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ORIGINAL ARTICLE

Effects of micronutrients on the detection of extracellular superoxide produced by the harmful raphidophyte *Chattonella antiqua* in culture

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Noxious *Chattonella* species cause many red tides and fish kills. The detection of extracellular O_2^- produced by these algae will be critical for the development of a rapid toxicity-evaluation method; however, such a development has been prevented by the mismatch of the extracellular O_2^- detection level between field and laboratory experiments. The aim of this study was to identify impediments to detect extracellular O_2^- in laboratory experiments. Our results indicate that Mn and Co in the medium decreased the detection levels of extracellular O_2^- produced by *Chattonella antiqua*, and the effect by Mn was stronger than that by Co. IC_{50} of the radical-scavenging activity of Mn was 17.5 μM, and little activity was detected at 8.75 μM or less. Furthermore, the level of extracellular O_2^- production by *C. antiqua* in Mn-depleted medium varied greatly under various culture conditions; the extracellular O_2^- production level of *C. antiqua* was high under conditions similar to those under which blooms develop in the field. Therefore, Mn is a key scavenger of extracellular O_2^-, and amounts of Mn must be carefully considered whenever extracellular O_2^- production levels of phytoplankton cultured with other kinds of media are measured.

KEYWORDS: *Chattonella*; fish mortality; harmful algal bloom; manganese; reactive oxygen species; superoxide

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INTRODUCTION

The raphidophyte *Chattonella marina* (Subrahmanyan) Y. Hara and Chihara var. *antiqua* (Hada) Demura and Kawachi (hereinafter, *C. antiqua*) is widely known as a noxious red-tide forming alga that leads to mass mortality of wild and aquacultured fish. The genus *Chattonella*, including *C. antiqua*, is widely distributed in the world (Imai and Yamaguchi, 2012), and blooms of these microalgae have caused many fish kills (Edvardsen and Imai, 2006 and references herein; Imai and Yamaguchi, 2012 and references herein; Nakashima *et al.*, 2019). In Japan, *C. antiqua* has frequently caused serious damage to wild and aquacultured fish ever since this species initially observed at Hiroshima Bay in 1969. More recently, mass mortality of aquacultured fish caused by red tides of this species have occurred frequently in coastal waters of western Japan, such as the Ariake and Yatsushiro Seas. Although a variety of hypotheses such as the physical clogging of gills by *Chattonella* cells, free fatty acids, reactive oxygen species (ROS) or varying combinations thereof have been proposed (Imai and Yamaguchi, 2012, and references therein; Hallegraeff *et al.*, 2017, and references therein), a mechanism by which *Chattonella* species kill fish is still unclear. To evaluate the toxicity of *Chattonella* and other microalgae in culture, the *in vitro* fish cell gill assay (Dorantes-Aranda *et al.*, 2013; Hallegraeff *et al.*, 2017; Imai and Yamaguchi, 2012) is widely known as a common and effective approach. However, this assay has been only used in cultures. Thus, it is essential to develop a rapid toxicity evaluation method for *Chattonella* species, which is used in common by field samples and laboratory experiments.

Recently, Diaz and Plummer (2018) reviewed production of extracellular ROS by phytoplankton, focusing on the production rates, taxonomic diversity, subcellular mechanisms, and functions of extracellular superoxide anion radical (O_2^-) and hydrogen peroxide (H_2O_2) production. ROS are known to damage to biomolecules such as lipids, proteins and DNA (Das and Roychoudhury, 2014); thus, these noxious substances seriously degrade cellular function (Apel and Hirt, 2004). Many studies (Kim *et al.*, 2006; Marshall *et al.*, 2005; Oda *et al.*, 1992, 1997) have reported that *Chattonella* species produce ROS such as O_2^-, H_2O_2, hydroxy radical and nitric oxide. In addition, it is suggested that an NADPH oxidase (NOX)-like enzyme located on the cell surface of *C. marina* cells may be involved in extracellular O_2^- generation (Kim *et al.*, 2000, 2007). Recently, Shikata *et al.* (2019) used RNA-sequencing analysis to find six putative genes that encode NOX in the genome of *C. antiqua*. Although a mechanism of fish kills by *Chattonella* species is still unclear as described above, a clear correlation between the cell's ability to produce O_2^- and toxicity has been reported (Ishimatsu *et al.*, 1996). In addition, Ishimatsu *et al.* (1996) observed that dead cells and culture filtrate exhibited no toxicity. Therefore, the detection of extracellular O_2^- produced by living *Chattonella* cells is a useful tool for the development of a rapid method of toxicity-evaluation of *Chattonella* species.

Chemiluminescence method (Godrant and Sarthou, 2009; Lee *et al.*, 1995; Nakashima *et al.*, 2006) is one of valid and feasible methods to detect extracellular O_2^- produced by living *Chattonella*. In addition, Lee *et al.* (1995) proposed that chemiluminescence method could be applicable for the detection of *Chattonella marina* cells in the early stage of its red tides because of high detection sensitivity. However, Shikata *et al.* (2020) observed that the extracellular O_2^- detection level of laboratory chemiluminescence assays is only one-fortieth of the detection level for field samples. Such a mismatch of O_2^- detection level between field samples and laboratory experiments has impeded the clarification of fish-killing mechanisms of *Chattonella* species and the development of a rapid toxicity-evaluation method using the chemiluminescence assay. Firstly, a big difference between the field and laboratory samples is amounts of nutrients. In many cases, amounts of nutrients in culture media are hundreds or thousands of times higher than that in seawater to prevent the limitation of the phytoplankton growth caused by nutrients deficiency. Thus, we suspect the presence of some sort of inhibitors dissolved in culture media affect the detection of extracellular O_2^- produced by *Chattonella* species, and we take particular note of Mn included in media because there are reports that manganese scavenges ROS (Archibald and Fridovich, 1982; Hansard *et al.*, 2011; Hussain and Ali, 1999; Wuttig *et al.*, 2013). On the other hand, a likely explanation is that several environmental factors such as light intensity, temperature, salinity and amounts of nutrients may make an enormous difference in the O_2^- detection level between field samples and laboratory experiments. In fact, Yuasa *et al.* (2020a, 2020b) reported that the extracellular O_2^- production by *C. antiqua* varied greatly as a function of culture conditions such as light intensity, light-intensity modulation and macronutrient limitation. Therefore, other environmental conditions such as temperature and salinity may also affect the extracellular O_2^- prodction by *Chattonella* species but it has not been investigated.

To detect extracellular O_2^- in laboratory experiments at the comparable level as those in field samples, in the present study, we investigated radical-scavenging activities of micronutrients in modified SWM-3 medium. In addition, we established a requirement for the composition of a medium that does not interfere with extracellular O_2^- detection. Furthermore, using the medium optimized for

Table I: Components of modified SWM-3 medium

$NaNO_3$	1 mM
NaH_2PO_4-$2H_2O$	50 μM
EDTA·2Na ($C_{10}H_{14}N_2Na_2O_8.2H_2O$)	115 μM
Fe (III)-EDTA ($C_{10}H_{12}FeN_2NaO_8.3H_2O$)	1 μM
$NaSiO_3$-$9H_2O$	27 μM
$MnCl_2$-$4H_2O$	35 μM
$CoCl_2$-$6H_2O$	100 nM
$ZnCl_2$	4 μM
H_2SeO_3	13 nM
H_3BO_3	1 mM
Cyanocobalamin (Vitamin B_{12})	740 pM
Thiamin Hydrochloride (Vitamin B_1 Hydrochloride)	30 μM
Biotin (Vitamin H)	4 nM
Tris (hydroxymethyl) aminomethane	3 mM

extracellular O_2^- detection, we evaluated the optimal temperatures and salinities for extracellular O_2^- production by growth experiment of *C. antiqua* under a crossed factorial design with 15 combinations of 5 temperatures (20.0, 22.5, 25.0, 27.5 and 30.0°C) and 3 salinities (25, 30 and 35).

METHOD

Algal species and culture conditions

An axenic strain of *C. antiqua* (strain NIES-1) was obtained from the National Institute for Environmental Studies (Ibaraki, Japan). The culture was maintained in an incubator in 100-mL flasks containing 50 mL of modified SWM-3 (Table I) with a salinity of 30 at 25°C under 200 μmol photons m^{-2} s^{-1} of cool-white fluorescent illumination on a 14:10 light:dark cycle. The media used in this study was removed $CuCl_2$-$2H_2O$, calcium pantothenate, nicotinic acid, *p*-aminobenzonic acid, inositol, folic acid and thymine from the original medium (Imai, 2012). Irradiance in the incubator was measured with a Quantum Scalar Laboratory Irradiance Sensor (QSL-2100/2101; Biospherical Instruments, San Diego, CA, USA). The natural seawater used for the culture medium was collected on April 20th in 2018 from the East China Sea in an area around Kamikoshikijima Island (lat 32°00′03″N, long 130°00′02″E), and the filtered seawater was aged in the laboratory for more than 1 year.

Detection of extracellular superoxide by chemiluminescence assay

Extracellular superoxide (O_2^-) was detected by chemiluminescence assay using L-012 sodium salt (8-Amino-5-chloro-2,3-dihydro-7-phenyl-pyrido[3,4-d]pyridazine sodium salt, FUJIFILM Wako Pure Chemical Corp., Osaka, Japan) as the chemiluminescent probe as described in a previous study (Yamasaki *et al.*, 2010). The probe was dissolved in ultrapure water and stored at −80°C until use. The reaction mixtures typically consisted of 970 μL of a *C. antiqua* cell suspension, 20 μL of phosphate buffered saline (PBS, pH 7.0) in the presence or absence of 200 U superoxide dismutase (SOD; Cu, Zn-SOD, FUJIFILM Wako Pure Chemical Corp.), which is known as a radical scavenger, and 10 μL of L-012 (final concentration, 10 μM). After the addition of L-012 into each flagellate cell suspension in a test tube (φ12 × 55 mm), the chemiluminescence was recorded immediately with a luminometer (AB-2270 Luminescencer Octa; ATTO Corp., Tokyo, Japan) for 30 s. As a control, we measured the chemiluminescence response in the modified SWM-3 medium containing the probe alone, without added flagellate cells. The O_2^- levels were calculated by subtracting the detected values of chemiluminescence in the presence of SOD from the detected values of chemiluminescence in the absence of SOD. The chemiluminescence assay was performed at room temperature in triplicate.

Radical-scavenging activity measurement of micronutrients

To investigate the effects of micronutrients in the modified SWM-3 medium on the detection of extracellular O_2^- produced by *C. antiqua*, we conducted the chemiluminescence assay to measure the radical-scavenging activities of micronutrients individually at concentrations identical to those in the modified SWM-3 medium (Table I). Note that we did not examine the radical-scavenging activities of iron because iron is an essential trace metal for the growth of *C. antiqua* (Imai and Yamaguchi, 2012; Iwaski, 1973; Okaichi and Montani, 2004). The reaction mixtures typically consisted of 960 μL of a *C. antiqua* cell suspension (2.7×10^3 cells mL^{-1}), 10 μL of test solution or ultrapure water as a control, 20 μL of PBS and 10 μL of L-012 (final concentration, 10 μM). After the addition of L-012 into each flagellate cell suspension in a test tube (φ12 × 55 mm), the chemiluminescence was recorded immediately with the same luminometer described above. The chemiluminescence assay was performed at room temperature and in triplicate.

To verify the O_2^- scavenging activity of Mn dissolved in ultrapure water, radical-scavenging activity (i.e. the inhibition rate of O_2^-) was also measured under the cell-free system (i.e. without addition of a *C. antiqua* cell suspension) and calculated by using the SOD Assay Kit-WST (Dojindo Laboratories, Inc., Kumamoto, Japan)

according to the manufacturer's instructions. In this method, O_2^-, which is generated by the xanthine/xanthine oxidase system, is detected by spectrophotometric measurement of a water-soluble formazan dye produced from a highly water-soluble tetrazolium salt, WST-1 (2-(4-Iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-*2H*-tetrazolium, monosodium salt) upon reduction with O_2^-. In addition, the natural seawater did not use for this experiment to exclude an effect of naturally occurring Mn and other trace elements.

Growth experiment and detection of extracellular O_2^- in the presence or absence of Mn

To investigate the effects of Mn on the growth and production of extracellular O_2^- by *C. antiqua*, in this study, we used two different media as follows: the complete modified SWM-3 medium and a Mn-depleted medium (modified SWM-3 without addition of $MnCl_2$-$4H_2O$). The growth experiment was conducted at 25°C in 100-mL flasks containing 50 mL of complete medium or Mn-depleted medium at a salinity of 30. A cell suspension of *C. antiqua* in early stationary phase ($2–3 \times 10^4$ cells mL^{-1}) was diluted to a density of 5×10^3 cells mL^{-1} with the complete medium or the Mn-depleted medium; then, 1 mL of each cell suspension was added into 49 mL of the same fresh medium. Three replicate flasks were used for each treatment. All flasks were gently mixed by hand twice a day and randomly rearranged to minimize the effects of light or temperature gradients in the incubator. Cell numbers of *C. antiqua* in 0.5-mL subsamples collected at 2-day intervals were counted microscopically. In addition, extracellular O_2^- was detected by chemiluminescence assay as described above.

Growth and detection of extracellular O_2^- under several combinations of water temperature and salinity

The growth experiment was conducted in 100-mL flasks containing 50 mL of Mn-depleted medium. We used a crossed factorial design with 15 combinations of 5 temperatures (20.0, 22.5, 25.0, 27.5 and 30.0°C) and 3 salinities (25, 30 and 35). A cell suspension of *C. antiqua* in early stationary phase ($2–3 \times 10^4$ cells mL^{-1}) was diluted to a density of 5×10^3 cells mL^{-1} with the Mn-depleted medium at each experimental salinity. Then, 1 mL of each cell suspension was added into 49 mL of the fresh Mn-depleted medium at each experimental salinity. Three replicate flasks were used for each treatment. All flasks were gently mixed by hand twice a day and randomly rearranged to minimize the effects of light or temperature gradients in the incubator. Cell numbers of *C. antiqua* in 0.5-mL subsamples collected at 2-day intervals were counted microscopically. In addition, extracellular O_2^- was detected by chemiluminescence assay as described above. The maximum growth rates during the growth experiment were determined for each flask from 3 consecutive data points using the method of Brand *et al.* (1981).

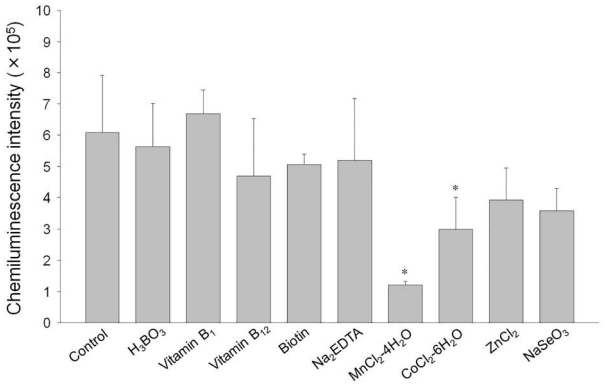

Fig. 1. Effects of micronutrients in the medium on the detection of extracellular O_2^- produced by *C. antiqua*. The extracellular O_2^- was detected with a chemiluminescence assay (see the Method section for details) and indicated as chemiluminescence intensity. Data are means ± SD of triplicate measurements. Asterisks (*) indicate significant differences from control at $P < 0.05$ (one-way ANOVA followed by Dunnett's *post hoc* test).

Statistical analyses

The data of radical scavenging activities of micronutrients (Fig. 1) were analyzed by one-way analysis of variance (ANOVA) and then tested using Dunnett's *post hoc* test. The data of radical scavenging activity of Mn were measured by using the SOD Assay Kit-WST (Fig. 2B), and the culture experiment under several combinations of water temperature and salinity (Table II) were analyzed by one-way ANOVA and then by Tukey's *post hoc* test. The analysis was performed using SPSS version 19.0 for Windows (SPSS, Inc., Chicago, IL, USA). A significance level of $P < 0.05$ was used for the test.

RESULTS

Radical-scavenging activity of micronutrients

To investigate the effects of each micronutrient in the modified SWM-3 medium on the detection of extracellular O_2^- produced by *C. antiqua*, the chemiluminescence assay was conducted. The total levels of extracellular O_2^- (i.e. integration value for 30 s) decreased in the presence

Table II: Effects of Mn-depleted medium on the maximum growth rate of C. antiqua under a crossed factorial design with 15 combinations of 5 temperatures and 3 salinities

	Salinity		
	25	30	35
Temperature (°C)	Maximum growth rate (divisions day^{-1})		
20.0	$0.64 \pm 0.19^{a,b,c}$	$0.50 \pm 0.15^{a,b}$	0.45 ± 0.15^{a}
22.5	$0.85 \pm 0.12^{c,d,e}$	$0.77 \pm 0.09^{a,b,c,d}$	$0.80 \pm 0.03^{b,c,d}$
25.0	1.13 ± 0.10^{e}	1.17 ± 0.03^{e}	$0.95 \pm 0.08^{c,d,e}$
27.5	1.16 ± 0.16^{e}	$1.09 \pm 0.11^{d,e}$	$1.08 \pm 0.04^{d,e}$
30.0	$0.85 \pm 0.09^{c,d,e}$	$0.87 \pm 0.04^{c,d,e}$	$0.74 \pm 0.08^{a,b,c}$

Data are means ± SD (bars) of triplicate measurements. Different lowercase letters differ significantly at $P < 0.05$ (one-way ANOVA followed by Tukey's *post hoc* test).

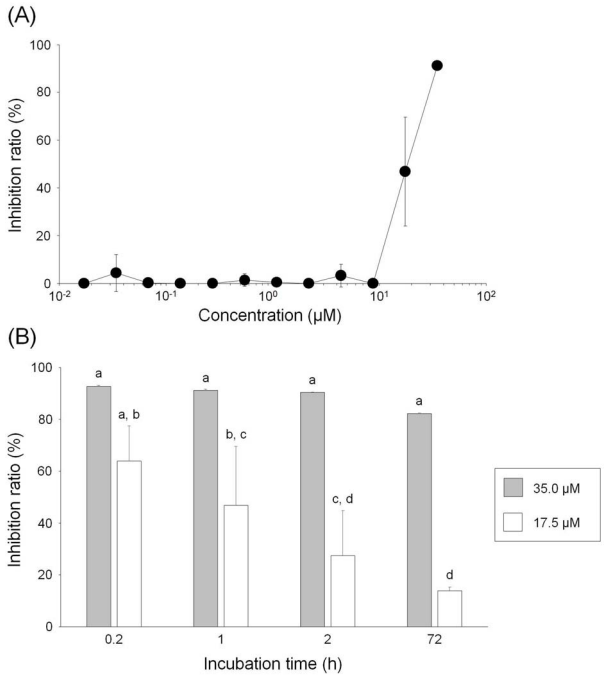

Fig. 2. Radical-scavenging activity of various concentrations of Mn (A) and two concentrations over four reaction times (B). Radical-scavenging activity was measured with the SOD Assay Kit-WST according to the manufacturer's instructions. Data are means ± SD of triplicate measurements. Bars with different lowercase letters differ significantly at $P < 0.05$ (one-way ANOVA followed by Tukey's *post hoc* test).

of Mn and Co (Fig. 1; $P < 0.05$), and Mn had particularly high radical-scavenging activity (Fig. 1). Although Zn and SeO_3 tended to slightly decrease total levels of extracellular O_2^-, differences from control were not significant. The other micronutrients did not affect detection.

In the next experiment, radical-scavenging activity of Mn was measured by using the SOD Assay Kit-WST under the cell-free system. IC_{50} of the radical-scavenging activity of Mn was 17.5 μM, and little activity was detected at concentrations of 8.75 μM (25% of the Mn concentration in the modified SWM-3) or less (Fig. 2A). Furthermore, with the Mn concentration the same as in the modified SWM-3 medium, the radical-scavenging activity remained high for 72 h, but with half that concentration, the activity decreased over time (Fig. 2B).

Effects of Mn on *C. antiqua* growth and detection levels of extracellular O_2^-

Chatonella antiqua cells grew and produced extracellular O_2^- in culture in the complete and Mn-depleted media (Fig. 3). Although the maximum cell density of *C. antiqua* was almost the same in both media, chemiluminescence intensity was dramatically higher in the Mn-depleted medium (Fig. 3B) than in the complete medium (Fig. 3A). The detection levels (total) of extracellular O_2^- in both media increased gradually and peaked on Day 8 in the complete medium and Day 6 in the Mn-depleted medium. After peaking, detection levels (total) of extracellular O_2^- in both media decreased gradually. Incidentally, the detection levels (total) of extracellular O_2^- in the Mn-depleted medium during the period of Day 2 to Day 10 were 37-fold higher (maximum: 50-fold, day 6; minimum: 25-fold, day 2) compared with the complete medium.

Similarly, although the growth of *C. antiqua* in both media was almost the same, detection levels (per cell) of extracellular O_2^- (i.e. integration value for 30 s per cell) in the Mn-depleted medium (Fig. 4B) were dramatically higher than that in the complete medium (Fig. 4A). The detection levels (per cell) of extracellular O_2^- of *C. antiqua* cultured in both media sharply increased and peaked on Day 4; after that, detection levels (per cell) of extracellular O_2^- in both media decreased gradually (Fig. 4). Incidentally, the detection levels (per cell) of extracellular O_2^- in the Mn-depleted medium during the period of Day 2 to Day 10 were 37-fold higher (maximum: 50-fold, Day 6; minimum: 22-fold, Day 8) compared with the complete medium.

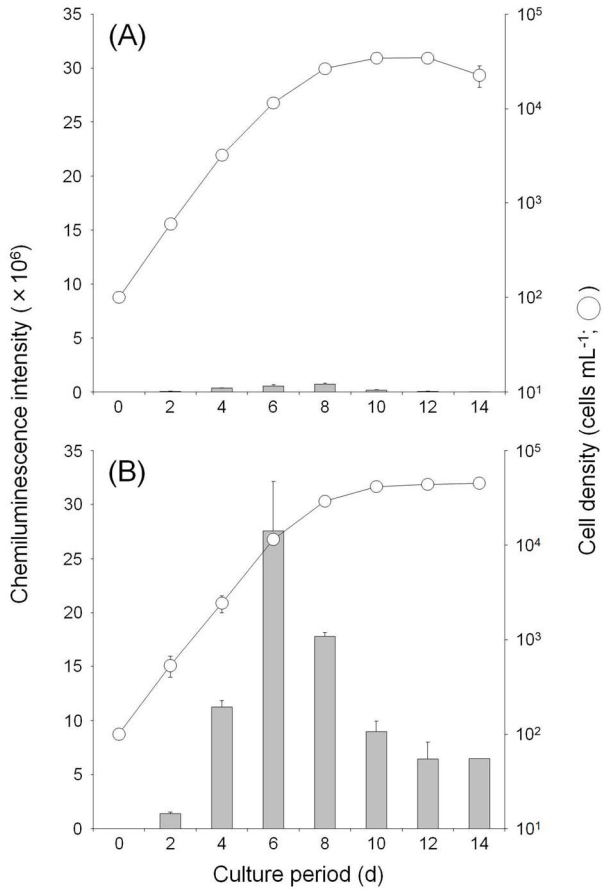

Fig. 3. The growth (cell density) of and detection levels (total) of extracellular O_2^- produced by *C. antiqua* cultured in complete medium (A) or Mn-depleted medium (B). *Chattonella antiqua* cells were counted microscopically. The extracellular O_2^- production by *C. antiqua* was detected with a chemiluminescence assay (see the Method section for details) and is indicated as chemiluminescence intensity. Data are means ± SD of triplicate measurements.

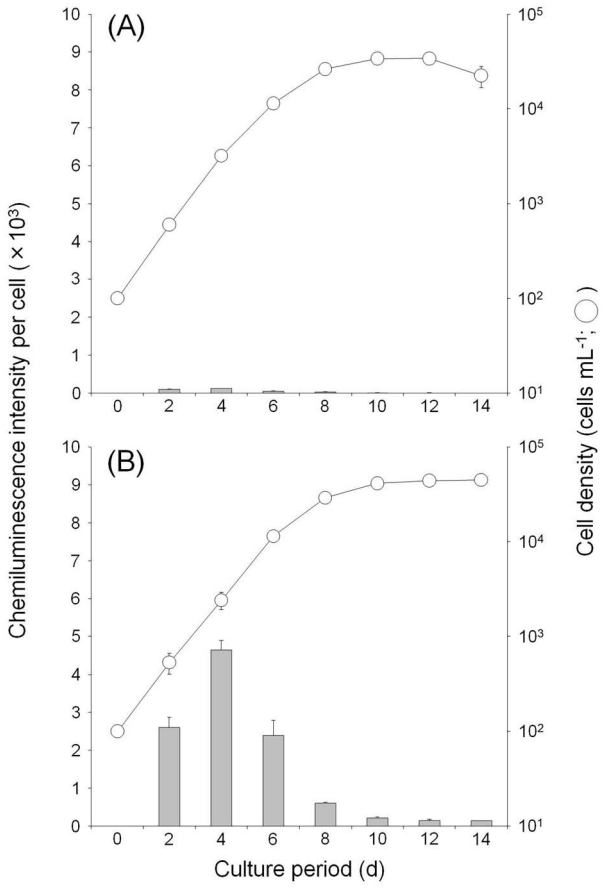

Fig. 4. The growth (cell density) of and detection levels (per cell) of extracellular O_2^- (per cell) produced by *C. antiqua* cultured in complete medium (A) or Mn-depleted medium (B). Cell numbers of *C. antiqua* were counted microscopically. The extracellular O_2^- production by *C. antiqua* was detected with a chemiluminescence assay (see the Method section for details) and is indicated as chemiluminescence intensity per cell. Data are means ± SD of triplicate measurements.

Effects of water temperature and salinity on *C. antiqua* growth and levels of extracellular O_2^-

The maximum growth rate of *C. antiqua* tended to be high at water temperatures from 25.0 to 27.5°C and salinities of 25–30 (Table II); the cell density of *C. antiqua* during the experiments tended to be high at water temperatures ranging from 22.5 to 27.5°C and salinities of 25–30 (Fig. 5D, G and J). In contrast, the maximum growth rate and the cell density of *C. antiqua* during the experiments tended to be low at 20.0 and 30.0°C (Fig. 5A and M, Table II), and the maximum growth rate was the lowest of all experimental conditions when *C. antiqua* cells were cultured at 20.0°C, salinity of 35 (Fig. 5A, Table II). Note, however, that *C. antiqua* cells continued to grow moderately at 20.0°C (Fig. 5A), while they reached the declining phase within a short time at 30.0°C (Fig. 5M).

The total levels of extracellular O_2^- by *C. antiqua* tended to be high at water temperatures ranging from 20.0 to 25.0°C and salinities of 25 and 30; it tended to be low at temperatures ≥27.5°C (Fig. 5B, E, H, K and N). The maximum total levels of extracellular O_2^- were observed at 25.0°C, salinity of 25 (Fig. 5H, Supplementary Table SI) and the minimum at 27.5°C, salinity of 35 (Fig. 5K, Supplementary Table SI). In contrast, per cell levels of extracellular O_2^- tended to be low at water temperatures ranging from 20.0 to 25.0°C and high at temperatures ≥27.5°C (Fig. 5C, F, I, L and O). The maximum per cell levels of extracellular O_2^- were observed at 30.0°C, salinity of 35 (Fig. 5O, Supplementary Table SI) and the minimum at 22.5°C, salinity of 25 (Fig. 5K, Supplementary Table SI).

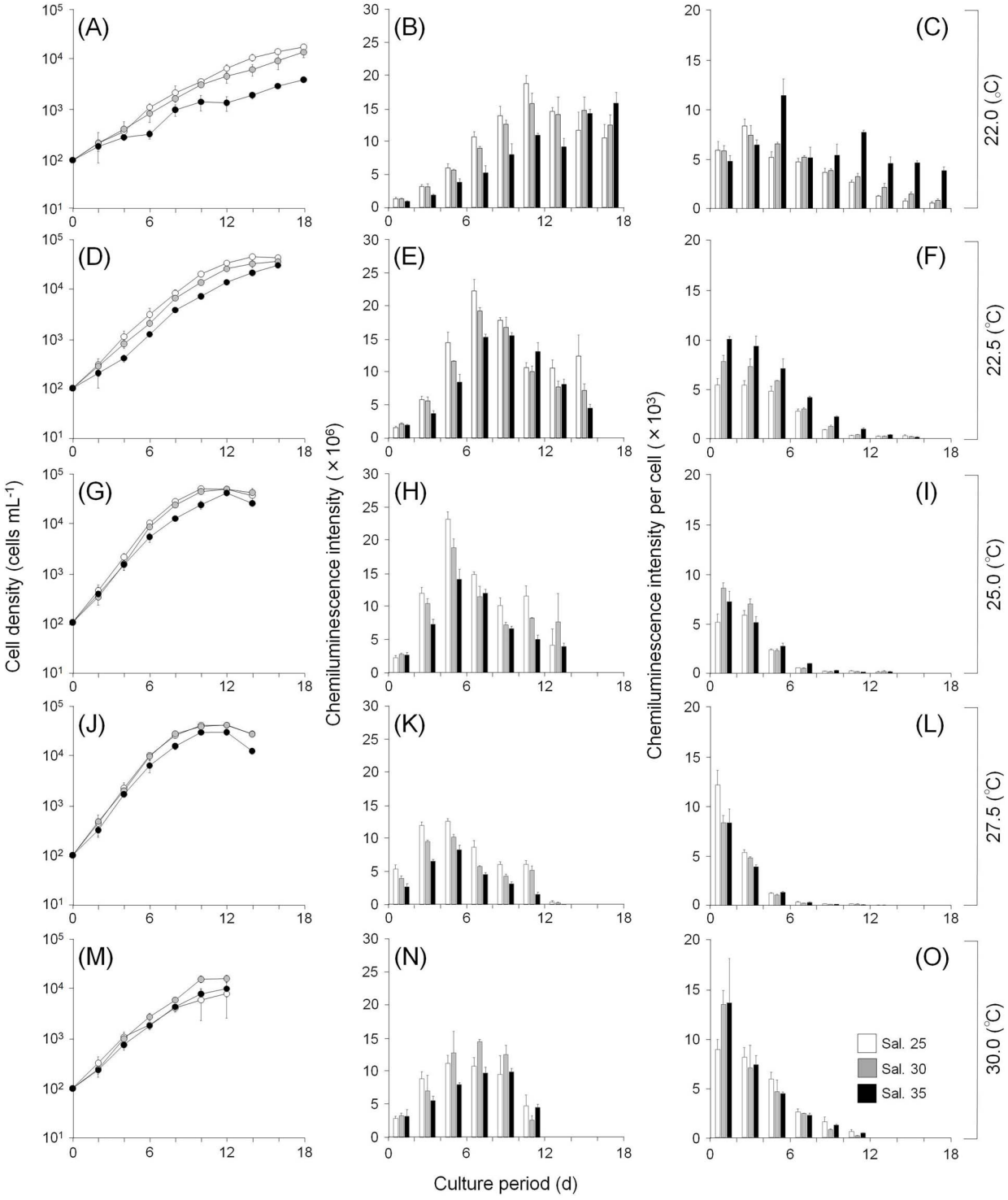

Fig. 5. Effects of Mn-depleted medium on *C. antiqua* growth and extracellular O_2^- levels (total and per cell) under a crossed factorial design with 15 combinations of 5 temperatures and 3 salinities. Cell numbers were counted microscopically (panels A, D, G, J and M). The extracellular O_2^- produced by *C. antiqua* was detected with a chemiluminescence assay (see the Method section for details) and is indicated as chemiluminescence intensity (total in panels B, E, H, K and N; per cell in panels C, F, I, L and O). Data are means ± SD of triplicate measurements.

DISCUSSION

Effects of micronutrients in the medium on detection levels of extracellular O_2^- produced by *C. antiqua*

Several studies reported that manganese (Archibald and Fridovich, 1982; Hansard *et al.*, 2011; Hussain and Ali, 1999; Wuttig *et al.*, 2013) and iron (Rose, 2012; Voelker and Sedlak, 1995) play a key role as superoxide scavengers in aquatic systems. In this study, we investigated the effects of micronutrients in the modified SWM-3 medium (Table I) on the detection of extracellular O_2^-. The detection levels (total) of extracellular O_2^- produced by *C. antiqua* were significantly decreased at Mn and Co concentrations identical to those in modified SWM-3 medium (Fig. 1, $P < 0.05$). Then, the effect by Mn was higher than that by Co (Fig. 1). In addition, the radical-scavenging activity of Mn was concentration-dependent, and little inhibition was detected at a concentration of 8.75 μM (25% of the Mn concentration in the modified SWM-3) or less (Fig. 2A). These results suggest that Mn in the modified SWM-3 medium either eliminates extracellular O_2^- or directly inhibits extracellular O_2^- production by *C. antiqua*. To verify the possibility of directly inhibition of extracellular O_2^- production by *C. antiqua*, radical-scavenging activity of Mn was measured by using the xanthine/xanthine oxidase system (i.e. cell-free reaction). The high inhibition ratio of O_2^- detection by Mn at a concentration of 35.0 μM continued for 72 h (i.e. the xanthine/xanthine oxidase system retained enzymatic activity after 72 h), but the inhibition ratio of O_2^- detection by Mn at a concentration of 17.5 μM decreased over time (Fig. 2B), suggesting that Mn scavenges the extracellular O_2^- produced by *C. antiqua* but does not inhibit extracellular O_2^- production by *C. antiqua*. Moreover, these results suggest that under the continuous supply of O_2^-, Mn cannot scavenge the extracellular O_2^- above a threshold level. In addition, Minakawa *et al.* (1996) measured total dissolvable manganese in the East China Sea and reported that the concentrations of Mn in samples collected from areas around Kyushu Island ranged from 2.4 to 5.0 nM. Therefore, the Mn concentration in natural seawater used in the present study is estimated to well below the IC_{50} (17.5 μM).

On the other hand, Itoh *et al.* (2009) performed quantitative analysis of trace metals in 6 coastal seawater samples around Okinawa and reported that the concentrations of Mn ranged from 146 pM to 110 nM (0.0080–6.03 μg L^{-1}) and of Co ranged from 59 to 577 pM (0.0035–0.034 μg L^{-1}). Though quantitative values of Mn and Co in samples of coastal areas were one two-hundredth or less of the concentration in the modified SWM-3, the utilization of metal chelators reported previously (Roe *et al.*, 2016) will be effective in minimizing the scavenging effect of O_2^- by trace elements such as Mn and Co. Furthermore, the composition and amounts of Mn and Co in media must be considered if detection of extracellular O_2^- produced by phytoplankton is necessary in the laboratory experiments. Note, however, that the inhibition of extracellular O_2^- detection by Mn and Co might be of concern only in modified SWM-3 medium. In other words, inhibition of extracellular O_2^- detection by micronutrients can change as a function of the amounts added, and it is possible that if other media were used in culture experiments, then other micronutrients might inhibit detection of extracellular O_2^-. Therefore, the dependence of the composition and amounts of micronutrients on the types of media must be thoughtfully considered whenever phytoplankton species, strains and/or regional differences are discussed in relation to the levels of extracellular O_2^- produced by phytoplankton.

Effects of Mn-depleted medium on *C. antiqua* growth and levels of extracellular O_2^- under various culture conditions

In this study, the maximum cell density of *C. antiqua* in the presence or absence of Mn was almost the same (Figs 3 and 4), consistent with Nakamura and Watanabe (1983), who reported that the addition of Mn^{2+}, Zn^{2+} and Co^{2+} had no effect on the growth of *C. antiqua*. As well as the previous study (Kim *et al.*, 2004), our results indicated that extracellular O_2^- production was maximal during exponential growth and subsequently decreased during the stationary phase. Though it is not possible to make a simple comparison of our results and the field samples (Shikata *et al.*, 2020) because the chemiluminescence method in these studies was not calibrated to superoxide concentrations by using the xanthine/xanthine oxidase system, total levels of extracellular O_2^- were 37-fold higher in *C. antiqua* cultured in Mn-depleted medium (Figs 3B and 4B) than in complete medium (Figs 3A and 4A), and the extracellular O_2^- detected with depleted medium appeared to be similar to the level detected in field samples (Shikata *et al.*, 2020). Thus, cultivation with Mn-depleted medium is useful for chemiluminescent extracellular O_2^- detection in laboratory experiments. To demonstrate that this method can recreate the extracellular O_2^- detection level of field samples, further chemiluminescence assay with detection limits or concentrations of O_2^- in the field and laboratory experiments are needed. Note that Mn-depleted media should be used for only experiments of O_2^- detection because *C. antiqua* can be successfully cultured in Mn-depleted medium for only a few generations (Data

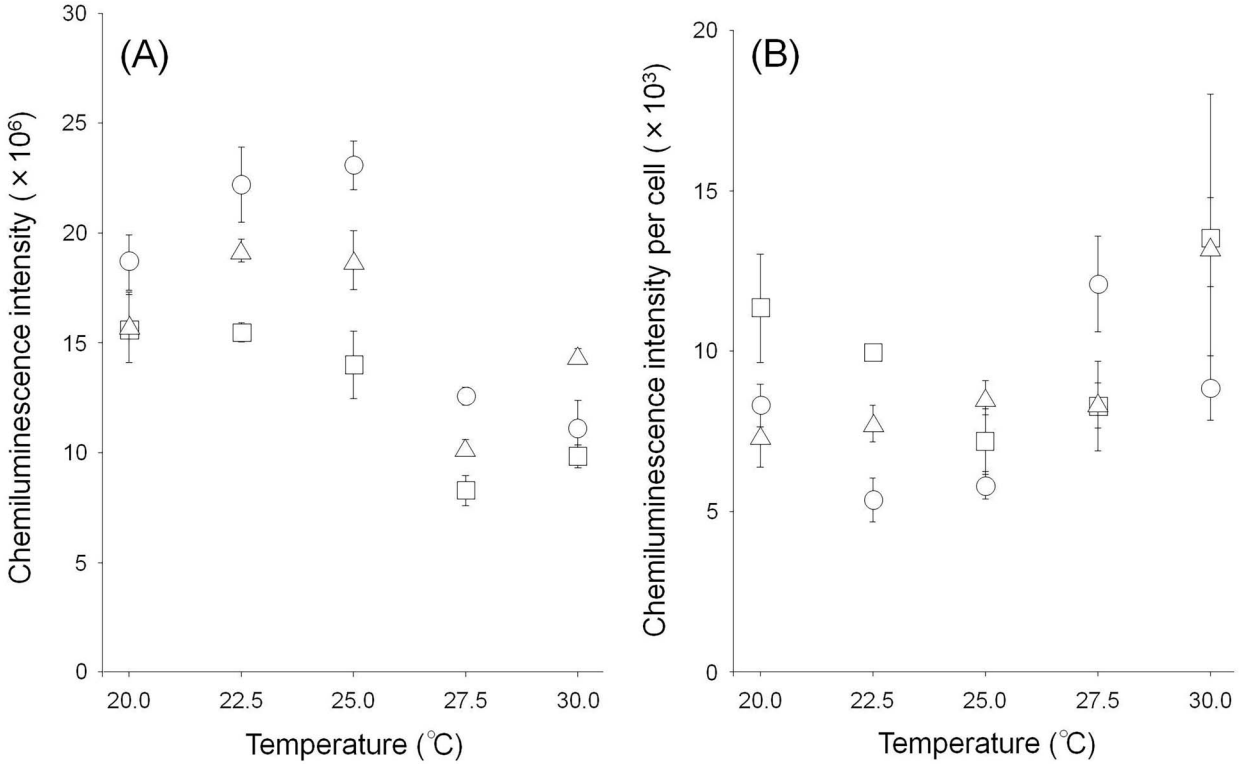

Fig. 6. Effects of Mn-depleted medium on the maximum extracellular O_2^- levels of total (A) and per cell (B) of *C. antiqua* under a crossed factorial design with 15 combinations of 5 temperatures and 3 salinities.

not shown). Though our results indicate that Mn is a key factor in a mismatch of O_2^- detection level between field samples and laboratory experiments, further study on the effect of Co will be necessary in the future.

Environmental factors such as light levels (Tzovenis *et al.*, 1997), nutrients (Chu *et al.*, 1996; Tzovenis *et al.*, 1997), temperature (Pahl *et al.*, 2010; Zhu *et al.*, 1997), pH (Pahl *et al.*, 2010) and salinity (Pahl *et al.*, 2010) affect the growth and the nutritional value of microalgae. Several studies reported that phytoplankton induce or augment the production of allelochemicals under nutrient-limiting conditions (Gross, 2003 and references herein; Legrand *et al.*, 2003 and references herein). Recently, several studies reported that the extracellular O_2^- production by *C. antiqua* varied greatly as a function of culture conditions such as light intensity, light-intensity modulation and macronutrient limitation (Yuasa *et al.*, 2020a, 2020b). In addition, Lee *et al.* (1995) reported that the comparatively higher growth and MCLA-dependent chemiluminescence of *C. marina* at 20.0–25.0°C suggest a relatively high probability of O_2^- release in the summer where outbreaks of the red tides of *C. marina* occur and cause mass fish death. To verify the possibility of toxicity change associated with a wide range of environmental conditions in the field, in the present study, we examined *C. antiqua* growth and levels of extracellular O_2^- under several different combinations of water temperature and salinity. As has been the case with past findings in laboratory experiments (Yamaguchi *et al.*, 1991) and field observations (Imai and Yamaguchi, 2012), in this study, the optimal conditions for both growth and extracellular O_2^- production were at 25.0°C and salinity of 25 (Fig. 5, Table II, Supplementary Table SI); the conditions under which *C. antiqua* produced high extracellular O_2^- levels in our laboratory study were similar to those under which blooms formed in the field. For instance, Nakashima *et al.* (2019) observed the mortality of cultured yellowtail *Seriola quinqueradiata* caused by *Chattonella* bloom (50 cells mL^{-1} and upwards) in the Yatsushiro Sea, Japan in summer 2016, and reported that water temperatures of the sea surface and water depth from 20 to 40 m were ranging from 25.8 to 30.9°C and 25.1 to 27.0°C, respectively. Therefore, the detection of extracellular O_2^- by a chemiluminescence method may provide useful data for the risk evaluation of fish mortality.

The growth of *C. antiqua* tended to be low at both 20.0 and 30.0°C (Fig. 5A and M, Table II), but total levels of extracellular O_2^- by *C. antiqua* tended to be high at 20.0°C and to be low at temperatures ≥27.5°C (Fig. 6A). The difference between the two temperature conditions

is that *C. antiqua* cells continued to grow moderately at 20.0°C (Fig. 5A), but at 30.0°C, they reached the declining phase within a short time (Fig. 5M); that is the extracellular O_2^- production by *C. antiqua* depends on the growth phase. Yuasa *et al.* (2020a) reported that the production of extracellular O_2^- in *C. antiqua* cells is regulated by photosynthesis because reducing power derived from electron transport might be required for the extracellular O_2^- production by NADPH oxidase (NOX). Thus, it is believed that the increase of extracellular O_2^- production during exponential growth under optimal growth conditions is due to active photosynthesis of *C. antiqua* cells. In contrast to total levels of extracellular O_2^-, the per cell levels of extracellular O_2^- tended to be low at 20.0–25.0°C and high at temperatures ≥27.5°C (Fig. 6B). Tillmann *et al.* (2009) suggested that stress and/or limiting growth conditions such as nutrient limitation may affect quantitative differences in excretion of lytic compounds. ROS are mainly produced at a low level in organelles under optimal growth conditions, but the rate of ROS production is dramatically elevated during stress (Suzuki and Mittler, 2006). In land plants, such as *Arabidopsis*, for example, temperature stresses enhanced the production of ROS and ROS-associated injury (Larkindale and Knight, 2002). Furthermore, Yuasa *et al.* (2020b) found that the levels of extracellular O_2^- in N- or P-deficient cultures of *C. antiqua* were high during the dark period when photosynthetic activity was low, and they suggested that a metabolic pathway independent of photosynthesis provides NADPH for extracellular O_2^- production under nutrient deficiency. Although the mechanism that controls this extracellular O_2^- production remains unclear, high-temperature stress at ≥27.5°C may briefly increase extracellular O_2^- production per cell of *C. antiqua* and rapidly shift the population toward the declining phase.

CONCLUSIONS

Mn and Co at concentrations identical to those in modified SWM-3 medium significantly decreased the detection levels of extracellular O_2^- produced by *C. antiqua* ($P < 0.05$); the inhibition by Mn was greater than that by Co. Consequently, Mn is a key scavenger of extracellular O_2^-, and a cultivation method with Mn-depleted medium is necessary for chemiluminescent extracellular O_2^- detection in laboratory experiments because this method can facilitate the more sensitive extracellular O_2^- detection. Furthermore, extracellular O_2^- production by *C. antiqua* varied with changes of water temperature and salinity; of particular interest, the total levels of extracellular O_2^- were high under conditions under which blooms form in the field. Consequently, our results clarified that the measurement of O_2^- reflects the combined influences of factors such as Mn and other metals that affect the technical detection limit of O_2^- and environmental factors that affect the physiological regulation of O_2^- production. Because certain metals included in culture media, such as Mn and Co, can eliminate the extracellular O_2^-, the composition and amounts of micronutrients in culture media require scrupulous attention if the cultures will be used to chemiluminescently detect extracellular O_2^- produced by phytoplankton. Future research used in the combination of the measurement conditions established in this study and the *in vitro* fish cell gill assay (Dorantes-Aranda *et al.*, 2013; Hallegraeff *et al.*, 2017; Imai and Yamaguchi, 2012) may contribute to the elucidation of the mechanisms of red-tide outbreaks and fish kills caused by *C. antiqua* as well as the establishment of a rapid toxicity evaluation method for *Chattonella* species in the field.

SUPPLEMENTARY DATA

Supplementary data is available at Journal of Plankton Research online.

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CONFLICT OF INTEREST

The authors declare that this study was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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