyzed using a Glutathione Assay Kit (Catalog No. CS0260; Sigma-Aldrich, Inc.). The samples were washed with 0.9% NaCl and were homogenized in trichloroacetic acid with a Teflon homogenizer at a ratio of 1:20 (w/v). The homogenate was centrifuged at 3,000 × *g* for 10 min at 4°C and supernatant was collected for the GSH assay according to the manufacturer's protocol. The GSH content was measured at an absorbance of 420 nm with a spectrophotometer, and the standard curves were generated with GSH equivalents (0, 150, and 350 μM).

Enzymatic activities of CAT and SOD were analyzed with enzymatic methods using SOD Assay Kit (Catalog No. 19160; Sigma-

Aldrich Chemie, Switzerland) and Catalase Assay Kit (Catalog No. CAT100; Sigma-Aldrich, Inc.), respectively. The CAT and SOD activities were calculated at an absorbance of 440 nm or 520 nm at 25°C with a spectrophotometer, respectively.

The ACH₅₀ activity of *C. polykrikoides*-exposed marine medaka was analyzed using sheep red blood cells (SRBCs; 1.5×10⁶ cells, National Institute of Toxicological Research, South Korea) following a previous method (Nam et al., 2020). Ethylene glycol tetraacetic acid (EGTA)-Mg-gelatin-veronal buffer (GVB; pH 7.2), was prepared before the assay by mixing gelatin (0.1%), EGTA (10 mmol · l⁻¹), and MgCl₂ (10 mmol · l⁻¹) and 25 μL of 6% serum was incubated in a 96-well plate with 5 μl of SRBCs and 10 mM phenol red-free Hank's buffer. The plate was incubated for 90 min at 25°C and 1 ml of 10 mM EDTA was added to the solution to stop the hemolytic reaction. The optical density of the mixture was scanned at a wavelength of 414 nm on the spectrophotometer. The volume of serum complement triggering 50% lysis of SRBCs (ACH₅₀; U · ml⁻¹) was measured.

The lysozyme activity of *C. polykrikoides*-exposed marine medaka was measured using a turbidimetric assay in a 96-well plate (Ellis, 1990). A substrate prepared with 0.3 mg · ml⁻¹ lysozyme-sensitive bacterium *Micrococcus lysodeikticus* (ATCC No. 4698, Sigma-Aldrich) and 0.05 M sodium phosphate buffer (pH 6.2) was employed for analyzing lysozyme activity. Samples were examined in triplicate for statistical assurance. For each test, 10 μl of marine medaka sample and 200 μl of *M. lysodeikticus* suspension were mixed and incubated at room temperature. After 5 min of incubation, the absorbance of the mixture was read at a wavelength of 450 nm on the spectrophotometer.

5. Statistical analyses

All data were analyzed using the statistical software package SPSS (ver. 17.0, SPSS Inc., Chicago, IL, USA) and expressed as mean ± standard deviation (S.D.). A two-way analysis of variance (ANOVA) was employed to investigate the effect of *C. polykrikoides* on the results of antioxidant defense system and immunity. If significant differences were calculated by the ANOVA test, an additional Tukey test was used to confirm whether *C. polykrikoides* treatments and time periods were significantly different. Results with *p* < 0.05 were considered significant.

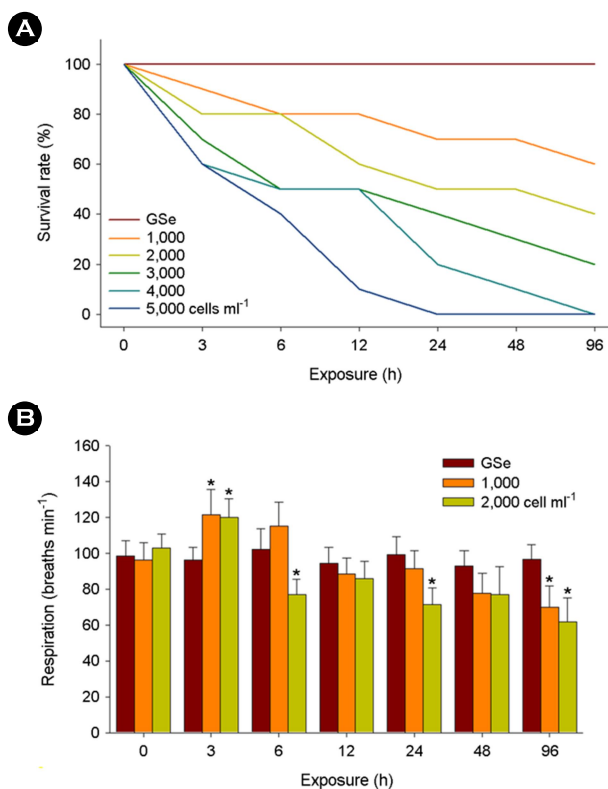


Fig. 1. Effects of different concentrations of *C. polykrikoides* on (A) survival rate (%) and (B) 5-min opercular respiration rate (breaths min⁻¹) of marine medaka for 96 h. Control was exposed only to GSe medium.

Results

1. Survival and respiration rate

For exposure of 1,000 and 2,000 cells ml⁻¹ of *C. polykrikoides*, no marine medaka died for 96 h in response to GSe medium, while concentration-dependent mortality was observed upon 1,000~5,000 cells ml⁻¹ of *C. polykrikoides* (Fig. 1A).

The opercular respiratory rates of marine medaka were significantly elevated at 3 h in both 1,000 and 2,000 cells ml⁻¹ of *C. polykrikoides* ($p < 0.05$), but the rates were significantly lowered at 6, 24, and 96 h in 2,000 cells ml⁻¹ of *C. polykrikoides*-exposed marine medaka ($p < 0.05$) (Fig. 1B). No significant modulation of the opercular respiratory rate was detected in response to GSe medium ($p > 0.05$).

2. Malondialdehyde and glutathione content

Significant increases in MDA content were detected in the liver

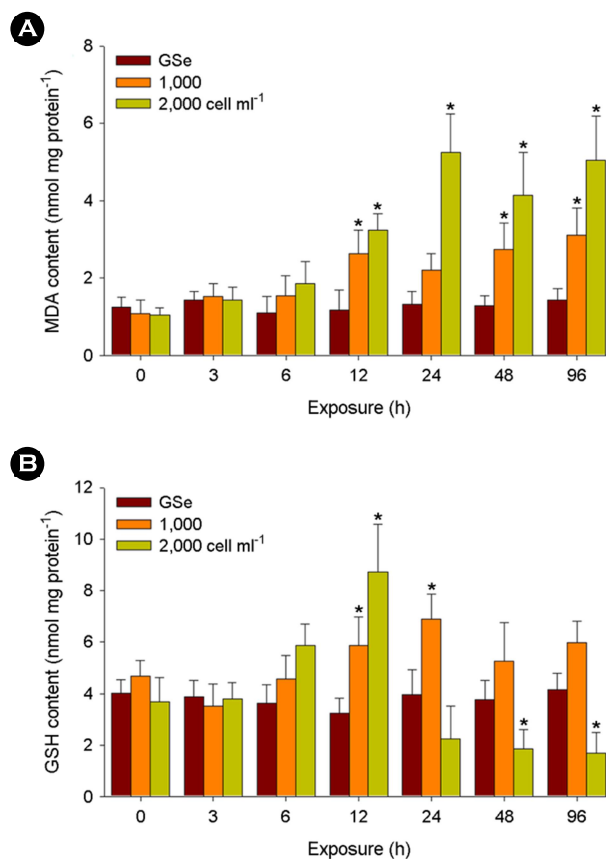


Fig. 2. Evaluation of (A) MDA and (B) GSH contents during 96 h exposure in the liver tissue of marine medaka exposed to different concentrations of *C. polykrikoides* (1,000 and 2,000 cells ml⁻¹). Control was exposed to GSe medium. Data are presented as the mean \pm standard deviation (S.D). Statistically significant values are marked with an asterisk ($p < 0.05$).

tissues exposed to 1,000 cells ml⁻¹ of *C. polykrikoides* at 12, 48, and 96 h and 2,000 cells ml⁻¹ at 12, 24, 48, and 96 h ($p < 0.05$) (Fig. 2A). No significant change was analyzed in response to GSe medium ($p > 0.05$).

In the case of GSH content, 1,000 cells ml⁻¹ of *C. polykrikoides* exposure resulted in its significantly higher levels at 12 and 24 h ($p < 0.05$), while the content significantly depleted after 48 h of exposure to 2,000 cells ml⁻¹ ($p < 0.05$) (Fig. 2B). No significant change was analyzed in response to GSe medium ($p > 0.05$).

3. Antioxidant defense parameter

In the liver tissue exposed to 1,000 cells ml⁻¹ of *C. polykrikoides*, CAT activity significantly elevated at 24, 48, and 96 h ($p < 0.05$), while the activity was significantly lowered by 2,000 cells ml⁻¹ at

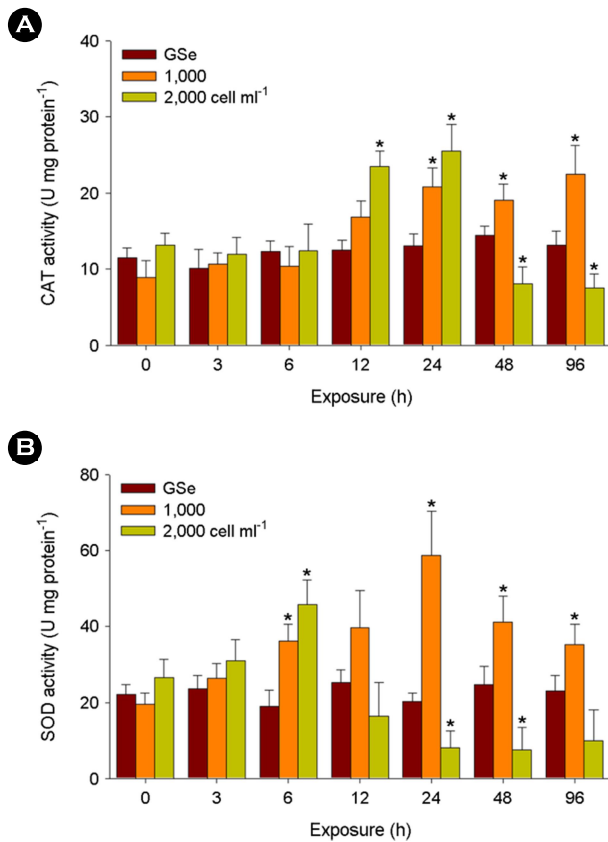


Fig. 3. Evaluation of (A) CAT and (B) SOD activities during 96 h exposure in the liver tissue of marine medaka exposed to different concentrations of *C. polykrikoides* (1,000 and 2,000 cells ml⁻¹). Control was exposed to GSe medium. Data are presented as the mean \pm standard deviation (S.D.). Statistically significant values are marked with an asterisk ($p < 0.05$).

48 and 96 h after significantly higher levels measured at 12 and 24 h ($p < 0.05$) (Fig. 3A). No significant change was analyzed in response to GSe medium ($p > 0.05$).

When exposed to 1,000 cells ml⁻¹ of *C. polykrikoides*, significantly higher activity of the SOD enzyme was detected at 6, 24, 48, and 96 h ($p < 0.05$) (Fig. 4A). In addition, a significantly higher level of SOD activity was measured in the 2,000 cells ml⁻¹ of *C. polykrikoides*-exposed liver tissue at 6 h ($p < 0.05$). The activity of SOD enzyme significantly lowered with exposure to 2,000 cells ml⁻¹ of *C. polykrikoides* at 24 and 48 h ($p < 0.05$). No significant change was analyzed in response to GSe medium ($p > 0.05$).

4. Immune parameter

Significant decreases in ACH₅₀ activity were observed in the liver

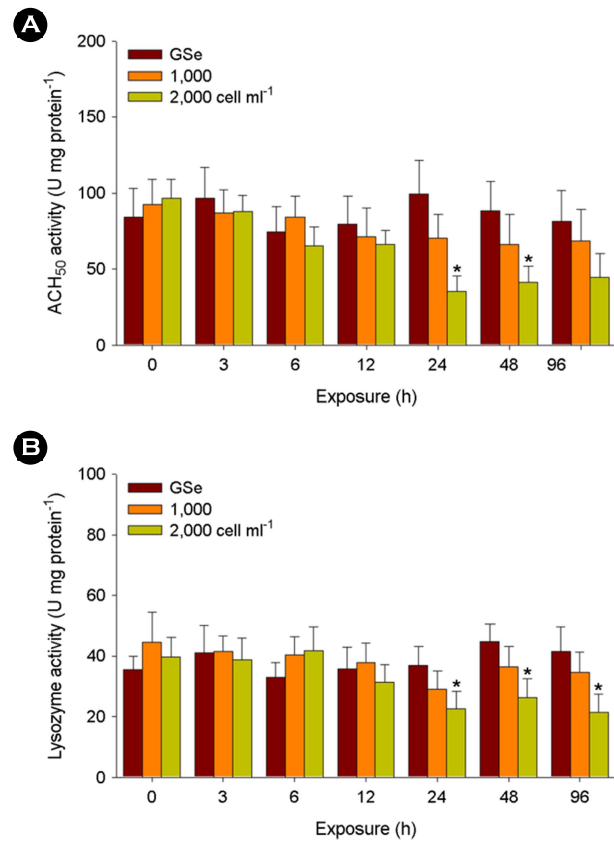


Fig. 4. Evaluation of (A) ACH₅₀ and (B) lysozyme activities during 96 h exposure in the liver tissue of marine medaka exposed to different concentrations of *C. polykrikoides* (1,000 and 2,000 cells ml⁻¹). Control was exposed to GSe medium. Data are presented as the mean \pm standard deviation (S.D.). Statistically significant values are marked with an asterisk ($p < 0.05$).

tissues exposed to 2,000 cells ml⁻¹ of *C. polykrikoides* at 24 and 48 h ($p < 0.05$) (Fig. 4A). No significant change was analyzed in response to GSe medium and 1,000 cells ml⁻¹ of *C. polykrikoides* ($p > 0.05$).

Similarly, 2,000 cells ml⁻¹ of *C. polykrikoides* exposure resulted in its significantly lower levels at 24, 48, and 96 h ($p < 0.05$), while no significant change was analyzed in response to GSe medium and 1,000 cells ml⁻¹ of *C. polykrikoides* ($p > 0.05$) (Fig. 4B).

Discussion

The result of survival rate revealed that the cell concentration and exposure period of *C. polykrikoides* was critical for the ichthyotoxicity in marine medaka. Similar concentration ranges of *C. polykrikoides* responsible for mortality were reported in multiple

fish species (Gobler et al., 2008; Shin et al., 2019; Haque et al., 2021). In marine medaka, *C. polykrikoides* significantly modulated their respiration rates in a short time course. Thus, mortality observed in marine medaka would be associated with alteration of the gill epithelium structure, disruption of ionic homeostasis, and suffocation through *C. polykrikoides* attachment. Thus, the increased mortality would be resulted from failure in gill function and respiration through direct ingestion and attachment/aggregation of *C. polykrikoides*, as we used a non-toxin-producing species. Detrimental effects on gill tissues of fish is critical for mortality, as gills are a crucial organ for absorption and transport of oxygen into whole body.

To date, negative physical and physiological damages induced by *C. polykrikoides* have been investigated in fish (Gobler et al., 2008), but limited information is available on molecular and biochemical effects of *C. polykrikoides* exposure. Treatment to *C. polykrikoides* significantly elevated MDA contents and the oxidative stress parameter, GSH content in marine medaka. Malondialdehyde is one of the byproducts of lipid peroxidation. Subsequently, the measurement of intracellular MDA content has been widely employed as a promising biomarker for assessing oxidative stress in fish (Lushchak, 2011). Therefore, increased MDA content observed in marine medaka indicated that treatment to *C. polykrikoides* may induce production of oxidative stressors (e.g., free radicals or pro-oxidant molecules) that can further trigger an induction of lipid peroxidation. In the flatfish *Paralichthys olivaceus* exposed to *C. polykrikoides*, concentration-dependent increase in lipid peroxidation was observed (Kim et al., 1999).

Glutathione is an important non-enzymatic antioxidant as a crucial protector against cellular redox-cycling fluctuations by exogenous oxidative stressors (Lushchak and Bagnyukova, 2006). The significant increase of GSH levels observed in marine medaka suggests a synthesis of new GSH that may be needed to maintain its required levels for the increase of the resistance capacity against oxidative stress as a free radical scavenger. However, prolonged depletion of GSH content can lead to vulnerability to oxidative stress and lethal damage (Townsend et al., 2003). The strongly modulated respiration rate would be considered a factor for intracellular oxidative stress, as low oxygen-triggered lipid peroxidation and subsequent oxidative stress has been well-characterized in fish (Lushchak and Bagnyukova, 2006).

The two antioxidant parameters, CAT and SOD enzymes are the first line of antioxidant defense upon oxidative stress. In detail, SOD converts two superoxide radicals ($O_2^{\cdot-}$) into hydrogen peroxide (H_2O_2) and CAT decomposes H_2O_2 into water (H_2O) and molecular

oxygen (O_2), resulting in the diminishment of the detrimental effects of free radicals (Lesser, 2006; Lushchak, 2011). Thus, increase enzymatic activities of CAT and SOD observed in marine medaka suggest stress response and adaptive metabolism against oxidative stress. In the gill cell line RTgill-W1 exposed to the raphidophyte *Chattonella marina*, significantly increased SOD enzyme activity was observed (Dorantes-Aranda et al., 2015). However, their parallel reduction of CAT and SOD enzymes may be explained as failure in regulating oxidative homeostasis.

The immune parameters, ACH_{50} and lysozyme play a crucial role in innate immunity, immunosuppression, and homeostasis in fish (Boshra and Sunyer, 2006; Saurabh and Sahoo, 2008). The significant decreases in enzymatic activities of ACH_{50} and lysozyme measured in marine medaka represent *C. polykrikoides*-triggered immune suppression. Since the liver is the important organ that synthesizes complement components (Boshra and Sunyer, 2006), the significantly decreased ACH_{50} activity implies a potential interruption in liver function. In fish, macrophages, neutrophils, and monocytes release lysozymes that directly lyse or degrade pathogen cell walls (Magnadóttir, 2006). Previously, the dinoflagellate *Alexandrium affine* (6,000 and 7,000 cells ml^{-1}) induced immunosuppression by significant decreases in lysozyme activity and total Ig level in the gill and liver tissues of red seabream, *Pagrus major* (Haque et al., 2021). Therefore, the decreased activity of lysozyme represents significant immune suppression and potentially higher vulnerability of the marine medaka to diseases and exogenous pathogens and may allow unlimited inflammation.

In summary, all the parameters of the antioxidant defense system and immunity analyzed in the liver tissue support oxidative stress induction and immunosuppression by *C. polykrikoides* in marine medaka. Our results also suggest that even environmental concentrations of HABs can inhibit respiration rate and may induce mass mortality in marine fish. Detection of early biological signals of aquatic animals in response to HABs will be useful to predict the occurrence of HABs and establish prevention plans in aquaculture and fisheries.

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Conflict of Interest

The authors declare that they have no conflicts of interest.

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