Changes in O_2^- and H_2O_2 production by *Chattonella antiqua* during diel vertical migration under nutrient stratification

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ABSTRACT: The red tide flagellate *Chattonella antiqua* produces high levels of reactive oxygen species (ROS) such as the superoxide anion (O_2^-) and hydrogen peroxide (H_2O_2). Production of ROS by this flagellate species depends on nutrient and water conditions during its growth phases. To clarify the effect of nutrient depletion on ROS production, we investigated the production pattern of ROS in a culture tank containing $1 m^3$ of medium with low nitrate and phosphate nutrient concentrations. The production of O_2^- and H_2O_2 by *C. antiqua* was lower when nutrients were limited than when they were fully supplied (f/2 medium), as indicated by reductions in nitrogen and phosphorus cell quotas. We also examined ROS formation by *C. antiqua* under stratified conditions subsequent to the nutrient depletion experiment. The vertical stratification of salinity (ΔS = 2.5 psu) and temperature (ΔT = 5°C) in the tank were analogous to natural conditions during red tides, and only the bottom layer was enriched with nutrients. *C. antiqua* showed clear diel vertical migration under stratified conditions. The ROS production due to photosynthesis was high during the light period at the surface, even though the ambient nutrient concentration of the surface was low, and it decreased in the dark bottom layer. The generation of H_2O_2 increased continuously for 4 d, but O_2^- generation peaked on the second day of stratification and then gradually decreased.

KEY WORDS: *Chattonella antiqua* · Diel vertical migration · Hydrogen peroxide · Nutrient stratification · Reactive oxygen species · Superoxide anion

INTRODUCTION

Chattonella antiqua is a dominant red tide-forming phytoplankton species worldwide and causes serious damage to fish farms. The mechanism of ichthyotoxicity of *C. antiqua* is not known in detail, but *Chattonella* spp. generate reactive oxygen species (ROS) such as the superoxide anion (O_2^-) and hydrogen peroxide (H_2O_2) (Shimada et al. 1991, 1993, Oda et al. 1992, 1995, Tanaka et al. 1992, 1994, Marshall et al. 2002). Since ROS are generally toxic to living organisms (Oda et al. 1989, 1997), those generated by *Chattonella* spp.

may cause gill tissue injury and, eventually, fish death (Kim et al. 2000, 2001).

To understand the ROS production mechanism, it is necessary to investigate ROS production during phytoplankton cell growth phases. Kim et al. (2004) conducted a large-scale tank experiment under light:dark conditions and demonstrated that ROS generation by *Chattonella antiqua* increased during the light period as a result of photosynthesis and decreased during the dark period. This finding indicated that ROS generation rates were closely related to growth metabolism during the cell division cycle of *C. antiqua* because cell

division occurred almost once per day during the exponential growth phase (Kim et al. 2004).

Chattonella antiqua is a motile phytoplankton characterized by diel vertical migration (DVM) and nocturnal nutrient uptake (Watanabe et al. 1991). The cells accumulate at the surface during the day for photosynthesis and sink to a deeper layer, where nutrients such as nitrate and phosphate are abundant, to take up the nutrients during the night. Under stratified conditions, the DVM ability gives the cells an ecological advantage over other non-motile phytoplankton species because the surface nutrients are generally almost depleted. The DVM of the flagellate population was examined in a culture tank at the National Institute for Environmental Studies (NIES), Japan (Kohata & Watanabe 1986, 1987, Watanabe et al. 1991, 1995, Kimura et al. 1999), in terms of nutrition and growth. Kim et al. (2004) suggested that the toxicity induced by ROS was presumably linked to cell migration through the cell cycle and nutrition although their study on ROS generation by *C. antiqua* was conducted in the NIES tank under fully mixed conditions

For the present study, we grew *Chattonella antiqua* in the NIES culture tank under limited nitrate and phosphate nutrient conditions to clarify the effect of nutrient depletion on ROS production through the growth metabolism during cell division cycles of *C. antiqua*. After the nutrients became depleted, vertical salinity and temperature stratification was established, and nutrients were enriched only in the bottom layer. These conditions are analogous to those observed under natural conditions during red tides. The purpose of this study was to investigate the effect of nutrient depletion on ROS production by *C. antiqua* and to clarify the relationship between the production of ROS and the uptake of nutrients during DVM under stratified conditions

MATERIALS AND METHODS

Experimental set-up of the culture tank. A clonal axenic culture strain of *Chattonella antiqua* (Hada) Ono (NIES-1, NIES Microbial Culture Collection) was precultured as described previously (Kohata & Watanabe 1988, Kim et al. 2004). The stock culture was maintained in f/2 medium (Guillard & Ryther 1962) under 80 µmol photons m^{-2} s^{-1} illumination on a 12:12 h light:dark regime. Temperature was kept constant at 23 ± 1°C.

A NIES culture tank (2 m high, 1 m internal diameter, working volume about 1 m^3, and airspace about 0.4 m^3) was used to grow *Chattonella antiqua* under axenic conditions (Kim et al. 2004). Before the experiments, the tank, the air and seawater filters, and all pipes were sterilized with steam (110°C, 0.5 kg cm^{-2}

pressure) for 30 min. The tank was then filled with 1 m^3 of medium, which was sterilized through 3 levels of filters (final filter, 0.1 µm Pall ULTIPOR, N66 Pall). Illumination (12:12 h light:dark) was provided by a daylight 5 kW xenon lamp with an irradiance of about 525 µmol photons m^{-2} s^{-1}. The light period was from 06:00 to 18:00 h.

Culture experiments were conducted under 3 nutrient conditions: (1) a fully supplied medium (FSM, f/2 medium) as a control, (2) a limited nutrient medium (LNM), and (3) a bottom-layer-enriched medium, after stratification had been achieved. The FSM experiment was conducted prior to the other two, and the LNM and stratification experiments were conducted sequentially in the same tank. In the FSM and LNM experiments, the medium was fully mixed by introducing sterilized air, regulated at about 200 ml min^{-1}, as fine bubbles from the bottom of the tank, and vertical mixing by thermal convection was induced by regulating the water temperature in the water jackets, which were used to keep the water temperature in the tank constant at 25 ± 1°C. In the LNM experiment, f/2 medium was used except that the initial concentration of dissolved inorganic phosphate (DIP; as $NaH_2PO_4 \cdot 2H_2O$) was adjusted to 1.0 µM, and that of dissolved inorganic nitrogen (DIN; as $NaNO_3$) was adjusted to 20 µM, and salinity was 30.8 psu.

A 2 l culture of *Chattonella antiqua* (about 2000 to 3000 cells ml^{-1}) prepared as described above at the exponential phase of growth was inoculated into the tank to achieve an initial cell concentration on Day 1 of 4.4 cells ml^{-1} in the FSM and 4.0 cells ml^{-1} in the LNM experiment.

We began the stratification experiment at 14:00 h on Day 10 (the 10th day after inoculation), which followed the LNM experiment, when the cell concentration was 590 cells ml^{-1} and DIP was <0.1 µmol l^{-1}. When aeration was stopped and water temperature in the regulating jackets was changed to produce stratification, *Chattonella antiqua* began to accumulate at the surface by vertical migration. Then, 100 l of the bottom water was discharged over a 2 h period. The next day (Day 11), 100 l of new medium enriched with DIN (440 µM) and DIP (19.3 µM) and with higher salinity (33.3 psu) was introduced into the bottom of the tank at 17:00 h to start the migration experiment. It took about 4 h to replace the bottom water and establish stratification, because the flow rate was regulated at less than 400 ml min^{-1} to avoid disturbing the interface. Water temperature was controlled at 27 ± 1°C at the surface and 22 ± 1°C in the bottom layer (ΔT = 5°C). Salinity was 30.8 psu at the surface and 33.3 psu in the bottom layer (ΔS = 2.5 psu). The large differences in salinity and temperature between surface and bottom layers in the tank prevented vertical mixing of the water col-

umn, and stratification was maintained throughout the migration experiment period regardless of the vertical migration of *C. antiqua* cells. Other experimental details were as described previously (Watanabe et al. 1991, Kim et al. 2004).

Samples were collected from the surface, middle, and bottom levels of the tank throughout the experiments. An aspirator was used for pumping samples to avoid damage to the cells. In the LNM and FSM experiments, 2 l water samples were collected once per day at 13:00 h from the middle level of the tank and used immediately to measure cell concentration, DIP and DIN in the medium, and ROS activities. During the stratification experiment (Days 11 to 16), 2 l each of surface and bottom waters were collected at 6 to 12 h intervals and used immediately to measure cell concentration, DIP and DIN in the medium, particulate carbon, and ROS activities.

Analytical methods. Cells were counted 3 times for each sample with a Coulter Multisizer II (Coulter Electronics). For particulate carbon and nitrogen measurements, part of the sampled water (500 ml) was filtered through precombusted (450°C for 4 h) Whatman GF/F 47 mm glass-microfibre filters (Whatman International). The filters were rinsed with 0.5 M ammonium formate and stored at –20°C until analysis (Watanabe et al. 1991, Kimura et al. 1999, Kim et al. 2004). After the filters had been dried in a forced-air oven at 80°C for 48 h, we measured particulate carbon and nitrogen on the filters with a CHN analyzer (MT-3, Yanaco; Kohata & Watanabe 1988). The DIP (as PO_4^{3-}) and the DIN (as NO_3^-) concentrations were analysed using the method of Murphy & Riley (1962) with a TRAACS 800 analyzer (Bran + Luebbe Corporation).

Measurements of O_2^-. To detect the O_2^- produced by *Chattonella antiqua*, we used 2-methyl-6(*p*-methoxyphenyl)-3,7-dihydroimidazo[1,2-α]pyrazin-3-one (MCLA) as a superoxide-specific chemiluminescent probe, as previously described (Lee et al. 1995, Oda et al. 1998). MCLA was dissolved in distilled water and stored at –30°C until use. MCLA was added to a cell suspension, and the chemiluminescence response was recorded immediately with a Luminoskan TL Plus (Thermo Labsystems). The amount of O_2^- generation detected by chemiluminescence responses was distinguishably high during the first 30 s of exposure, so the readings of the responses were accumulated for that time period (Nakamura et al. 1998). Typical reaction mixtures comprised 500 µl of cell suspension, 166 µl of MCLA solution (final concentration, 5 µg ml⁻¹), and 15 µl of f/2 medium. Reagent background was measured with the same reaction mixture except that 15 µl of superoxide dismutase (SOD; final concentration, 100 units ml⁻¹) was used instead of 15 µl of f/2 medium at the beginning of every

sampling time. MCLA produced a slight chemiluminescence response in f/2 medium, but a similar level of response was also observed in distilled water under the same experimental conditions. Therefore, the value in f/2 medium without cell suspension was subtracted from each value as non-specific background. All chemiluminescence analyses were done immediately after the sampling in triplicate at 27°C.

Measurement of H_2O_2. H_2O_2 was detected by a *p*-hydroxyphenylacetate (PHPA) assay (Hyslop & Sklar 1984) within 1 h after the sampling for triplicate samples at 27°C. After the addition of PHPA (final concentration, 1 mM) and horseradish peroxidase (final concentration, 100 mg ml⁻¹) to the cell suspension in f/2 medium, the increase in fluorescence intensity during the first minute of incubation was measured with a fluorescence spectrophotometer (Shimadzu RF-540) at an excitation wavelength of 317 nm and an emission wavelength of 400 nm in the presence or absence of 500 units ml⁻¹ catalase. The catalase-inhibited increase in fluorescence was considered to represent actual H_2O_2. The production of H_2O_2 was estimated using a standard curve of H_2O_2 in cell-free f/2 medium. The standard solution of H_2O_2 in f/2 medium was prepared from reagent-grade H_2O_2 (Santoku Chemical Industries). Under these assay conditions, the increase in fluorescence was proportional to the production of H_2O_2 (Zhou et al. 1997).

RESULTS

Effect of nutrient depletion on O_2^- and H_2O_2 production during growth phases

To elucidate the effect of nutrient depletion on O_2^- and H_2O_2 production, cell growth, O_2^- production, and H_2O_2 production of *Chattonella antiqua* cells grown in LNM were compared with those of cells grown in FSM (Fig. 1). O_2^- and H_2O_2 activities in LNM were weak, whereas those in FSM increased with cell growth (Fig. 1B,C). After inoculation of cells into the tank, growth was measured by counting live cells at 1 d intervals. The specific growth rate (µ) was calculated from the cell density changes (Kohata & Watanabe 1988). The values were averaged over Days 6 and 10 when the cell quota in LNM was lower than that in FSM as described below, being 0.57 d⁻¹ for FSM and 0.51 d⁻¹ for LNM. Averaged values were close to 0.67 d⁻¹ that was obtained during the exponential growth phase under a good-growth condition in the same tank (Kohata & Watanabe 1988). The pH increased from 8.09 to 8.65 from the beginning of the LNM experiment to Day 10, showing that the air bubble from the bottom of the tank supplied sufficient air to cell growth. Ambient nutrient concentrations in
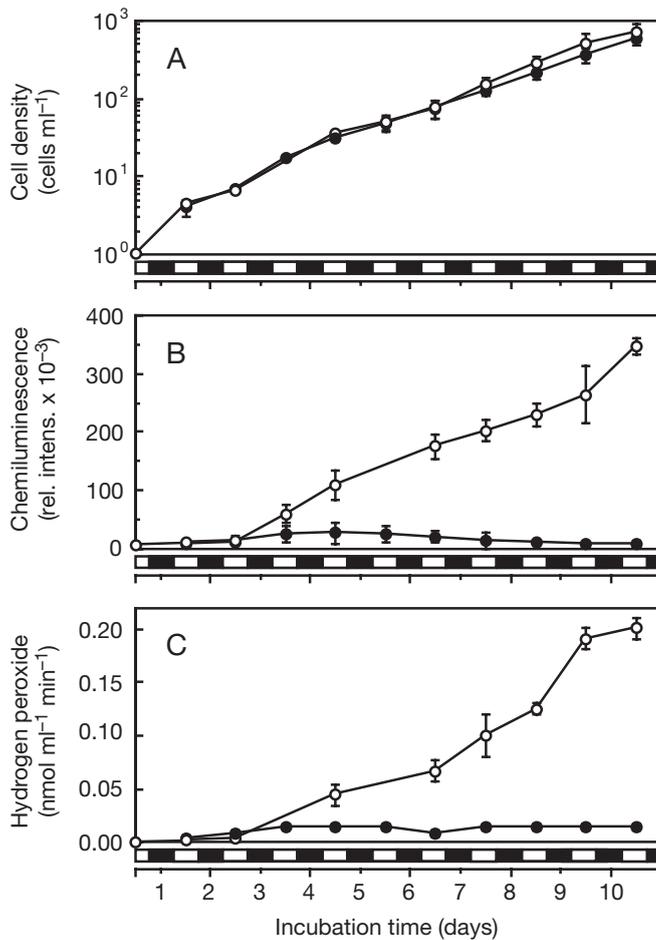
Fig. 1. *Chattonella antiqua*. Cell growth, superoxide anion (O_2^-) generation, and production of hydrogen peroxide (H_2O_2) between cells grown under the fully supplied (○) and limited-nutrient (●) culture conditions in the National Institute for Environmental Studies tank. (A) Cell densities, (B) O_2^- generation detected by chemiluminescence responses, and (C) production of H_2O_2. Vertical bars in the figure are standard deviations of triplicate measurements ($N = 3 \pm SD$). The black and white segments on the lower bars indicate dark and light periods, respectively, during the 12:12 h light:dark cycle
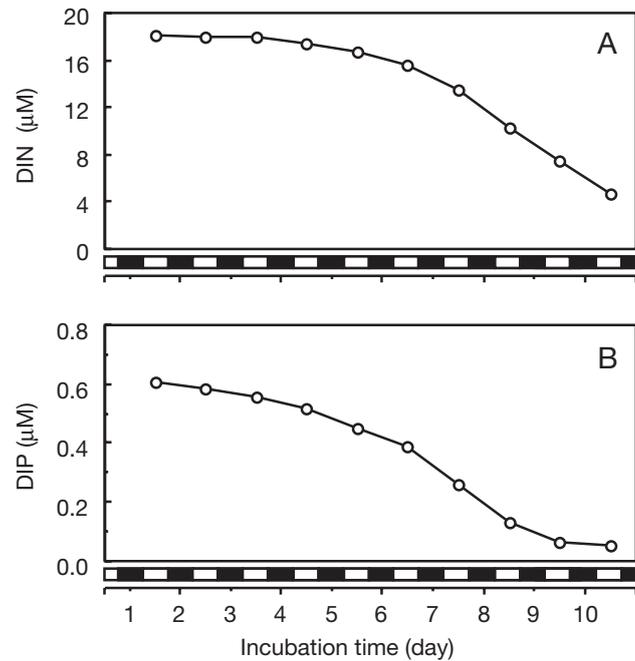

Fig. 2. (A) Ambient dissolved inorganic nitrogen (DIN) and (B) dissolved inorganic phosphate (DIP) concentrations in the liquid nutrient medium of *Chattonella antiqua* grown under nutrient-limited conditions. The black and white segments on the lower bars indicate dark and light periods, respectively, during the 12:12 h light:dark cycle

LNM decreased from initial DIN and DIP concentrations of 18.1 and 0.6 µM l^{-1} to 4.7 and 0.05 µM l^{-1} on Day 10, respectively (Fig. 2), during the cell growth phase. DIP decreased gradually from the first day, but DIN was relatively constant until Day 7, when it began to decrease steeply. The concentration of DIP in the medium was completely depleted below detection limit by Day 9 (Fig. 2B), but the cell density continued to increase (Fig. 1A). We also calculated the daily changes in nitrogen and phosphorus quotas of *C. antiqua* cells by dividing the decrease in ambient nutrient concentration by cell density during cell growth. The nitrogen and phosphorus cell quotas decreased to 22 and 1.2 pmol $cell^{-1}$, respectively (Fig. 3).
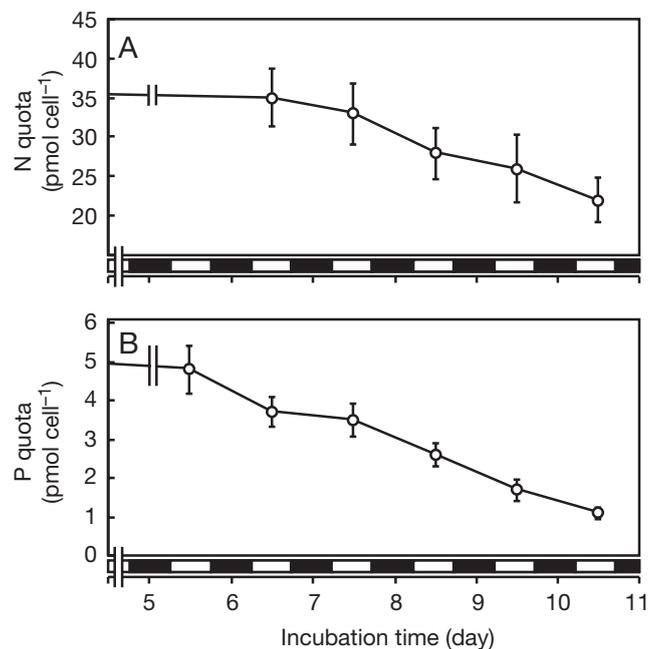

Fig. 3. *Chattonella antiqua*. (A) Nitrogen and (B) phosphorus cell quotas of cells grown under nutrient-limited conditions. $N = 3 \pm SD$. The black and white segments on the lower bars indicate dark and light periods, respectively, during the 12:12 h light:dark cycle

ROS activities during diel vertical migration

At the beginning of the stratification experiment, nutrient-rich seawater with high salinity was introduced into the bottom layer. The DIN and DIP in the bottom layer reached peak values of 392 and 15.4 µM, respectively, at 17:00 h on Day 11 (Fig. 4), which was when the stratification became established in the tank. Under the stratified conditions, *Chattonella antiqua* cells began to move downward before the lights were turned off and formed a dense layer on the bottom of the tank. The cells of low N cell quota reached the nutrient-rich bottom layer and took up nutrients such as DIN and DIP rapidly, thus decreasing nutrient concentrations in the bottom layer (Fig. 4).

The timing of ascending migration and cell division by *Chattonella antiqua* overlapped. The cells accumulated in the surface layer (Fig. 5A) during the daytime, when cell density at the surface was 5.9×10^3 cells ml^{-1}, or about 2.4 times that observed during the stationary phase in the NIES tank (Kohata & Watanabe 1988, Kim et al. 2004). Particulate carbon of *C. antiqua* cells changed similarly to cell density (Fig. 5A,B).

O_2^- and H_2O_2 production by *Chattonella antiqua* was monitored under stratified conditions to investi-

gate the production pattern during DVM. The intensity of the chemiluminescence response of O_2^- and the production of H_2O_2 generally recovered from the lowest values in LNM after nutrients were added to the bottom layer (Fig. 5C,D). However, the time of maximum production of O_2^- and H_2O_2 was markedly different between the surface and bottom layers; both O_2^- and H_2O_2 production were high during the light period and low during the dark period in the surface
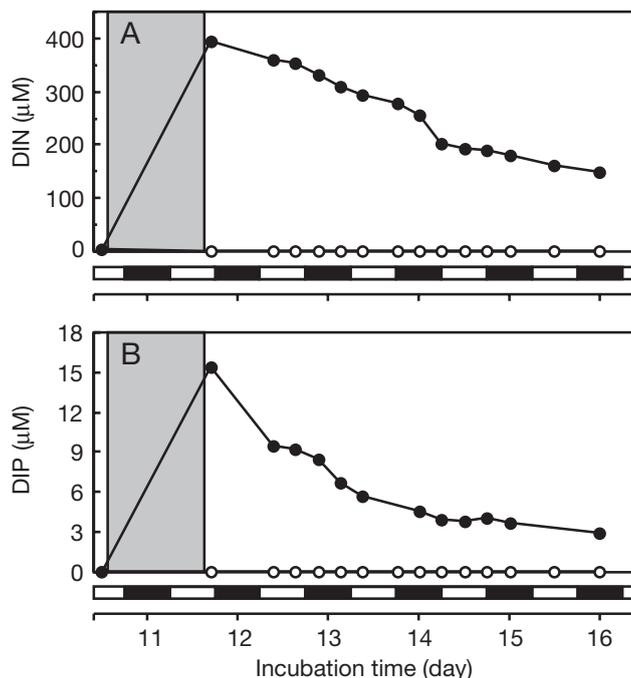

Fig. 4. (A) Dissolved inorganic nitrogen (DIN) and (B) dissolved inorganic phosphate (DIP) concentrations at the surface (O) and in the bottom (●) layer under stratified conditions. Shaded areas denote the period during which stratification was being established. The black and white segments on the lower bars indicate dark and light periods, respectively, during the 12:12 h light:dark cycle
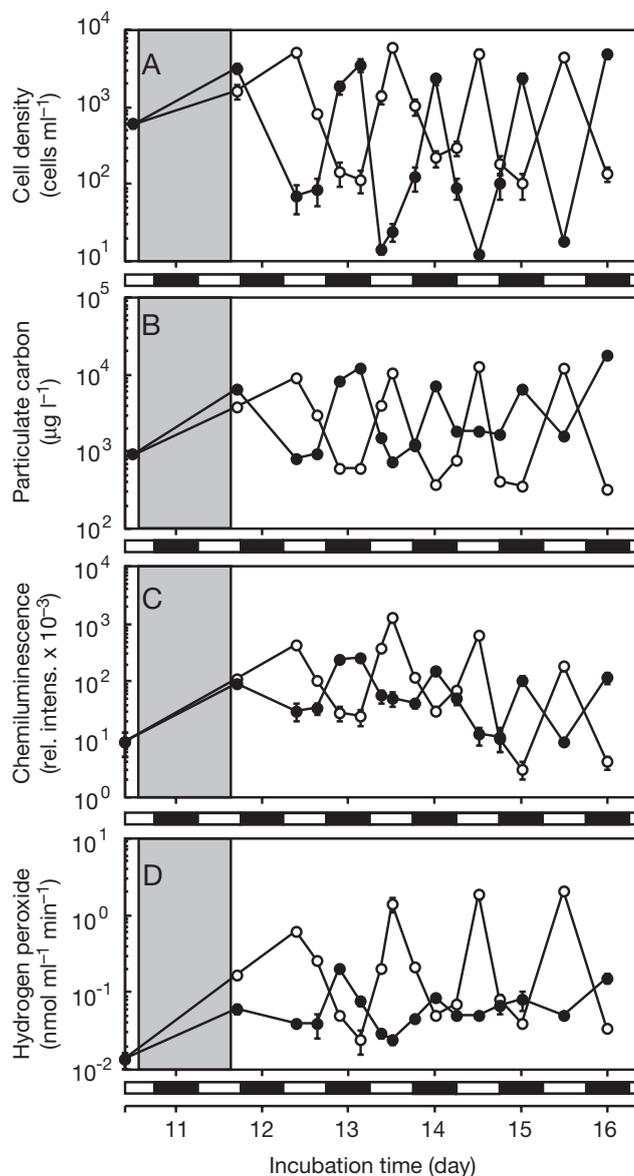

Fig. 5. *Chattonella antiqua*. Vertical distribution of (A) cell densities, (B) particulate carbon, (C) O_2^- production, and (D) H_2O_2 production under stratified conditions at the surface (O) and in the bottom (●) layer. Shaded areas denote the period during which stratification was being established. The black and white segments on the lower bars indicate dark and light periods, respectively, during the 12:12 h light:dark cycle. $N = 3 \pm$ SD for (A), (C), and (D); $N = 1$ for (B)

layer (Fig. 5C,D), reflecting the DVM shown by the cell density and carbon content changes (Fig. 5A,B). The daytime production of O_2^- in the surface layer reached maximum values on Day 13 and decreased thereafter, whereas daytime H_2O_2 production in the surface layer gradually increased during DVM (Fig. 5C,D).

The changes in ROS generating activities shown by O_2^- and H_2O_2 production in the surface and bottom layers were normalized by dividing the total activity by the particulate carbon value to obtain the generation per particulate carbon content (GPC) so that we could compare the ROS production during DVM independent of changes in cell density. The GPC values for O_2^- and H_2O_2 changed in clearly different ways during DVM (Fig. 6). The GPC value for O_2^- was sometimes higher in the surface layer than in the bottom layer. Both values reached a maximum on Day 13 and decreased thereafter. However, the GPC of H_2O_2 in the surface layer was always greater than that in the bottom layer. The surface GPC of H_2O_2 showed clear diel changes, with local peaks at 19:00 h on Days 13 and 14, and the daytime values tended to increase during the experimental period (Fig. 6B).
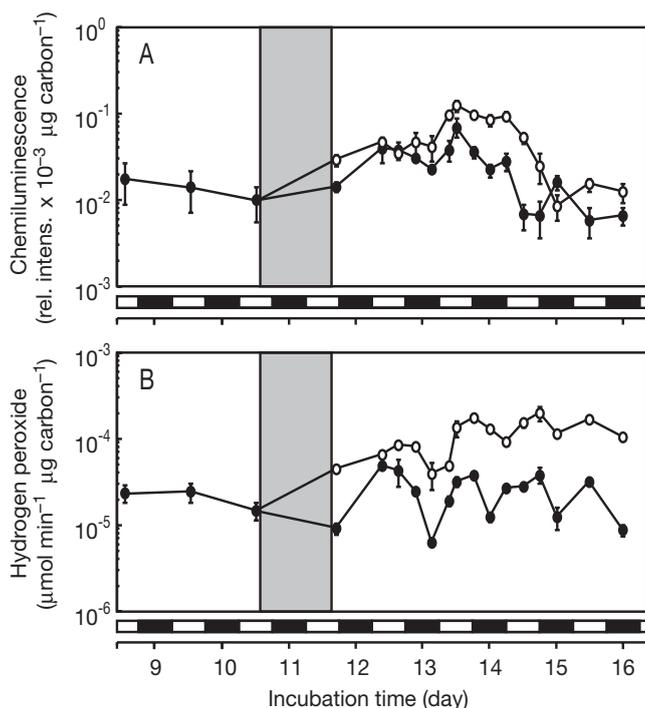

Fig. 6. Generation per particulate carbon content (GPC) values for (A) O_2^- and (B) H_2O_2 of *Chattonella antiqua* in the surface (O) and bottom (●) layers under stratified culture conditions. Shaded areas denote the period during which stratification was being established. The black and white segments on the lower bars indicate dark and light periods, respectively, during the 12:12 h light:dark cycle

DISCUSSION

Chattonella antiqua cells grown in LNM had lower nitrogen and phosphorus cell quotas and weak O_2^- and H_2O_2 responses during the growth phase, showing that nutrient depletion negatively affected ROS production (Figs. 1 & 3). Cell growth kinetics have been intensively analysed as functions of ambient nutrient concentrations (e.g. Dugdale 1967) and cell quotas (e.g. Droop 1973, Amano et al. 1998). In most cell-quota growth models, the cell quota depends on the nutrient uptake rate, which is a function of ambient nutrient concentrations. A typical uptake function is a Monod-type equation, which has a half-saturation constant as a control parameter (Nakamura 1985a,b). We regulated the initial phosphate concentration in the tank in LNM at lower than the half-saturation constant of *C. antiqua* (1.9 µM for phosphate, Nakamura 1985b) to reduce the uptake rate (Fig. 2B). The minimum cell quota, at which phytoplankton cells cease growing, of *C. antiqua* is 11.0 pmol cell^{-1} for nitrogen and 1.0 pmol cell^{-1} for phosphorus, determined from semicontinuous culture experiments (Nakamura & Watanabe 1983). Although the difference between the specific growth rates of *C. antiqua* in LNM (0.51 d^{-1}) and in FSM (0.57 d^{-1}) was small (Fig. 1A), the N and P cell quotas in LNM decreased to minimum cell quota (Fig. 3), indicating that the physiological conditions of flagellate cells in LNM differed from those in FSM. The N and P cell quotas in FSM remained almost constant at around 37 and 5.5 pmol cell^{-1}, respectively (data not shown). The effect of nutrient depletion was also observed in cell size changes of *C. antiqua*. Cell size (estimated diameter as sphere) in LNM decreased gradually from 36.3 µm at the beginning to 30.8 µm on Day 10 of the LNM experiment in the tank, but values in FSM were relatively constant (36.5 ± 1 µm) during cell growth (data not shown).

Generally, energy generation in biological organisms requires uptake of both nitrogen and phosphorus. Nitrogen is required for synthesis of various biomolecules, and phosphorus is used in the oxidative reaction of fuel molecules during the synthesis. During biosynthesis, ATP and NADPH are generated as electrons flow from the reduced forms of these carriers to O_2. In particular, the partly reduced form, O_2^-, is generated. The generation of ROS is one of the earliest events in the positive control of cell growth in response to growth factors under growth-promoting conditions (Murrell et al. 1990, Shibanuma et al. 1990). Furthermore, the negative effect of nutrient depletion on ROS production was confirmed by its recovery when the cells were placed in a nutrient-rich medium (Fig. 5C,D).

Night-time nitrogen uptake has been observed in a natural phytoplankton population dominated by dinoflagellates (Cullen & Horrigan 1981), and dinoflagel-

lates forming blooms in stratified waters are able to increase their population by nitrogen uptake at depth at night and photosynthesis near the surface during the day through DVM (Dortch & Maske 1982). In the present study, we also found both DVM and nocturnal uptake of nutrients by *Chattonella antiqua* in the culture tank, when vertical stratification of nitrogen and phosphorus was established. Both DIN and DIP clearly decreased over time in the bottom layer under stratified conditions (Fig. 4). Because *C. antiqua* migrates vertically, ascending to the surface photic layer during the day and descending to deeper layers at night, the cells can access nutrients provided that the nutricline is shallower than the depth to which they descend (Fig. 5A). DVM and nocturnal nutrient uptake by *C. antiqua* were shown experimentally by Watanabe et al. (1991). They also suggested that DVM was the most important factor affecting the dominance of *C. antiqua* from the observation in an enclosure in the Seto Inland Sea, Japan (Watanabe et al. 1995).

The higher ROS production during the daytime could be due to photosynthesis in the surface layer under stable stratification (Fig. 5). This diel pattern in ROS production is similar to the results obtained previously under fully mixed conditions in the same tank (Kim et al. 2004). The sharp daytime peaks in ROS production (Fig. 5C,D) reflected cell accumulations at the surface. The GPC, which is independent of changes in cell density, of O_2^- reached a maximum during the daytime on Day 13. The GPC of H_2O_2 reached local maxima at 19:00 h on Days 13 and 14. Although the detailed mechanisms of ROS production by flagellate cells is unknown, various chemical reactions in higher plants occur during photosynthesis to form O_2^-, H_2O_2, and ·OH (Halliwell & Gutteridge 1984). Kim et al. (2004) documented that, similar to higher plants, under fully mixed conditions ROS production by *Chattonella antiqua* was affected by photosynthesis; in addition, they showed that ROS production expressed as GPC was relatively high in the daytime and decreased at night. The results obtained under stratified conditions showed the same diel trend in ROS production, indicating that ROS production is strongly related to photosynthetic processes.

The PHPA method provides absolute production for H_2O_2, the most stable ROS, and enables us to compare data sets obtained under different conditions. The GPC for H_2O_2 of *Chattonella antiqua* was higher at the surface than in the bottom layer. ROS production by the cells remaining in the bottom layer during the day was low (Fig. 6) because these cells can be considered to be less active. In contrast, the GPC for H_2O_2 of migrating cells ranged between 0.01 and 0.20 nmol min^{-1} µg carbon^{-1} (Fig. 6), which corresponded to the diel changes under fully mixed conditions (0.04 to 0.24 nmol min^{-1}

µg carbon^{-1}; Kim et al. 2004). These results suggest that the toxicity induced by H_2O_2 production is linked to cell migration through the cell cycle and nutrition.

The O_2^- and H_2O_2 production in the surface recovered from relatively low levels after nutrients were added to the bottom layer under stratified conditions (Fig. 5C,D). However, the pattern of O_2^- production was different from that of H_2O_2 production in the surface layer. Daytime O_2^- production peaked on Day 13 and decreased thereafter, whereas daytime H_2O_2 production increased throughout the experimental period. Kim et al. (2004) reported that O_2^- production by *Chattonella antiqua* reached a maximum during the exponential growth phase and decreased subsequently during the stationary phase; the maximum H_2O_2 generation occurred during the early stationary growth phase. They also showed that GPC values for H_2O_2 during the stationary phase were similar to those obtained during the exponential phase. However, the GPC values for O_2^- during the stationary phase were clearly lower than during the exponential phase. Under stratified conditions, pH was more than 8.6 at the surface, which is similar to the pH during the early stationary growth phase in the fully mixed experiment (Kim et al. 2004). Thus, growth conditions at the surface during the stratified experiment resembled those of the early stationary phase during the fully mixed experiment. Both O_2^- and H_2O_2 production by actively migrating cells are consistent with those observed during the fully mixed experiment with respect to both their pattern of change and their magnitude when this resemblance is taken into account.

In conclusion, our results demonstrate that: (1) ROS generation such as O_2^- and H_2O_2 production by *Chattonella antiqua* is affected by nutrient availability during cell growth phases; (2) ROS (O_2^- and H_2O_2) production by *C. antiqua* under nutrient stratified conditions was high in the daytime, suggesting that ROS production is related to photosynthetic processes; and (3) *C. antiqua* cells accumulated at the surface during diel migration resembled those in early stationary phases under fully mixed conditions with respect to both the patterns of change and GPC values of O_2^- and H_2O_2.

Despite many investigations of the growth response of phytoplankton to limited nutrient conditions (Cullen & Horrigan 1981, Paasche et al. 1984, Cullen 1985, Watanabe et al. 1991), little information has been available on the effect of nutrient depletion on ROS production. The present investigation clarifies the relationship between growth and ROS production under both nutrient-depleted conditions and nutrient stratification. Ichthyotoxicity of *Chattonella antiqua* under natural conditions may be caused by phenomena similar to our observations on ROS production by *C. antiqua* under experimental stratified conditions.

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