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Effects of the algicides $CuSO_4$ and NaOCl on various physiological parameters in the harmful dinoflagellate *Cochlodinium polykrikoides*

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Abstract The marine dinoflagellate *Cochlodinium polykrikoides* has spread worldwide and is responsible for harmful algal blooms. The chemical biocides, copper sulfate ($CuSO_4$) and sodium hypochlorite (NaOCl), are known to be effective in removing bloom-forming or biofouling organisms. Here, we assessed the biocidal efficiency and toxicological properties of NaOCl and $CuSO_4$ on the physiological and catalase responses of *C. polykrikoides*. The endpoints used were cell counts, pigment content, chlorophyll autofluorescence (CAF), and antioxidant catalase (CAT) activity. The test organism showed a dose-dependent decrease in growth rate against the algicides; 72-h median effective concentrations (EC_{50}) were 0.584 and 0.633 mg L^{-1} for NaOCl and $CuSO_4$, respectively. The decrease in pigment levels and CAF intensity showed that NaOCl and $CuSO_4$ might affect the photosynthetic processes of the exposed cells. Furthermore, a considerable increase in CAT activity in the cells was detected, indicating that the algicides might generate reactive oxygen species, thereby markedly damaging the cells. These results suggest that the test algicides are very effective in removing *C. polykrikoides* by inducing cellular stress and inhibiting cell recovery at higher concentrations.

Keywords Algicide · *Cochlodinium polykrikoides* · Copper sulfate · Harmful algal bloom · Sodium hypochlorite

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Introduction

Harmful algal blooms (HABs) are significant and pose a growing major threat to public and ecosystem health, affecting fisheries and creating associated economic loss in developed and developing countries (Anderson 2007). *Cochlodinium polykrikoides* Margalef is a harmful dinoflagellate that is widely distributed in tropical and warm temperate waters throughout the world (Richlen et al. 2010). The species causes fish kills by producing massive amounts of mucous and depleting oxygen supplies (Kim et al. 2000). Consequently, blooms of this species lead to severe economic losses and environmental impacts, particularly in countries such as South Korea and Japan (Kim et al. 2004; Lee 2008). The causative organism spreads through oceanic regions and has recently expanded its geographic range to include Europe, India, Middle Eastern countries, and North America (Kudela and Gobler 2012). Several physical, chemical, and biological mitigation measures have been developed and used by several countries for controlling HABs (Kim 2006). Some chemicals (e.g., copper sulfate ($CuSO_4$), hydrogen peroxide (H_2O_2), magnesium hydroxide, ozone, and sodium hypochlorite (NaOCl)) with algicidal properties have been used for the control and removal of HABs (Kim et al. 2000; Sengo and Anderson 2004; Qian et al. 2010).

Because they are very cost effective, both $CuSO_4$ and NaOCl have long been used as biocides in the freshwater industry (Haas 1999; USEPA 2009). These chemicals can be used for cleaning swimming pools, on aquaculture farms, and even for removing HABs (Kim et al. 2007). In particular, NaOCl is a strong oxidizing biocide (Hilgren et al. 2007) and reacts with water to form hypochlorous acid (HOCl) (White 1999), which is a strong reactive oxygen species (ROS) (Halliwell 2006) that leads to damage in the physiological and biochemical activities of exposed organisms (Phe et al. 2005), whereas $CuSO_4$ is a non-oxidizing biocide

(Gant et al. 2007) that releases Cu(II) ions when dissolved in water. Although copper is considered an essential micronutrient for algal growth, high concentrations can lead to denaturation of nucleic acids, alteration of proteins, and permeabilization of the cell membrane (Verhoeven and Eloff 1979). Moreover, free and excess copper is very toxic because it generates ROS in the cells (Cervantes and Gutierrez-Corona 1994).

Even under optimal conditions, ROS such as superoxide radical (O_2^-), H_2O_2, hydroxyl radical (OH^-), and HOCl are generated as by-products of normal metabolism in different subcellular compartments, including chloroplasts, mitochondria, and plasma membranes that are linked to the electron transport systems (Asada 1999; Gómez et al. 2004; Trachootham et al. 2008). However, biotic or abiotic stress might produce an excessive concentration of ROS, resulting in oxidative damage in cells (Lushchak 2011). To mitigate and repair the damage initiated by ROS most organisms, including plants and algae, have developed complex antioxidant systems, such as low molecular weight compounds and antioxidant enzymes (Ogawa 2005). Catalase (CAT), a tetrameric heme-containing enzyme, can catalyze the breakdown of H_2O_2 to water and molecular oxygen. CAT, along with superoxide dismutase (SOD), represents the first line of defense against free radicals (Radocanović et al. 2010). Considering that algicides induce severe cellular stress to generate high amounts of ROS, the evaluation of antioxidant enzyme activity might provide insights into the mode of action of the biocide chemicals (Trivedi et al. 2012). Moreover, biochemical markers are considered to be sensitive indicators of environmental and subcellular stress under both laboratory and field conditions (Hinton and Lauren 1990).

The effects of potential algicides on algal growth have been analyzed based on measuring the inhibition of algal growth, pigment content, and photosynthetic rate (Gregg and Hallegraef 2007; Song et al. 2010; Viriyatum 2013); however, the possible modes of action of biocides and biochemical responses of the target organisms have not been clearly explained. In this study, we evaluated the efficiency of the oxidizing biocide NaOCl and non-oxidizing biocide $CuSO_4$ in controlling HABs by targeting the harmful dinoflagellate *C. polykrikoides*, with emphasis on changes in the physiological and antioxidant enzyme CAT activities caused by both the algicides.

Materials and methods

Cell culture, growth conditions and maintenance

Cochlodinium polykrikoides (CP-01) was obtained from the National Fisheries Research and Development Institute (NFRDI) of Korea, cultured in f/2 medium (Guillard and Ryther 1962), and maintained at 20 °C using a 12:12-h light/dark cycle with a photon flux density of 65 μmol photons m^{-2} s^{-1}.

Preparation and treatment of the algicide

NaOCl was commercially obtained (Cat. No. 425044, Sigma-Aldrich Co., USA), and a stock solution (1,000 mg L^{-1}) was prepared using autoclaved distilled water (ADW). The chlorine (Cl) concentrations in NaOCl were determined spectrophotometrically using diethyl-phenylenediamine (DPD) at 515 nm (APHA 1998). Similarly, $CuSO_4$ (Cat. No. C1297, Sigma, USA) was commercially obtained, and a stock solution (1,000 mg L^{-1}) was prepared using ADW. Copper concentrations from the dissolved $CuSO_4$ were analyzed using a NexION 300X ICP-MS (PerkinElmer, USA).

For the algicide assay, we used exponential growth phase cultures (200 mL) with an initial cell density of 1,300±0.5× 10^4 cells mL^{-1} and individually treated with NaOCl (0.1, 0.5, 1.0, 2.0, and 3.0 mg L^{-1}) and $CuSO_4$ (0.5, 1.0, 2.0, 3.0, and 5.0 mg L^{-1}) at nominal doses and considering the demand of the medium, as determined previously. Control cultures were maintained as per OECD guidelines (OECD 2011). Test doses also considered the concentrations observed in environmental discharges elsewhere (Calderon 2000; Watson and Yanong 2006). Samples were drawn after 0, 6, 12, 24 and 72 h of exposure to the algicides. The cultures were harvested and washed with filtered sterilized seawater prior to the assay.

Cell count and median effective concentration

Cell counts in each test flask were determined using a plankton-counting chamber (HMA-S6117, Matsunami Glass, Japan) and were plotted against the exposure times.

Percent inhibition (or survival percentage) and 72-h median effective concentration (EC_{50}) were calculated, following the recommendation by the Organisation for Economic Co-operation and Development testing guidelines (OECD 2011). Percent inhibition was calculated based on the following equation:

$$\%I = (\mu C - \mu T/\mu C) \times 100$$

where $\%I$ = percent inhibition in average specific growth rate, μC = mean value for μ in the control, and μT = mean value for growth rate in the treated samples.

The 72-h EC_{50} values were estimated using a sigmoidal dose–response curve and plotted using Origin version 8.5 (MicroCal Software Inc., USA) based on the sigmoidal four-parameter equation (Teisseyre and Mozrzymas 2006):

$$\log EC_{50} = a + (b-a)\left[1 + 10^{(x-c)d}\right]^{-1},$$

where *a* is the response value at 0 or minimum asymptote, *b* is the response value for infinite concentration or maximum asymptote, *c* is the midrange point, *d* is the steepness of the curve or the Hill slope, and *x* is the dilution coefficient.

Analysis of pigment and chlorophyll autofluorescence

Chlorophyll *a* (Chl *a*) and carotenoid (CAR) were measured by concentrating 10 mL of the culture at different time intervals. The pigments were extracted with 90 % acetone after overnight incubation in the dark. The supernatants extracted were measured using a DU730 Life Science UV/vis spectrophotometer (Beckman Coulter, Inc., USA). The Chl *a* and CAR concentrations were estimated according to Parsons et al. (1984).

Chlorophyll autofluorescence (CAF) was measured using a fluorescent microscope (Axioskop, Carl Zeiss, Germany) at ×400 magnification. An ultraviolet dichroic (G365/395–488 nm) source was used for excitation, and emission was collected by setting the detection bandwidth between 630 and 750 nm. Digital image analysis was performed using ImageJ 1.29× (National Institutes of Health (NIH), USA). Mean fluorescence intensity (MFI) was expressed in terms of pixel gray value, ranging from 0 to 270. The reported MFI values indicate the average MFI values obtained from a minimum of 50 individual cells.

Analysis of catalase activity

CAT activity was measured according to Aebi (1984), which was based on H_2O_2 degradation by CAT in the samples. Five milliliters of the algal culture was centrifuged at 4,200 rpm for 10 min. Two milliliters of extraction buffer (1.0 M phosphate buffer) was added to the pellet. The cells were homogenized using a Teflon pestle (BelArt F19922-0001, Scienceware, USA) in ice, and then the tube was placed in a water bath at 40 °C for 5 min (modified from Soto et al. 2011) for extraction. The homogenate was centrifuged at 4,200 rpm for 10 min, and the supernatant was collected for the assay.

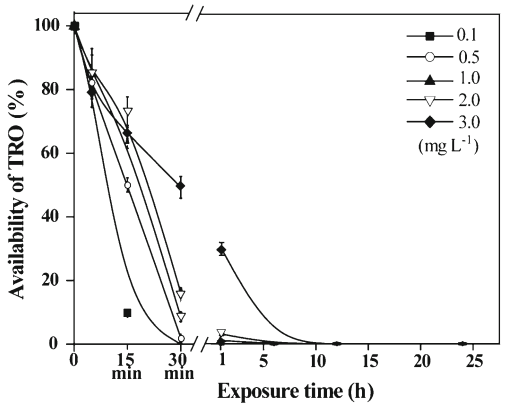

Fig. 1 Percentage availability of total residual oxidants (*TRO*) in f/2 medium at different time intervals. Below detection limit (<0.01 mg L^{-1})

Table 1 Copper concentrations detected in f/2 medium and copper-spiked *Cochlodinium* cultures

Spiked nominal Cu^{2+} dose (mg L^{-1})	Measured Cu^{2+} (mg L^{-1})
0.0 (f/2 medium)	BDL
0.1 (*Cochlodinium* culture)	0.0806
1.0 (*Cochlodinium* culture)	0.8965
5.0 (*Cochlodinium* culture)	3.7120

BDL below detection limit (<0.01 mg L^{-1})

Added to 100 μL of the supernatant were 1.6-mL 1.0 M phosphate buffer, 0.2-mL 0.3 % H_2O_2, and 3 mM EDTA, and the mixture was shaken well for 3 min. CAT activity was calculated using an extinction coefficient of 0.036 per millimolar per centimeter and calculated per cell. One unit of the enzyme was considered as the amount necessary to decompose 1.0 μL of H_2O_2 per minute at 25 °C. The absorbance of the supernatant was read at 240 nm in a DU730 Life Science UV/Vis spectrophotometer.

Statistical analysis

All data presented are mean values of triplicates. One-way analysis of variance (ANOVA) with post hoc Dunnett's

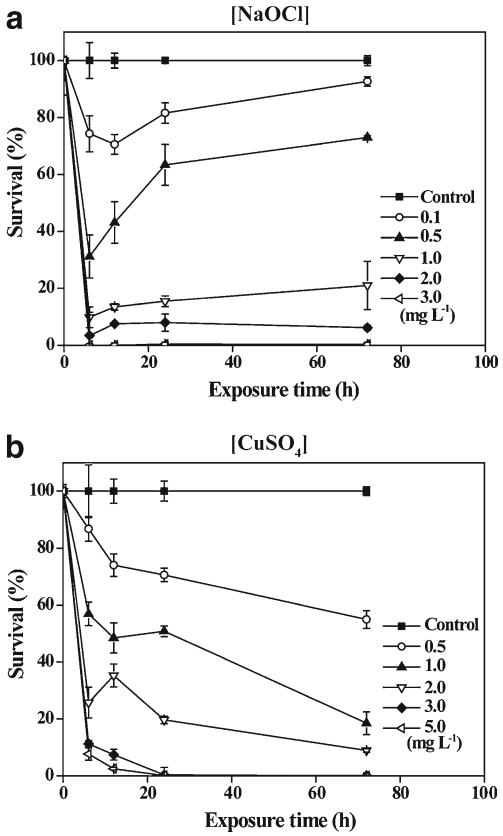

Fig. 2 Survival of *C. polykrikoides* after exposure to algicides: **a** NaOCl and **b** $CuSO_4$

multiple comparison test using Graphpad InStat (Graphpad Software, Inc., USA) was used for comparisons between non-treated and treated cultures. $P<0.001$ was accepted as significant. The correlation between MFI and Chl *a* was tested using Pearson's correlation coefficient (R^2) and an Excel spreadsheet (Microsoft, USA).

Results and discussion

Stability of NaOCl and $CuSO_4$

The concentration of NaOCl measured immediately after adding it to the algal suspension was 15–20 % less than the nominal doses (0.1, 0.5, 1.0, 2.0, and 3.0 mg L^{-1}; Fig. 1). In addition, after 60 min of exposure, the available total residual oxidants (TROs) were from ~10 to 15 % for 2.0 and 3.0 mg L^{-1}, respectively, whereas they were below detectable limit (BDL; >0.01 mg L^{-1}) at lower concentrations. A possible reason is that NaOCl is highly reactive and volatile; it reacts with the inorganic and organic substances present in the medium (Jegatheesan et al. 2009). Therefore, the TRO level or free (chlorine) Cl_2 concentration is usually BDL after 1–3 h of exposure (Abdul-Baki 1974). However, Cl_2 damages cell membranes immediately (within 15 min) after addition, making the cell unable to regrow (Phe et al. 2005; Patil and Jagadeesan 2011). These merits (i.e., acute effects with little residues left in water) allow the use of Cl_2 as a potential algicide for the control of HAB in the environment.

$CuSO_4$ is a non-oxidizing biocide that forms Cu(II) ions, which in turn, form several complexes with the organic and inorganic compounds present in the medium. In addition, $CuSO_4$ is highly soluble in water and is strongly bioaccumulative in cells (García-Villada et al. 2004). In the present study, the measured concentrations of Cu(II) did not show much change from the nominal concentrations (Table 1), suggesting that it persists in the environment (Raman and Cook 1990).

Biocide efficiency and toxic effects

The individual biocidal efficiency of NaOCl and $CuSO_4$ against *C. polykrikoides* was quantified using percent survivability with the increase in NaOCl and $CuSO_4$ doses (Fig. 2). Percent reduction in cell counts was significant ($P<0.001$) over all time intervals for each algicide exposure. Similarly, Chl *a* and carotenoid levels decreased significantly ($P<0.001$) after 6- and 72-h exposures (Fig. 3). Hence, the results clearly

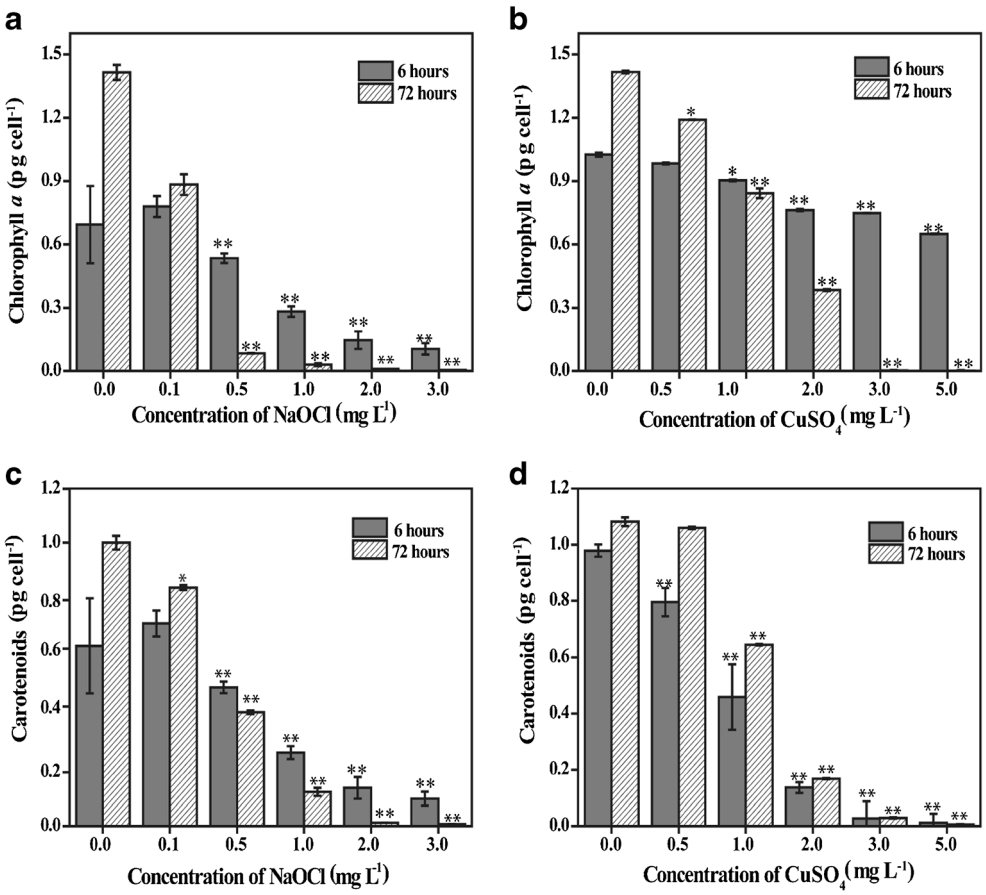

Fig 3 Variation in chlorophyll *a* (**a**, **b**) and carotenoid (**c**, **d**) levels of *C. polykrikoides* after 6 and 72 h of exposure to algicides NaOCl and $CuSO_4$. Significant differences as determined by Dunnett's multiple comparison test are represented as $*P<0.05$, $**P<0.001$ level compared to the control

show that NaOCl and $CuSO_4$ have detrimental, biocidal effects on the exposed *C. polykrikoides* cells.

As for numerical comparisons, 72-h EC_{50} was calculated using growth rates, which were 0.584 mg L^{-1} for NaOCl and 0.633 mg L^{-1} for $CuSO_4$. In addition, the minimal effective concentration was estimated by determining EC_5, EC_{10}, and EC_{20} values (Table 2), which represent the minimal algicide concentration that evokes an effect on the test species. The estimates show that *C. polykrikoides* was highly sensitive to exposure to algicidal agents. The present data are in accordance with those detected from the dinoflagellate *Prorocentrum minimum* (Guo and Ki 2012; Ebenezer and Ki 2013). Previous reports and our findings suggest that the two tested algicides are highly efficient in controlling dinoflagellate bloom (Han et al. 2001; Jeong et al. 2002; Oliveira-Filho et al. 2004).

Effect of algicides on CAF levels

Microalgae exhibit autofluorescence properties resulting from the presence of photosynthetic pigments (Trampe et al. 2011); therefore, measuring CAF has been reported to be a rapid, sensitive method by which the physiological status of microalgae can be assessed (Nancharaiah et al. 2007). In the present study, CAF as MFI per cell decreased significantly ($P<0.05$) with the increase in exposure time and algicide dose (Fig. 4). A positive correlation was observed between MFI and Chl *a* levels for both NaOCl ($R^2=0.964$, $P<0.01$) and $CuSO_4$ ($R^2=0.935$, $P<0.01$), respectively. After 6- and 12-h exposures, the MFI for 1.0, 2.0, and 3.0 mg L^{-1} NaOCl decreased significantly ($P<0.05$) by approximately 25–46 % compared to that for the control; however, no significant decrease was observed for low doses (0.1 and 0.5 mg L^{-1}). Subsequently, after 24- and 72-h exposures, the percent reduction at 0.1 and 0.5 mg L^{-1} NaOCl was between 28 and 60 % compared to that for the control (Fig. 4), and no fluorescence was observed for the high doses (1.0–3.0 mg L^{-1}).

As for $CuSO_4$, after 6- and 12-h exposures, the MFI for 2.0, 3.0, and 5.0 mg L^{-1} showed a significant decrease ($P<0.05$) by approximately 10–34 % compared to that for the control (Fig. 4), although the low doses (0.5 and 1.0 mg L^{-1}) showed no significant decrease. However, after 24 and 72 h of exposure, the percent reduction for 0.5 and 1.0 mg L^{-1} $CuSO_4$ was between 35 and 67 % of that of the control. A previous study by Ebenezer and Ki (2013) showed that the oxidative biocide

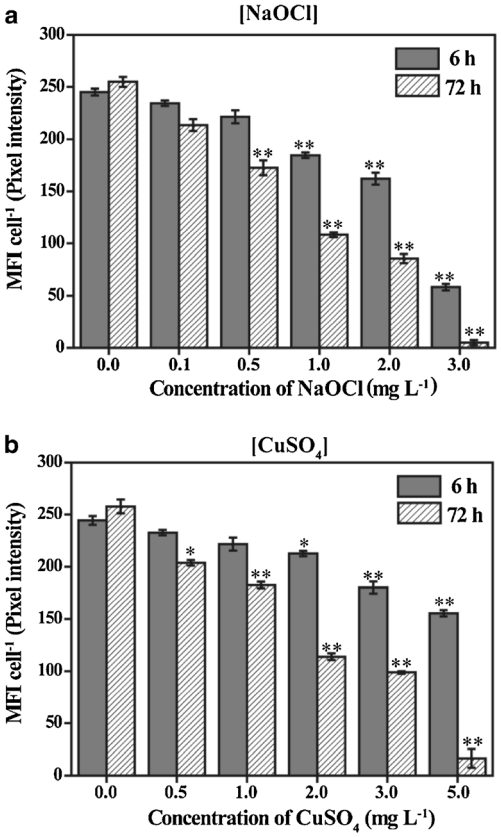

Fig. 4 Variation in MFI of *C. polykrikoides* after 6 and 72 h of exposure to algicides NaOCl (**a**) and $CuSO_4$ (**b**). *Error bars* represent ± SD; $n \geq 50$. Significant differences as determined by Dunnett's multiple comparison test are represented as $^*P<0.05$, $^{**}P<0.001$ level when compared to the control

Cl_2, for which the test doses ranged from 1.0 to 3.0 mg L^{-1}, showed a marked reduction of MFI of *P. minimum*. In addition, Ma et al. (2011) reported that 0.2 mg L^{-1} of Cl_2 completely suppressed the photosynthetic activity of the diatom *Phaeodactylum tricornutum*. Moreover, $CuSO_4$ showed a significant reduction of CAF in the freshwater microalgae *Pseudokirchneriella subcapitata* and *Chlorella* sp. at 0.05–0.25 mg L^{-1}, after a 72-h exposure (Stauber et al. 2002). The bleaching property of NaOCl can affect pigments, especially carotene and proteins related to it (Albrich et al. 1981), whereas $CuSO_4$ interferes with carbon fixation, affecting the photosynthetic apparatus (Stiborová et al. 1986). NaOCl and $CuSO_4$ have been widely used as algicides; however, the mode of toxicity of each chemical is different. Nevertheless, NaOCl and $CuSO_4$ might affect the photosynthetic efficiency of the test dinoflagellates, most

Table 2 The effective concentrations (mg L^{-1}) after 72 h of exposure to algicides of *C. polykrikoides*

Chemicals	EC_5	95 % confidence interval	EC_{10}	95 % confidence interval	EC_{20}	95 % confidence interval	EC_{50}	95 % confidence interval
NaOCl	0.07	0.05–0.08	0.14	0.11–0.16	0.31	0.28–0.33	0.58	0.55–0.62
$CuSO_4$	0.09	0.06–0.11	0.20	0.17–0.21	0.49	0.46–0.52	0.66	0.63–0.68

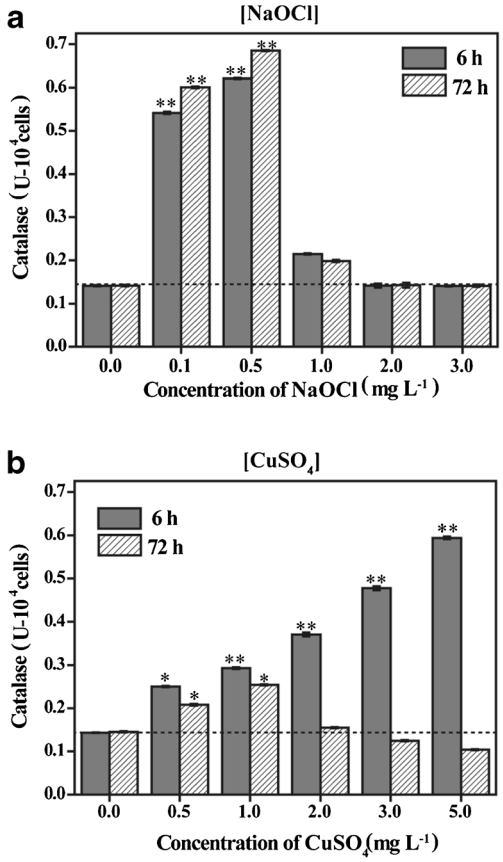

Fig. 5 Variation in catalase activity in *C. polykrikoides* after 6 and 72 h of exposure to algicides NaOCl (**a**) and $CuSO_4$ (**b**). Significant differences as determined by Dunnett's multiple comparison test are represented as **P*<0.05, ***P*<0.001 level when compared to the control

likely damaging their photosystems (Oukarroum et al. 2012; Ebenezer and Ki 2013).

Effect of algicides on antioxidant CAT activity

In general, the amount of ROS generated is equal to the amount eliminated in robust cells; however, when the cell undergoes oxidative stress, the ROS concentration within the cell is more than the amount eliminated, which leads to the destruction of cellular mechanisms (Lushchak 2011). The algicides NaOCl and $CuSO_4$ are known to induce ROS production in chemical-exposed cells (Letelier et al. 2005; Halliwell 2006). The deleterious effects of free radicals produced because of ROS generation can be controlled by specific antioxidant systems (Lushchak 2011). CAT is an antioxidant enzyme that decomposes H_2O_2 to protect cells from ROS damage (Radocanović et al. 2010). In the present study, considerable variations in CAT activity were observed depending on algicide dose and exposure time (Fig. 5). For cells exposed to NaOCl, the CAT activity increased significantly ($P<0.001$) by up to seven times at lower doses (0.1 and 0.5 mg L^{-1}) compared to that in the control. An increased activity after 6 h of exposure to NaOCl was observed, but the activity remained stable even after 72 h of exposure. Moreover, higher doses (1.0–3.0 mg L^{-1}) did not show any significant change in CAT activity compared to that for the control. A similar trend was observed in SOD and reduced glutathione (GSH) activities (unpublished data). The increased CAT activity at lower doses could be due to the enzyme's involvement in degrading ROS; however, at higher doses, increased ROS concentrations could lead to inactivation of antioxidant enzymes (Shao et al. 2008). For this possible reason, the higher doses in the present study might have inactivated the CAT activity. Previous reports also state that low levels (0.05–0.13 mg L^{-1} NaOCl) induce CAT

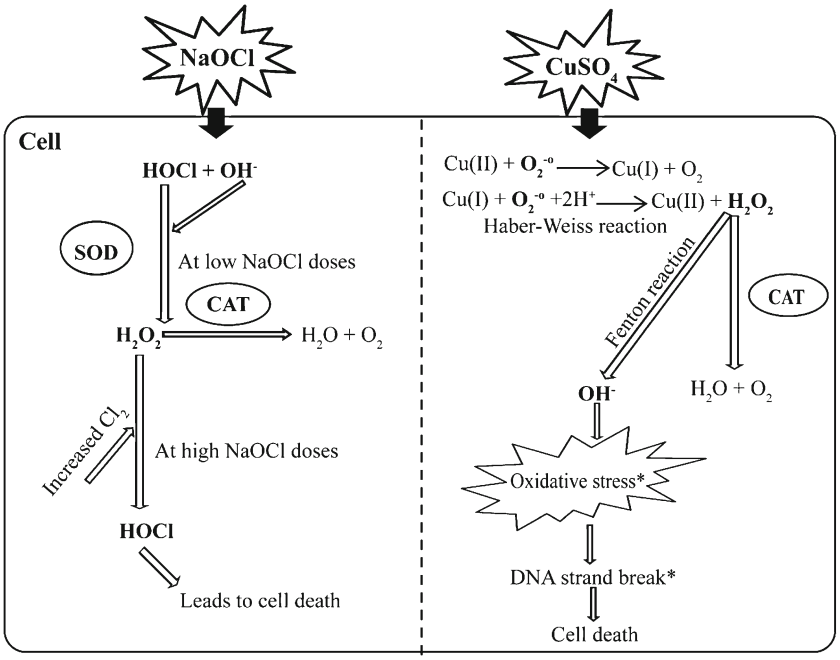

Fig. 6 A schematic summary of catalase (CAT) activity, including superoxide dismutase (SOD), in the dinoflagellate *C. polykrikoides* exposed to the algicides NaOCl and $CuSO_4$. *Asterisk* denotes that these were not observed in the present study but were included from data reported elsewhere

protein radical formation (Bonini et al. 2007). Similarly, higher levels of HOCl have the ability to destroy CAT (Mahawar et al. 2011), which explains why no CAT activity was observed at high NaOCl doses in the present study.

As for $CuSO_4$, the CAT activity showed a dose-dependent increase after 6 h of exposure and the trend remained the same even after 12 h of exposure (Fig. 5b). However, after 24 and 72 h of exposure, CAT activity showed a marginal increase at lower concentrations (0.5 and 1.0 mg L^{-1}) compared to that of the control, although no significant change in activity was observed at higher doses (2.0, 3.0, and 5.0 mg L^{-1}). It is reported that Cu(II) ions produce H_2O_2 and superoxide radical through a Haber–Weiss reaction (Santo et al. 2008; Letelier et al. 2005). As CAT is involved in the dissipation of H_2O_2, an increase in CAT activity after 6 and 12 h of exposure, thus, is explainable; however, at higher concentrations, H_2O_2 forms a complex with Cu(II) that leads to a break in the DNA, resulting in cell death (Li and Trush 1993). Moreover, Cu(II) ions at concentrations around 1.0 mg L^{-1} have been found to induce oxidative stress and damage to DNA in the marine dinoflagellate *P. minimum* (Guo et al. unpublished). In the present study, we could not observe CAT activity after 24 and 72 h of exposure to $CuSO_4$ because of a decrease in the viable cell numbers. The pathways leading to cell death when exposed to the algicides as recorded in the present study is explained using a schematic diagram (Fig. 6).

In conclusion, it is obvious that both NaOCl and $CuSO_4$ are algicidal agents that are highly effective in reducing the *C. polykrikoides* blooms, as revealed by a marked reduction in growth rate and Chl *a* levels in the test species. It can also be noted that the *Cochlodinium* cell count and Chl *a* levels in *C. polykrikoides* did not recover following exposure to higher doses of the algicides. From our data, it is clear that NaOCl causes immediate damage to the target cell; hence, the use of this algicide at the wake of a bloom could prevent the bloom from expanding. On the other hand, $CuSO_4$ showed a dose-dependent decrease in cell counts. Copper-based algicides are one of the approved algicides for use in swimming pools and on aquaculture farms according to the World Health Organization (WHO); however, their persistence in the marine environment must be taken in to account. Both algicides induced ROS production and were successful in inactivating antioxidant enzyme activity at higher concentrations; however, these chemicals can also be a threat to non-target organisms, and as such, their presence and concentrations in the marine environment must be continuously monitored.

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