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Physiological and biochemical responses of the marine dinoflagellate *Prorocentrum minimum* exposed to the oxidizing biocide chlorine

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ABSTRACT

Toxic effects of the commonly used biocide chlorine (Cl_2) on the marine dinoflagellate *Prorocentrum minimum* were assessed using growth-, pigment- and enzyme activity-based endpoints. Cell count, chlorophyll *a* levels, carotenoids, and chlorophyll autofluorescence were monitored up to 72 h after exposure to Cl_2, and these parameters showed a dose- and time-dependent decrease. The 72-h median effective concentration (EC_{50}) based on growth rate was 1.177 mg L^{-1}. Cl_2 dose above 0.5 mg L^{-1} were toxic to *P. minimum* after 6-h exposure to Cl_2; the effect increased with increase in exposure time as revealed by a significant reduction in growth rate and decreased chlorophyll fluorescence. Moreover, the activities of antioxidant enzymes, including superoxide dismutase and catalase, were altered proportionally with increasing Cl_2 dose. The results of this study show that Cl_2 concentrations as observed in power-plant discharges and in drinking-water systems cause physiological and biochemical damage to the microalgae.

1. Introduction

Chlorine (Cl_2) is one of the commonest biocides used for the control and removal of harmful and slime-forming organisms in drinking water supplies and in once-through cooling electricity-generating plants (Calderon, 2000; Ebenezer et al., 2012). It is also commonly used in swimming pools to remove algae and to control harmful algal blooms (HABs) in aquaculture farms (Anderson et al., 2001). As Cl_2 is an active oxidizing agent, it affects the biochemical and physiological processes of organisms by damaging the cell membrane, protein, and nucleic acids (Phe et al., 2005). Cl_2 concentrations of 0.02–0.5 mg L^{-1} are generally observed in freshwater as well as marine environments (USEPA, 1999; Environment agency, 2011). These low concentrations of Cl_2 have been reported to affect non-target organisms, including bacteria, microalgae, and zooplankton, in the discharge area (Lattemann and Höpner, 2008).

Microalgae are unicellular, photosynthetic organisms of varying shapes and size, abundant in the aquatic environment, and they form the base of the food chain in the aquatic ecosystem. These organisms respond rapidly to changing environmental conditions (Sarthou et al., 2005). Owing to their diversity and sensitivity in aquatic environments, microalgae are useful bioindicators of environmental changes, for both short- and long-term environmental monitoring as well as ecotoxicology assessments (Franklin et al., 2001). Microalgae-based ecotoxicology assessment generally measures the growth rate, and chlorophyll-level variations between the non-treated and treated samples (OECD, 2011). Even though it provides a quantitative measure of the effect of a toxicant to the algae, little is known regarding the mechanism of toxicity (Franklin et al., 2001). In addition to the aforementioned endpoints, measurement of physiological activity, such as chlorophyll autofluorescence, has been used to differentiate live and dead algal cells (Franklin et al., 2001). Monitoring of cellular defense mechanisms (in terms of biochemical enzyme activity) is an added advantage to ecotoxicity assessment (Torres et al., 2008), and can shed light on the mode of action of a toxicant on the algae.

All aerobic organisms have the ability to generate different reactive oxygen species (ROS), including superoxide anion, hydrogen peroxide, and hydroxyl radicals, through numerous ways due to the reduction of molecular oxygen (Heyno et al., 2011). Low levels of ROS are useful to the organism as signaling molecules for altering gene expression and modulating the activity of stress-related proteins; however, various environmental stresses lead to higher concentrations of all ROS species (Apel and Hirt, 2004). Increased ROS concentration can be harmful to the organism, because it causes peroxidation of lipids, oxidation of proteins, damage to nucleic acids, and enzyme inhibition (Mishra et al., 2011). Cells have their own protective mechanisms against oxidative damage; detoxification or scavenging of ROS is achieved by a well-organized antioxidative system, including several

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lower-molecular-weight compounds and enzymes (Tripathi et al., 2006). Superoxide dismutase (SOD) is the first line of defense of the cell against ROS (Ken et al., 2005). Moreover, other antioxidant enzymes such as catalase (CAT) and low-molecular-weight antioxidants, including glutathione and carotenoids, are reported to counteract the oxidative stress in both plants and microalgae (Meriga et al., 2004; Wang et al., 2012).

In the aquatic environment, Cl_2 hydrolyses rapidly; it combines with other chemical compounds, and usually exists in a combined state (USEPA, 1994). Cl_2 easily dissolves in water to form hypochlorous acid (HOCl) which is an ROS (White, 1998; Lu et al., 2012). Evaluation of ROS production and determination of antioxidant enzyme activity may act as rapid and simple biomarkers for Cl_2 toxicity assessment.

In the present study, the marine dinoflagellate *Prorocentrum minimum* was used, because it is widely distributed in temperate and subtropical waters (Heil et al., 2005). *P. minimum* has been reported to cause diarrhetic shellfish poisoning and have harmful effects on aquatic organisms and humans (Hackett et al., 2004). Recently, the species has been used as a test organism for aquatic toxicity assays, genomics, and evolutionary studies (Lin et al., 2006; Guo and Ki 2012), because these cells grow well under laboratory conditions and have distinct genomic features (Lin et al., 2006). The objectives of the present study were (1) to investigate the impact of the oxidizing biocide chlorine (Cl_2) on the physiological and biochemical activities of *P. minimum*, and (2) to compare the sensitivity of biochemical markers in parallel with direct cell counts and pigment concentration in ecotoxicology assessments.

2. Materials and methods

2.1. Experimental organism and culture conditions

The *P. minimum* strain (D-127) was obtained from the Korea Marine Microalgae Culture Center (Pukyung National University, Busan, Korea). The cells were cultured in f/2 medium (Guillard and Ryther, 1962). The cultures were maintained at 20 °C, under 12:12-h light:dark cycle with a photon flux density of approximately 65 μmol photons^{-1} m^{-2} s^{-1}.

2.2. Test chemical preparation and toxicity assay

Sodium hypochlorite was commercially obtained (Cat. No. 425044, Sigma, MO). The initial concentration in the bottle was determined to be 14,293 mg L^{-1} using iodometry (APHA, 1998). The stock and working solutions were prepared using autoclaved distilled water (ADW) for which the demand was below detection limit (0.01 mg L^{-1}). Cl_2 solution of nominal doses, 0.1, 0.5, 1.0, 2.0, and 3.0 mg L^{-1}, were prepared by diluting the stock solution with ADW. The Cl_2 concentration was determined spectrophotometrically using diethyl-phenylenediamine (DPD) at 515 nm (APHA, 1998). To ensure that the exact nominal concentration as described above is bioavailable to our test species, we determined the Cl_2 demand of the f/2 medium (APHA, 1998) prior to our experiments. The demand was found to be 0.7 mg L^{-1}, and media with the Cl_2 concentrations mentioned above were prepared accordingly.

Cl_2 at nominal doses that took account of the Cl_2 demand of the medium as described above was added to triplicate 200-mL cultures of *P. minimum* during the exponential phase of growth. The initial cell concentration was $1.0 \pm 0.1 \times 10^5$ cells mL^{-1}. The samples were drawn for growth-based assays and biochemical assays at 0, 6, 12, 24, and 72 h. The Cl_2 concentration was determined at 5, 15, 30 min, 1, 6, 12, and 24 h using DPD method (APHA, 1998) as shown in Table 1.

2.3. Cell count and median effective concentration (EC_{50})

Cell counts in each test flask were determined using a hemocytometer (Marienfeld GmbH, Lauda, Germany). Cell counts were plotted against exposed time.

The 72-h EC_{50} (median effective concentration) and the percentile inhibition were calculated as recommended by OECD (2011) testing guidelines. The value of 72-h EC_{50} was estimated using a sigmoidal dose–response curve and plotted using Origin version 8.5 (MicroCal Software Inc., Northampton, MA) based on the sigmoidal 4-parameter equation (Teisseyre and Mozrzymas, 2006);

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

where a is the response value at zero or minimum asymptote, b is the response value for infinite concentration or maximum asymptote c is the mid-range point, d is the steepness of the curve or the Hill slope, and x is the dilution co efficient.

2.4. Pigments and chlorophyll autofluorescence analyses

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, Fullerton, CA). The chl *a* and CAR concentration was estimated according to a method described by Parsons et al. (1984).

Chlorophyll autofluorescence was measured using a fluorescent microscope (Axioscope, Carl Zeiss, Oberkochen, Germany) at 400 × magnification. UV dichoric (G365/ 395–488 nm) source was used for the excitation and the emission was collected by setting the detection bandwidth between 630 and 750 nm. Digital image analysis was performed using the image analysis software ImageJ 1.29 × (NIH, Bethesda, MD). Mean fluorescence intensity (MFI) was expressed in terms of pixel gray value, which ranges from 0 to 270. The reported MFI values indicate average of MFI values obtained from a minimum of 50 individual cells.

2.5. Biochemical assays: superoxide dismutase (SOD) and catalase (CAT)

SOD was measured by monitoring the inhibition of photochemical reduction of nitroblue tetrazolium chloride (NBT) (Beauchamp and Fridovich, 1971). Five milliliters of the algal culture was centrifuged at 4200 rpm for 10 min. Five milliliters of 100 mM phosphate buffer was added to the pellet. The cells were homogenized using a Teflon pestle tissue homogenizer in ice, and the tube was then placed in a water bath at 40 °C for 5 min (modified from Soto et al. (2011)). The mixture was centrifuged at 4200 rpm for 10 min. 2.6 mL of reaction mixture (0.1 M phosphate buffer, 130 mM methionine solution, 750 μM Na_2EDTA solution, and 20 μM riboflavin solution) was added to the supernatant. The tubes were incubated under controlled light conditions (65 μmol photons m^{-2} s^{-1}) for 30 min. The absorbance was read at 560 nm. One unit of SOD (U) was defined as the amount of enzyme resulting in 50% inhibition of photochemical reduction of NBT. SOD levels were represented as units per 10^5 cells (U/10^5 cells).

CAT is a tetrameric heme-containing enzyme, which catalyzes the breakdown of hydrogen peroxide to water and molecular oxygen (Aebi, 1984). Five milliliters of the algal culture was centrifuged at 4200 rpm for 10 min. Two milliliters of extraction buffer (1 M phosphate buffer) were added to the pellet. The cells were homogenized using a Teflon pestle tissue homogenizer in ice. The tube was placed in a water bath at 40 °C for 5 min (modified from Soto et al., 2011). The homogenate was centrifuged at 4200 rpm for 20 min. To 100 μL of the supernatant, 1.6 mL of 1 M phosphate buffer, 0.2 mL of 0.3% H_2O_2, and 3 mM EDTA were added in a test tube and the mixture was shaken well for 3 min. Enzyme activity was calculated using an extinction coefficient of 0.036 per mM cm^{-1} and expressed as (unit/mg protein). One unit of enzyme is the amount necessary to decompose 1 μL of H_2O_2 per minute at 25 °C. The absorbance of the supernatant was read at 240 nm.

2.6. Statistical analysis

All data presented are mean values of triplicates. One-way analysis of variance (ANOVA) with post hoc Student's Newmann–Keuls test using Graphpad InStat (Graphpad Software, Inc., CA) was used for comparisons between non-treated and treated cultures. $P < 0.05$ was accepted as significant. The correlation between MFI and chl *a* was tested using Pearson's correlation coefficient (R^2) in an Excel spreadsheet (Microsoft Corporation, Redmond, WA).

Table 1
Total residual oxidant levels at different time intervals.

Initial chlorine dosed (mg L^{-1})	Residual oxidant (mg L^{-1})							
	0 min	5 min	15 min	30 min	60 min	6 h	12 h	24 h
Control	0	0	0	0	0	0	0	0
0.1	0.098	0.003	BDL[a]	BDL	BDL	BDL	BDL	BDL
0.5	0.485	0.219	0.009	BDL	BDL	BDL	BDL	BDL
1.0	0.991	0.754	0.023	BDL	BDL	BDL	BDL	BDL
2.0	1.857	1.724	1.704	1.585	0.690	0.457	BDL	BDL
3.0	2.884	2.857	2.069	1.003	0.897	0.533	BDL	BDL

[a] BDL: Below detection limit (< 0.01 mg L^{-1}).