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Synergistic effects of elevated carbon dioxide and sodium hypochlorite on survival and impairment of three phytoplankton species

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Sodium hypochlorite (NaOCl) is widely used to disinfect seawater in power plant cooling systems in order to reduce biofouling, and in ballast water treatment systems to prevent transport of exotic marine species. While the toxicity of NaOCl is expected to increase by ongoing ocean acidification, and many experimental studies have shown how algal calcification, photosynthesis and growth respond to ocean acidification, no studies have investigated the relationship between NaOCl toxicity and increased CO_2. Therefore, we investigated whether the impacts of NaOCl on survival, chlorophyll *a* (Chl-*a*), and effective quantum yield in three marine phytoplankton belonging to different taxonomic classes are increased under high CO_2 levels. Our results show that all biological parameters of the three species decreased under increasing NaOCl concentration, but increasing CO_2 concentration alone (from 450 to 715 µatm) had no effect on any of these parameters in the organisms. However, due to the synergistic effects between NaOCl and CO_2, the survival and Chl-*a* content in two of the species, *Thalassiosira eccentrica* and *Heterosigma akashiwo*, were significantly reduced under high CO_2 when NaOCl was also elevated. The results show that combined exposure to high CO_2 and NaOCl results in increasing toxicity of NaOCl in some marine phytoplankton. Consequently, greater caution with use of NaOCl will be required, as its use is widespread in coastal waters.

Key Words: high CO_2; marine phytoplankton; ocean acidification; sodium hypochlorite; synergistic effect

INTRODUCTION

The anthropogenic input of CO_2 into the atmosphere and ocean has been on a constant rise since the Industrial Revolution, resulting in decreased pH and changes in ocean carbon chemistry (Orr et al. 2005). Under the Intergovernmental Panel on Climate Change (IPCC) IS92a scenario, the pH of the ocean will drop 0.2-0.3 units by the end of this century (Caldeira and Wickett 2003), which will drive massive alterations to the lives of marine organisms (Kroeker et al. 2013). Undoubtedly, many studies have shown the combined effects of increased CO_2 and other environment parameters, such as iron availability on marine organisms (Endo et al. 2013), changes in the depth of euphotic zone on phytoplankton (Gao et al. 2012*b*), and the importance of substrates for spawning coral settlement (Doropoulos and Diaz-Pulido 2013). The projected CO_2 increases combined with the presence of other chemicals, such as sodium hypochlorite (NaOCl) may further result in synergistic effects that will impact

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the survival and physiological condition of marine phytoplankton. However, despite the importance of the chemical interaction between NaOCl and CO_2, no studies have investigated the combined effects of increased CO_2 and NaOCl on marine phytoplankton.

NaOCl is one of the most widely used antifouling agents in power plant cooling and ballast water treatment systems (Gregg and Hallegraeff 2007, Saleem et al. 2012). Given that many power plants are located near coastal regions due to the use of seawater as a coolant, fouling organisms such as barnacles, oysters, and bryozoans often colonize surfaces in their coolant systems, which then interfere with water flow and heat transfer efficiency (Taylor 2006, Polman et al. 2013). Moreover, the number of large transport vessels has expanded on the world's oceans due to increased global economic activity, resulting in approximately 3,500 mega tons of ballast water being used globally each year for stability and maneuverability of ships during voyages (Endresen et al. 2004). As a result, many exotic marine species have invaded new coastal regions through ballast water, causing serious disturbances to marine ecosystems (Gray et al. 2006, Smayda 2007). UV radiation, ozone (O_3) treatment, heat exposure, and various chemicals are all used to prevent biofouling and the introduction of exotic species. Also chemical treating these waters with NaOCl has received considerable attention as a simple and efficient method of reducing fouling (Allonier et al. 1999, Gregg and Hallegraeff 2007).

When NaOCl is added to water, it dissociates into sodium hydroxide (NaOH) and hypochlorous acid (HOCl), as shown in Eq. (1). HOCl further dissociates into hypochlorite (OCl^-) and hydrogen ions (H^+) as shown in Eq. (2). This dissociation is reversible and strongly dependent on pH (Sarbatly and Krishnaiah 2007). Generally, 25% of the HOCl will occur as free chlorine at pH 7.7 in 20°C seawater under a 30 psu condition, but the proportion of HOCl increases as pH decreases (Sugam and Helz 1976). According to the Eq. (2) equilibrium, almost 99% of the HOCl will be free chlorine at pH 5.2, and about 99% will be OCl^- at pH 9.3.

$$NaOCl + H_2O \rightarrow HOCl + NaOH \quad (1)$$

$$HOCl \leftrightarrow OCl^- + H^+ \quad (2)$$

HOCl is 80-200 times stronger than OCl^- in terms of pathogen disinfection (White 1992). A pH reduction of 0.2-0.3 units will increase the proportion of HOCl by 7% (Table 1). Despite this small change, increased CO_2 (lower pH than present day) will increase the proportion of HOCl, which might also increase NaOCl toxicity to organisms.

In this study, we examined the short-term effects of increased levels of CO_2 and NaOCl, both alone, and in combination, on the abundance, Chl-*a* content, and photosynthetic efficiency of three phytoplankton species. Temporal fluctuations in natural environment conditions and population sizes cannot be realistically simulated under laboratory conditions. The typical diel variations in aqueous partial pressure of carbon dioxide (pCO_2) depend on the tidal cycle, photosynthesis during the day, and respiration at night. Anthropogenic NaOCl effluents also vary over tidal, daily, and seasonal scales. Therefore, this study was designed to simulate the mean temperature during late spring / early summer, and the pCO_2 values of the present day and the predicted value for the year 2100.

Table 1. Seawater carbon chemistry of two CO_2 levels

	Normal CO_2	High CO_2
pH (NBS)	8.0	7.8
A_{T_mea} (μmol kg^{-1})	2,266.7 ± 3.6	2,263.9 ± 0.8
$DIC_{\_mea}$ (μmol kg^{-1})	2,060.0 ± 3.0	2,131.3 ± 2.5
pCO_{2_cal} (μatm)	450.5 ± 4.0	714.7 ± 13.8
$HCO_3^{-}{}_{\_cal}$ (μmol kg^{-1})	1,889.4 ± 3.1	1,997.8 ± 3.7
$CO_3^{2-}{}_{\_cal}$ (μmol kg^{-1})	155.6 ± 1.2	109.7 ± 1.7
HOCl (%)	15.2	22.2
OCl^- (%)	84.8	77.8

Data are represented as mean ± standard deviation (n = 3). High CO_2 level was manipulated with adding CO_2 saturated seawater into natural seawater.
A_T, total alkalinity; DIC, dissolved inorganic carbon.

MATERIALS AND METHODS

Experimental procedure

Three species of marine phytoplankton, *Thalassiosira eccentrica* (Ehrenberg) Cleve, *Akashiwo sanguinea* (Hirasaka) Hansen et Moestrup and *Heterosigma akashiwo* (Hada) Hada, were used in this experiment. One of the most common and wide-spread diatoms, *T. eccentrica*, was isolated from Hampyeng Bay, Korea, in February 2009. The red-tide forming dinoflagellate, *A. sanguinea* (strain, AS-LOHABE01), and the raphidophyte, *H. akashiwo* (strain, HA-LOHABE), were obtained from Chonnam National University, Korea. The three species were cultured in filtered (GF/F filters; Whatman, Maidstone, UK)

and autoclaved natural seawater to which 132 μL L^{-1} of f/2 medium was added. Irradiance (50 μmol photons m^{-2} s^{-1}) and temperature (20°C) were maintained constant during the experiments.

The NaOCl solution was produced by electrolysis from GF/F filtered seawater with an immediate reaction from a 4.5 A electrical current supply. Total residual chlorine (TRC) concentration was determined by the iodometric method (Clesceri et al. 1999). In this experiment, the same seawater was used, as the NaOCl TRC concentrations vary depending on the presence of organic matter, temperature, salinity, and the chemical speciation of elements (Kester 1986).

Two levels of seawater CO_2 concentration, normal (450 μatm) and high (715 μatm) CO_2 were prepared for the experiment based on IPCC's IS92a scenario. Specifically, high CO_2 seawater was established by spiking CO_2-saturated seawater into normal seawater (dilution ratio of CO_2-saturated SW to normal SW = 4/1,000). The pH difference was about 0.2 units initially (calibrated using standard NBS buffers at pH 7 and 10; checked using pH meter; PHM 210; Radiometer Analytical SAS, Lyon, France), and total alkalinity (A_T) and dissolved inorganic carbon (DIC) were quantified by potentiometric acid titration (Metrohm 765; Metrohm, Herisau, Switzerland) (Millero et al. 1993, Hernández-Ayón et al. 1999). Other carbon parameters (pCO_2, HCO_3^-, and CO_3^{2-}) were calculated using CO_2SYS basic software and two measurable parameters (A_T and DIC, in this study) (Lewis and Wallace 1998).

To evaluate the synergistic effects of high CO_2 and NaOCl on the three phytoplankton species, each species was exposed to the two levels of CO_2 and four NaOCl concentrations (0, 1.0, 2.0, and 3.0 ppm for *T. eccentrica*; and 0, 0.5, 1.0, and 1.5 ppm for *A. sanguinea* and *H. akashiwo*) for 8-h, both alone and in combination. All experiments were conducted in three replicates 75 mL polycarbonate bottles under 20°C and 30 psu conditions. The cultures were manually and gently shaken before sub-sampling, which was done by taking 5 mL plankton samples at 0, 1, 2, 4, and 8-h. Sub-samples of 3 mL were used to measure chlorophyll *a* (Chl-*a*) and were immediately preserved with Lugol's solution for cell counts. Two mL sub-samples were used for photosystem II (PSII) measurements.

Phytoplankton abundance and survival

Preserved phytoplankton cells from single and combined CO_2 and NaOCl experiments were enumerated using a Sedgwick-Rafter counting chamber (1 mL) and an inverted microscope (Axiostar plus; Carl Zeiss, Oberkochen, Germany). Survival (%) was calculated by dividing the number of live individuals by the total number of individuals in each treatment. Additionally, the 1-h lethal median concentration (LC_{50}) was determined by the Probit analysis method (Finney 1971).

Chl-*a* measurement

To determine the Chl-*a* content, *in vivo* chlorophyll fluorescence was measured using a Turner 10AU fluorometer (Turner Designs, Sunnyvale, CA, USA). GF/F filtered seawater was used to determine the cell-free control of *in vivo* fluorescence. *In vivo* fluorescence values were converted to Chl-*a* concentrations using the previously determined a linear calibration curve between two parameters ($r^2 = 0.74$).

Effective PSII quantum yield measurement

PSII photochemical efficiency was assessed at set time intervals (0, 1, 2, 4, and 8-h) using a Phyto-PAM (Walz, Effeltrich, Bavaria, Germany). The Phyto-PAM applies light emitting diodes with a peak emission at 650 nm, saturating flashes, and actinic irradiance. The fluorescence of a cell-free control was determined using seawater filtered with a Whatman GF/F filter to correct the background fluorescence. The effective quantum yield (Φ_{PSII}) was measured using light-adapted samples, at 50 μmol photons m^{-2} s^{-1}.

$$\Phi_{PSII} = (F_m' - F_t)/F_m' \quad (3)$$

F_m' and F_t represent the maximum fluorescence and the steady state fluorescence for the light-adapted samples, respectively.

Statistical analyses

The Shapiro-Wilk's test was used to determine the normality of the distribution at the 0.05 level, and equal variances were tested using the Levene's test. Although for the majority of test cases, the assumptions of normality were not met even after arcsine transformation, statistical differences in untransformed data (initial / final) were examined by general linear model (GLM) and two-way analysis of variance (ANOVA) was used to assess the effects of NaOCl and high CO_2 level, both alone, and in combination. Additionally, non-parametric Kruskal-Wallis test and Shapiro-Wilk's test were used to identify the statistical significant differences among the treatments

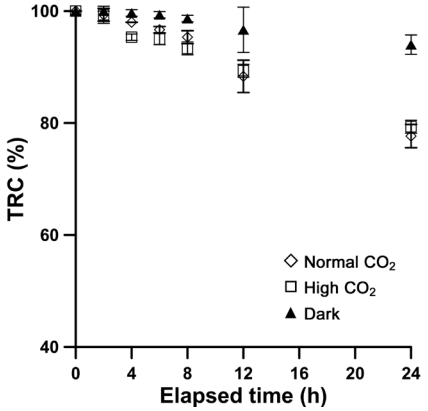

Fig. 1. Changes in the total residual chlorine (TRC) concentration over time with the normal and high CO_2 level seawaters at fluorescent light, and with normal CO_2 level seawater in the dark.

(two levels of CO_2 and four concentrations of NaOCl) on the abundance, survival, Chl-*a*, and Φ_{PSII} values. The confidence levels for all analyses was set at 95% ($p < 0.05$). All statistical analyses were conducted using SPSS version 17.0 software (SPSS Inc., Chicago, IL, USA).

RESULTS

Carbon chemistry and TRC concentration

The carbon chemistry of high CO_2 seawater was altered as the pCO_2, dissolved inorganic carbon (DIC) and bicarbonate (HCO_3^-) concentrations increased, and carbonate (CO_3^{2-}) concentration decreased compared to those in normal CO_2 seawater (Table 1). The pH for the normal and high pCO_2 seawater was maintained at 8.0 and 7.8 during the experimental period, respectively. TRC concentrations decreased by 6% in the dark and by 22% under white fluorescent light after 24-h (Fig. 1). The proportion of HOCl and OCl^- were 15.2 and 84.8% in normal CO_2 seawater (pH 8.0) (Table 1). The proportion of HOCl was increased by 7% in high CO_2 seawater (pH 7.8) compared to normal CO_2 seawater. No significant difference in the decreasing rate of the TRC was detected between the normal and high CO_2 levels throughout the experimental period, in spite of the different proportion of HOCl and OCl^- (Fig. 1).

Phytoplankton abundance and survival

The abundances of *Thalassiosira eccentrica* (diatom), *Akashiwo sanguinea* (dinoflagellate), and *Heterosigma akashiwo* (raphidophyte) were not significantly different between normal and high CO_2 levels during 8-h incubation period (Fig. 2).

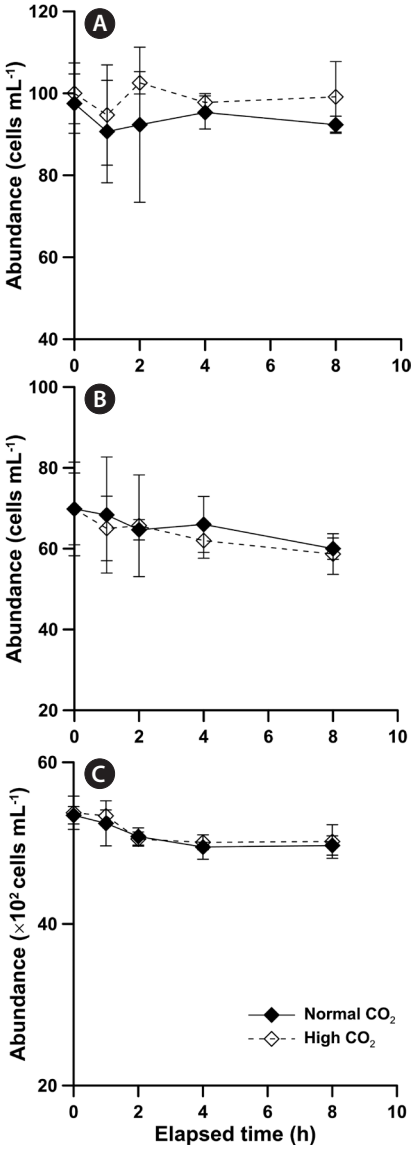

Fig. 2. Abundance of experimental organisms over time with the normal and high CO_2 level seawaters. (A) *Thalassiosira eccentrica*. (B) *Akashiwo sanguinea*. (C) *Heterosigma akashiwo*. Data are represented as mean ± standard deviation (n = 3).

The abundance of *T. eccentrica* was 96.3 ± 7.6 and 98.7 ± 8.3 cells mL^{-1} in the normal and high CO_2 seawaters on initial time, respectively, but on the final sampling time, the abundance was slightly decreased ranging from 0.3-4.2% in both CO_2 seawaters, with no significant differences compared to initial abundance (Fig. 2A). In both CO_2 seawater conditions, slightly decreases in abundance were also detected in *A. sanguinea* and *H. akashiwo* after

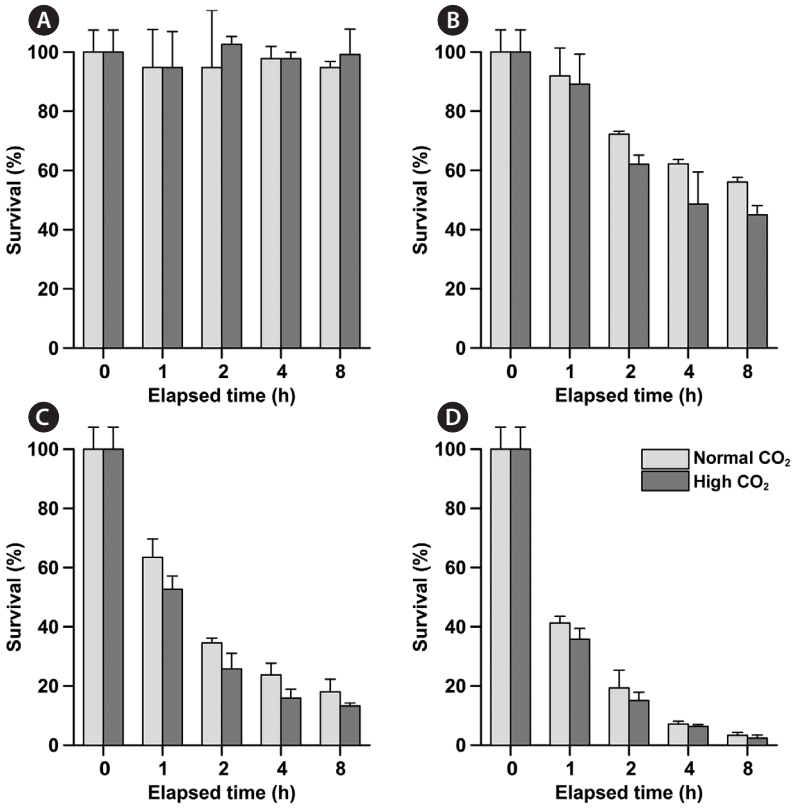

Fig. 3. Survival of *Thalassiosira eccentrica* over time with the normal and high CO_2 level seawaters as a function of the sodium hypochlorite (NaOCl) concentration. (A) 0 ppm. (B) 1.0 ppm. (C) 2.0 ppm. (D) 3.0 ppm. Data are represented as mean ± standard deviation (n = 3).

8-h and there were no significant differences between initial and final abundance (Fig. 2B & C).

Phytoplankton survival (%) was dependent upon NaOCl concentrations and varied between species. The 1-h LC_{50} values for *T. eccentrica*, *A. sanguinea*, and *H. akashiwo* for NaOCl were 2.6, 1.2, and 0.9 ppm in normal CO_2 seawater, respectively (Table 2). The lowest and highest 1-h LC_{50} values were observed for *H. akashiwo* and *T. eccentrica*. Survival (%) of *T. eccentrica*, *A. sanguinea*, and *H. akashiwo* decreased with increasing NaOCl concentration, showing 56.1, 1.4, and 9.4% survival at 1.0 ppm NaOCl after an 8-h exposure (Figs 3-5). After the 8-h exposure to 1.5 ppm NaOCl, most *A. sanguinea* and *H. akashiwo* cells were dead, but some *T. eccentrica* survived at even higher NaOCl concentrations.

Table 2. The 1-h LC_{50} (ppm) values of three phytoplankton species exposure to four different concentrations of sodium hypochlorite (NaOCl) under normal and high CO_2 level seawaters

Species	LC_{50} (ppm)	
	Normal CO_2	High CO_2
Thalassiosira eccentrica (Ehrenberg) Cleve	2.6 ± 0.1	2.4 ± 0.1
Akashiwo sanguinea (Hirasaka) Hansen et Moestrup	1.2	0.9 ± 0.1
Heterosigma akashiwo (Hada) Hada	0.9 ± 0.1	0.8 ± 0.1

Data are represented as mean ± standard deviation (n = 3).

The LC_{50} values of *T. eccentrica*, *A. sanguinea*, and *H. akashiwo* after one hour exposure to NaOCl under high CO_2 seawater were all slightly lower than those observed under normal CO_2 seawater (Table 2). Survival (%) of *T. eccentrica* was significantly lower under high CO_2 in the 2.0 ppm NaOCl treatment than that in normal CO_2 seawater ($p < 0.05$). However, no significant differences were observed between the two CO_2 levels at the other NaOCl concentrations (Fig. 3). Additionally, survival (%) of *H. akashiwo* was significantly lower at 0.5 and 1.0 ppm NaOCl in high CO_2 seawater than that in normal CO_2 seawater (Fig. 5). In contrast, *A. sanguinea* showed no difference in survival (%) between normal and high CO_2 seawater at all NaOCl concentrations tested (Fig. 4).

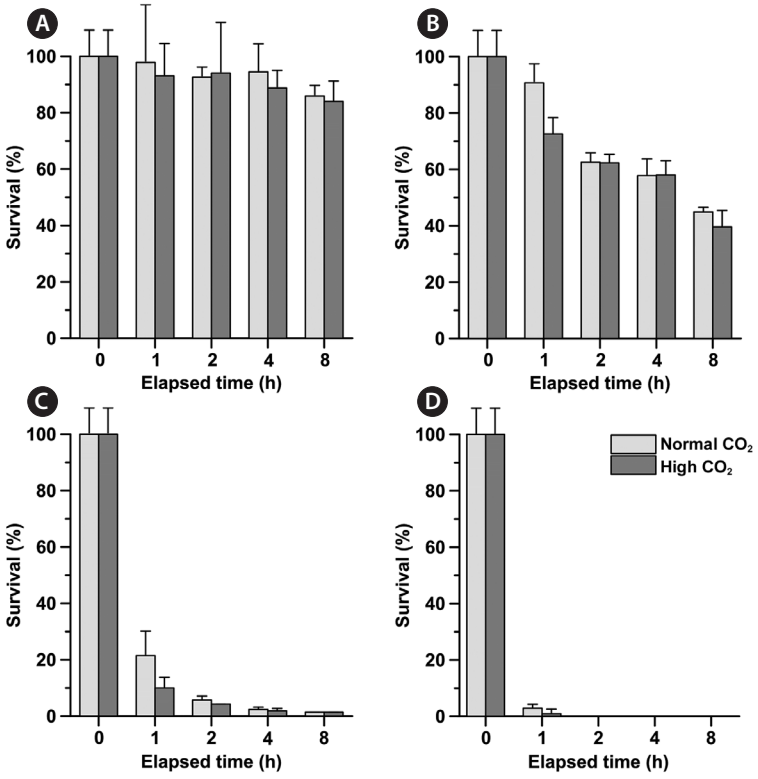

Fig. 4. Survival of *Akashiwo sanguinea* over time with the normal and high CO_2 level seawaters as a function of the sodium hypochlorite (NaOCl) concentration. (A) 0 ppm. (B) 0.5 ppm. (C) 1.0 ppm. (D) 1.5 ppm. Data are represented as mean ± standard deviation (n = 3).

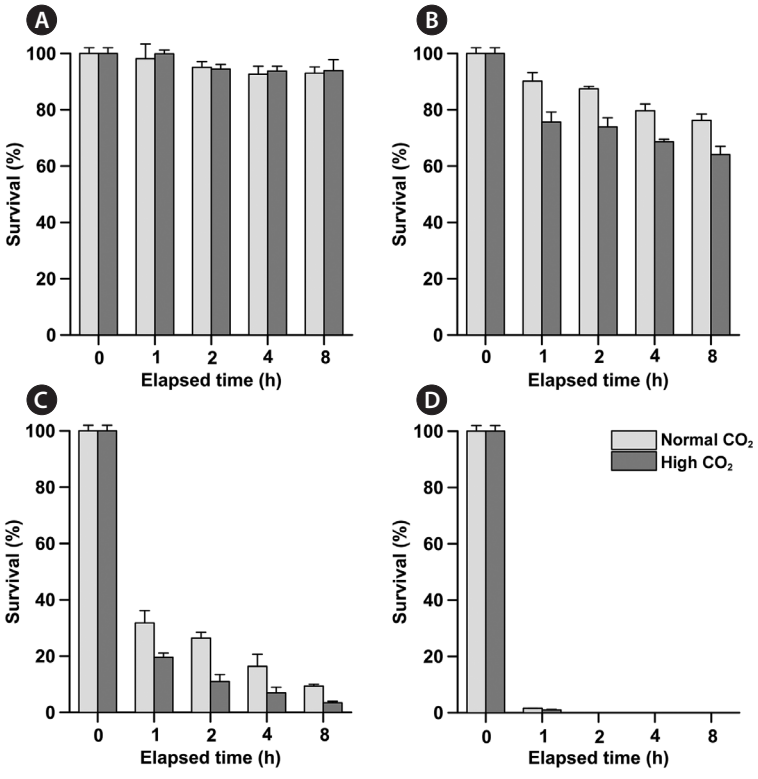

Fig. 5. Survival of *Heterosigma akashiwo* over time with the normal and high CO_2 level seawaters as a function of the sodium hypochlorite (NaOCl) concentration. (A) 0 ppm. (B) 0.5 ppm. (C) 1.0 ppm. (D) 1.5 ppm. Data are represented as mean ± standard deviation (n = 3).

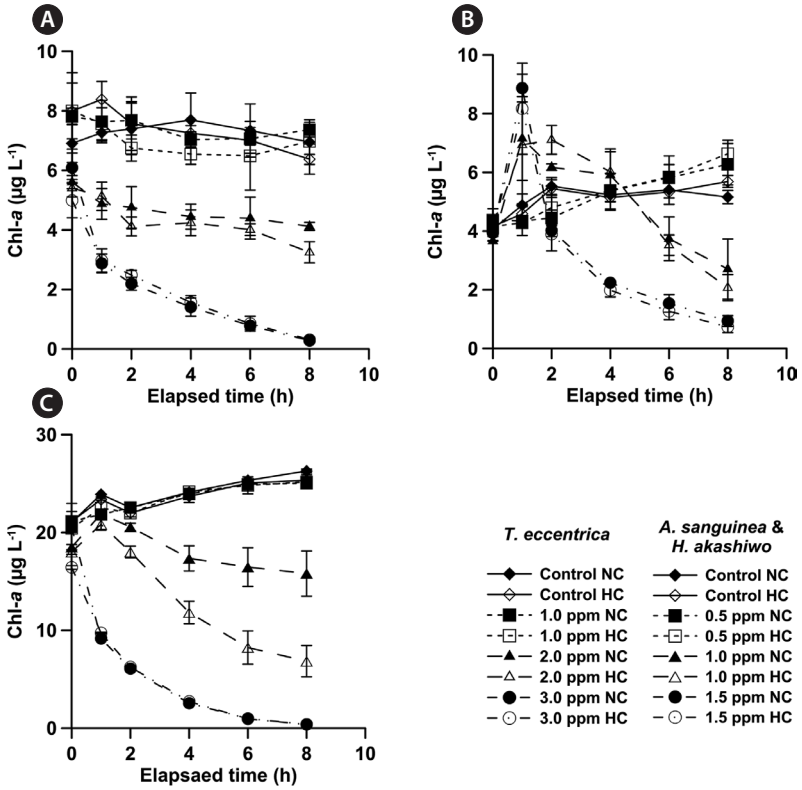

Fig. 6. Chlorophyll *a* (Chl-*a*) content over time with the normal CO_2 (NC) and high CO_2 (HC) level seawaters as a function of the sodium hypochlorite (NaOCl) concentration. (A) *Thalassiosira eccentrica*. (B) *Akashiwo sanguinea*. (C) *Heterosigma akashiwo*. Data are represented as mean ± standard deviation (n = 3).

Chl-*a* content and Φ_{PSII}

The Chl-*a* and Φ_{PSII} of the three species were similar in both normal and high CO_2 seawaters (Figs 6 & 7). Chl-*a* contents of *T. eccentrica* decreased towards the end of the experiment under both CO_2 seawaters. In contrast, Chl-*a* contents of *A. sanguinea* and *H. akashiwo* were slightly higher at the end of the experiment. However, differences between initial and final periods were not observed to be significant.

Regardless of CO_2 concentration, Chl-*a* contents of *T. eccentrica*, *A. sanguinea*, and *H. akashiwo* was significantly lower at NaOCl concentrations >2.0, 1.0, and 1.0 ppm, respectively (Fig. 6). At the end of the experiment, the Chl-*a* content of *T. eccentrica* at 2.0 ppm NaOCl in high CO_2 seawater decreased by 20% relative to that in normal CO_2 seawater. Also, Chl-*a* content of *H. akashiwo* at 1.0 ppm NaOCl in high CO_2 seawater was lower by >50% relative to that in normal CO_2 seawater. However, Chl-*a* contents of *T. eccentrica* and *H. akashiwo* was not different with CO_2 level at the other NaOCl concentrations. In contrast to *T. eccentrica* and *H. akashiwo*, Chl-*a* content of *A. sanguinea* exhibited no significant differences between normal and high CO_2 seawaters at any NaOCl concentration.

No significant changes in the Φ_{PSII} of *T. eccentrica*, *A. sanguinea* and *H. akashiwo* were observed after 8-h exposure to the lowest (1.0, 0.5, and 0.5 ppm, respectively) NaOCl concentrations (Fig. 7). However, Φ_{PSII} of *T. eccentrica* decreased from 0.65 to 0.30 upon exposure to 2.0 ppm NaOCl, and decreased from 0.65 to 0.05 upon exposure to 3.0 ppm NaOCl after 8-h. Furthermore, the Φ_{PSII} of *A. sanguinea* and *H. akashiwo* declined from 0.72 to 0.51 and 0.62 to 0.53 upon exposure to 1.0 ppm NaOCl after 8-h. The Φ_{PSII} values for *T. eccentrica*, *A. sanguinea*, and *H. akashiwo* decreased to <0.1 at 3.0, 1.5, and 1.5 ppm NaOCl, respectively. Although Φ_{PSII} values were slightly increase after 6-h exposure to NaOCl, these cells did not recover their original health status (Fig. 7). For all NaOCl concentrations, Φ_{PSII} of three species were not significantly different between normal and high CO_2 levels during 8-h incubation period (Table 3).

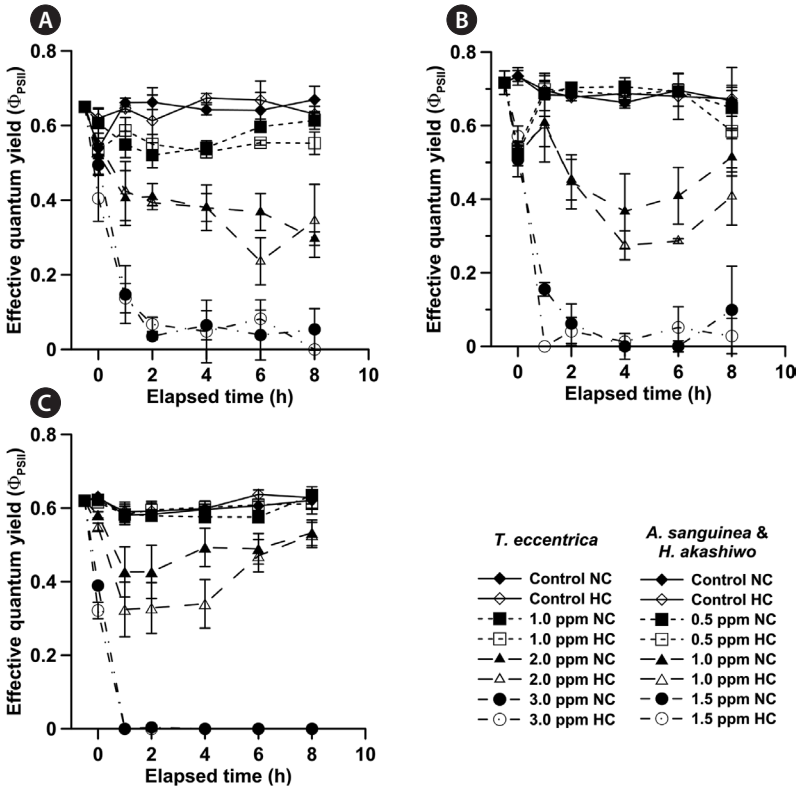

Fig. 7. Photosynthetic effective quantum yield (Φ_{PSII}) over time with the normal CO_2 (NC) and high CO_2 (HC) level seawaters as a function of the sodium hypochlorite (NaOCl) concentration. (A) *Thalassiosira eccentrica*. (B) *Akashiwo sanguinea*. (C) *Heterosigma akashiwo*. Data are represented as mean ± standard deviation (n = 3).

Table 3. The results of the General Linear Model or two-way ANOVA for determining the effects of NaOCl and CO_2 level, both alone, and in combination on survival, chlorophyll *a* (Chl-*a*), and effective quantum yield (Φ_{PSII})

Species	Parameter	NaOCl (d.f. = 3)	CO_2 (d.f. = 1)	NaOCl × CO_2 (d.f. = 3)
Thalassiosira eccentrica	Survival	0.000**	0.782	0.028* (F = 3.940)
	Chl-*a*	0.000**	0.707	0.277 (F = 1.406)
	Φ_{PSII}	0.000**	0.817	0.173 (F = 1.883)
Akashiwo sanguinea	Survival	0.000**	0.906	0.556 (F = 0.718)
	Chl-*a*	0.000**	0.990	0.225 (F = 1.617)
	Φ_{PSII}	0.000**	0.580	0.659 (F = 0.544)
Heterosigma akashiwo	Survival	0.000**	0.805	0.000** (F = 12.846)
	Chl-*a*	0.000**	0.599	0.000** (F = 26.813)
	Φ_{PSII}	0.000**	0.884	0.621 (F = 0.606)

*$p < 0.05$, **$p < 0.001$.

DISCUSSION

Our study provides the first insight into the effect of elevated CO_2 on NaOCl toxicity in marine phytoplankton. Despite the importance of the chemical interaction between NaOCl and CO_2, their synergistic effect on marine organisms remains to be assessed. The 1-h LC_{50} values for *Thalassiosira eccentrica*, *Akashiwo sanguinea*, and *Heterosigma akashiwo* toward NaOCl were lower in high CO_2 water than those in normal CO_2 water (Table 2). Our data confirmed that the toxicity of NaOCl increased due to ocean acidification for at least two of the investigated species and pH is a non-trivial factor related to the use of NaOCl in coastal zones.

Growth and photosynthesis of marine phytoplankton usually increase with increasing CO_2 (Schippers et al. 2004, Gao et al. 2012*a*, McCarthy et al. 2012). However, we found that the abundance, Chl-*a* and Φ_{PSII} of the three phytoplankton species remained unchanged by increased CO_2 indicating that the three species were not significantly affected by increases in CO_2 (Fig. 2). It is possible that our observation period was not long enough to determine the effect of enhanced CO_2 on growth. Moreover, coastal phytoplankton species may already have adapted to small changes close to the equilibrium pH; some phytoplankton species seem to get their CO_2 requirement fulfilled at current CO_2 levels (Hinga 2002).

NaOCl in water is converted to NaCl over time in sunlight and particularly by UV radiation. However, long-term exposure to NaOCl has significant adverse impacts on marine organisms at any concentration (Rajamohan et al. 2007, Añasco et al. 2008). For example, previous studies have reported that TRC concentrations from NaOCl of 30 to 106 ppb will kill 50% of the exposed individuals in seven dinoflagellate species (*Gymnodinium catenatum*, *Cochlodinium polykrikoides*, *Akashiwo sanguinea*, *Lingulodinium polyedrum*, *Prorocentrium micans*, *Alexandrium affine*, and *G. impudicum*) after only one hour (Jeong et al. 2002). Moreover, the 1-h LC_{50} values for the diatoms *Skeletonema costatum* and *Thalassiosira rotula* toward NaOCl are 3,128 to 3,433 ppb (Jeong et al. 2002). Our experiments showed that the three phytoplankton species responded differently to NaOCl. In our study, the 1-h LC_{50} value for *A. sanguinea* in 1.2 ppm NaOCl was much higher than that reported by Jeong et al. (2002) but was similar to the 1-h LC_{50} value observed for the diatom *T. eccentrica*. Also, the NaOCl tolerance decreased in the following sequence: diatom (*T. eccentrica*) > dinoflagellate (*A. sanguinea*) > raphidophyte (*H. akashiwo*) (Table 2). These differences in NaOCl toxicity may be related to the size and density of the cells (Franklin et al. 2002, Echeveste et al. 2010). Generally, small cells have a larger surface area than that of bigger cells. The cell sizes of *T. eccentrica*, *A. sanguinea*, and *H. akashiwo* were 15-110, 35-85, and 8-25 μm, respectively. Consequently, different tolerances to NaOCl toxicity may be related to the significant increase in the surface area of the three species. Moreover, much higher LC_{50} values for *T. eccentrica* compared to those for *A. sanguinea* and *H. akashiwo* may have resulted from the presence of siliceous frustules on *T. eccentrica* (Branco et al. 2010, Sánchez-Marín et al. 2010).

We used Φ_{PSII} as an indicator of phytoplankton health, as it is a rapid and immediate measure of photosynthetic capacity, regardless of cell density and size. There was a discrepancy between the survival (%) and Φ_{PSII} measurements of NaOCl effects. An 8-h exposure to the lowest NaOCl concentrations caused a significant reduction in survival (%) of *T. eccentrica*, *A. sanguinea*, and *H. akashiwo* but did not exhibit a noticeable effect on Φ_{PSII}. Additionally, Φ_{PSII} in *A. sanguinea* and *H. akashiwo* increased upon exposure to 1.0 ppm NaOCl after 4 h, likely due to the contribution of chlorophylls from dead cells to the F_0, but did not contribute to F_m in a live / dead cell mixture (Franklin et al. 2009). This result indicates that the Φ_{PSII} parameter was a relatively insensitive indicator of sublethal conditions, such as those due to NaOCl toxicity (Lumsden and Florence 1983).

Chl-*a* content of *A. sanguinea* increased rapidly after a 1-h exposure to the 1.0 and 1.5 ppm NaOCl treatments. This result may have been due to the production of pheophytin following exposure to NaOCl, with the fluorometer detecting the fluorescence of both live cells and pheophytin containing cells (Arar and Collins 1997). Cells with pheophytin emit greater fluorescence than that of live cells (Schreiber et al. 1994). Therefore, the *in vivo* fluorescence detected by the fluorometer was an overestimate when cells with pheophytin were present. However, this temporary increase in the Chl-*a* content was negligible.

Our study has demonstrated that NaOCl and elevated CO_2 act synergistically to reduce survival and Chl-*a* in the some species. The maximum CO_2 levels estimated for the end of the century (2100) were 0.2 pH units lower than those of today. However, NaOCl has been used extensively in coastal zones, where pH can fluctuate more widely (pH 7.4-8.4) than the range used in this study (Hofmann et al. 2011, Booth et al. 2012). Based on these findings, the toxicity of NaOCl may vary in coastal zones in the future. Furthermore, the synergistic effects of NaOCl due to increased CO_2 levels in seawater were species specific. Thus, further research on various marine organisms un-

der more widely pH ranges will be required.

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