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Nutrient Uptake and Growth Kinetics of *Chattonella antiqua* (Hada) Ono (Raphidophyceae) Isolated from Korea

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The red tide-causing flagellate *Chattonella antiqua* can cause mass fish kills by their clogging in fish gills. This study examined the nutrient requirements of *C. antiqua* isolated from Korea. *C. antiqua* displayed maximum growth at the day five, followed by a decrease in cell density. Nitrate and nitrite were the preferred nitrogen sources, along with adenosine diphosphate for phosphorus compounds. In medium that contained ammonium, a significant decrease in cell density was observed. Half-saturation constants, Ks, calculated from the maximum growth rate were 4.94 μM for NO_3 and 0.79 μM for PO_4. The growth of *C. antiqua* was not within the function of the N:P ratio (R^2 = 0.29). With an N:P ratio as low as 10, the increase in cell density was apparent, with a higher division rate. At levels above 50 μM of $NaNO_3$ or 8 μM of NaH_2PO_4, the growth rates were somewhat decreased. Phosphate was the limiting factor for *C. antiqua* growth since the starvation of phosphate had brought about a rapid decrease in cell density in semi-continuous culture. Studies about the temporal modification of the efficiency of nitrate or phosphate uptake may be necessary to explain the bloom dynamics of *C. antiqua.*

Key Words: *Chattonella antiqua,* N:P ratio, nutrition, Raphidophyceae, red tide

INTRODUCTION

Red tide-causing *Chattonella* species are found worldwide, including in the Pacific Ocean and the Mediterranean Sea (Hallegraeff *et al.* 1998; Imai *et al.* 1998; Imai 2000). The blooms resulting from *Chattonella* species cause widespread fish kills (Hallegraeff *et al.* 1998).

Physiological clogging and damage to gills by mucus extraction and hemolytic fatty acids and/or the brevetoxin produced by *Chattonella* have been suggested as the fish killing mechanism of the bloom (Kahn *et al.* 1998; Bourdelais *et al.* 2002). Alternatively, other evidence indicates the production of superoxide radicals as the major mechanism of fish mortality by raphidophycean flagellates, *Chattonella* (Kim *et al.* 2000a; Kuroda *et al.* 2005) and *Heterosigma* (Yang *et al.* 1995); such fish killing mechanism coincides with the blooming of *Cochlodinium* (Kim *et al.* 2000a, 2000b).

Blooms of *Chattonella* species were found in inland seas with bountiful nutrients from freshwater estuaries during periods of higher water temperatures (about 25°C) (Nakamura *et al.* 1989). It has been suggested that high light intensity (> 100 μE m^{-2} s^{-1}) and low salinity (> 20‰) are the elements that promote the growth of the species (Yamaguchi *et al.* 1997).

Chattonella species form brownish or yellow-greenish cysts of 25-35 μm in diameter (Yamaguchi and Imai 1994) at the end of the summer season (Imai and Itoh 1986; Imai 1989). It is known that the cysts of *Chattonella* species maturate at the bottom of the sea in temperatures as low as 11°C and germinate in those as high as about 20-25°C during the summer (Imai *et al.* 1998).

In Korea, the first recorded incidence of a *Chattonella*-caused bloom was in Jindong Bay during April of 1983 with a density of 200-1,100 cells mL^{-1}, and the species was identified as *C. antiqua* (Park *et al.* 1987). *C. antiqua* (Hada) Ono was first described in the Seto Inland Sea in Japan in 1969 (Hada 1974). Fortunately, Korea has not yet experienced serious economic damage from *C. antiqua* blooms (Lee *et al.* 2005).

The occurrence of *Chattonella* red tides has been monitored over an extended area, and it occurs concurrently with *Cochlodinium* red tides in Korea (Lee *et al.* 2005). It is very interesting that *Chattonella* red tide shares the same

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temporal and spatial space, as well as fish killing mechanism, with the most notorious *Cochlodinium* red tide on the Korean coasts. Increasing worries about mass fish kills caused by *Chattonella* indicate the need for an in-depth study regarding *Chattonella* growth physiology with isolates from the Korean coasts. Results may be useful for understanding *Chattonella* blooms and the development of monitoring and mitigation methodologies for red tide.

MATERIALS AND METHODS

Organism and culture conditions

The nutrient requirements of *Chattonella antiqua* were investigated in laboratory culture experiments. A strain of *C. antiqua* (NF-F-CAN1) was isolated from Jangheung, Chonnam, Korea during a bloom in early August 2003. The medium f/2 without silica, made in artificial seawater L1 medium, was employed as the basal medium. Acclimated cells were inoculated into triplicate test tubes containing nitrogen (N) or phosphorus (P) compounds for examination. Control tubes did not contain any N or P sources.

Unless otherwise stated, incubations were carried out at 22°C under a 12:12 hr light-to-dark (LD) cycle regime. Illumination was provided by cool-white fluorescent lamps at a light intensity of 1,200 Lux. All equipment and glassware were washed with 30% v/v hydrochloric acid (HCl) to remove ammonium and metals, then thoroughly rinsed with distilled water and autoclaved.

Statistical values were calculated for each batch from the measurement data. The data are presented as the mean standard deviation. Tests for significance in the analytical study were performed using the Students' t-test.

Effects of nutrients on growth

To test the ability of *Chattonella antiqua* to use various N- and P-containing components for growth, batch culture experiments were conducted with initial concentrations of 400 cells mL^{-1}. Cells for inoculation were pre-incubated for a week in either N- or P-depleted medium. Nutritional needs were examined using three different inorganic nitrogen sources (nitrate, $NaNO_3$; nitrite, $NaNO_2$; and ammonium, NH_4Cl) and four organic or inorganic phosphorus sources (adenosine triphosphate, ATP; adenosine diphosphate, ADP; glycerophosphate, GYP; and orthophosphate, NaH_2PO_4). These compounds were selected because they cover a diverse structural range and are likely major sources of N and P in the natural environment (van Boekel 1991) and also because there is a preference for inorganic N and organic P sources among many phytoplankton species (Nakamura and Watanabe 1983b; Yamaguchi *et al.* 2001).

The concentrations of the N and P sources were 250 μM and 25 μM, respectively, which corresponded to 12.5% and 25% of the concentrations of N and P. All compounds were added individually to the autoclaved N- or P-depleted f/2 medium in capped test tubes (25 x 150 mm), after sterilization by filtration through a 0.2-μm pore-size disposable syringe filter (Sterivex-GS, Millipore, Bedford, MA, USA).

Acclimated cells were inoculated into triplicate test tubes containing the N or P compounds to be examined. Each subsequent day, 10 μL aliquots of suspension were removed and the cell density (D) was determined with a microscope. Cell concentrations were determined by counting cell numbers under a microscope between 09:00 and 12:00 hours in the morning, a period when no cell division was confirmed with a microscope.

Specific growth rates (SGR) were calculated by using the following equation with a modification from Stein (1973):

$$SGR = \ln(D_1 / D_0) \; / \; (t_1 - t_0)$$

Where D_1 is the number of the cells after a certain time (t_1) and D_0 is the cell number at the beginning (t_0) is equal to 400 cells mL^{-1} for this experiment.

Uptake kinetics

The N- and P-starved culture was distributed into six flasks and diluted with N-and P-free f/2 medium. The cell density in the inoculation was adjusted to be 400 cells mL^{-1}. Nitrate was added serially to give final concentrations of 5, 10, 20, 50 or 100 μM. Phosphate was added serially to give final concentrations of 2, 4, 8, 16 or 32 μM. Concentrations of nitrate and phosphate were determined at the beginning and at the end of the incubation using the method proposed by Strickland and Parsons (1972).

Nutritional requirements were calculated with an adaptation of the Monad formula (Eppley *et al.* 1969) as follows:

$$\mu = \mu_{max} [S \; / \; (Ks + S)]$$

$$S = \mu_{max} (S / \mu) - Ks$$

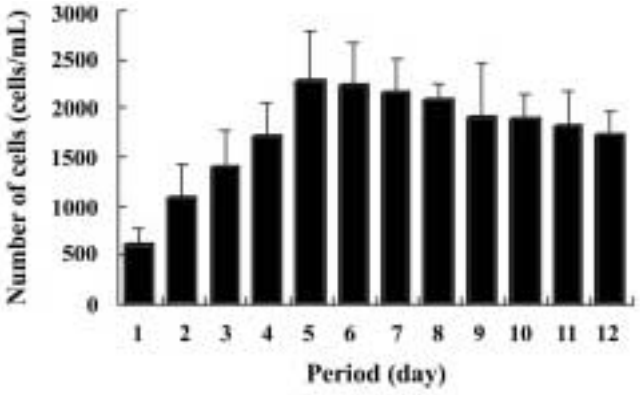

Fig. 1. Population growth of *Chattonella antiqua* in f/2 media.

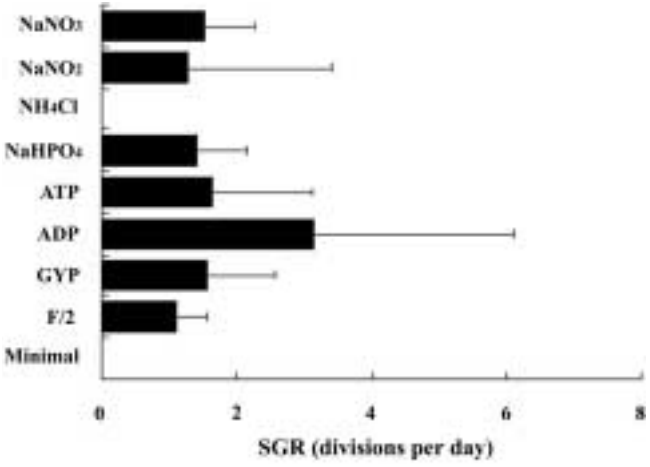

Fig. 2. Specific growth rates of *Chattonella antiqua* with a supplement of nitrogen or phosphorus source.

Where μ is the average rate of growth (divisions/day), μ_{max} is the maximum rate of growth (divisions/day), Ks is the half-saturation constant (μM), and S is the ambient nutrient concentration (μM).

Effects of N:P ratio

A comparison of the growth based on the N:P ratio was performed with an initial concentration of 400 cells mL^{-1}. To 250 mL of cells grown in N- and P-depleted f/2 medium for one week, a mixture of an N source ($NaNO_3$) and a P source (NaH_2PO_4) was supplied in the following amounts: 5, 10, 20, 50, and 100 μM for NO_3, and 2, 4, 8, 16, and 32 μM for PO_4. Cell densities were measured daily. A batch of cells growing in N- and P-depleted media was used as a control for the comparison.

Semi-continuous growth experiment

A culture of exponentially growing *Chattonella antiqua*, at a concentration of 800 cells/mL, was equally distributed into each of the following: 700 mL each of NO_3-limited medium, NH_4-limited medium, or PO_4-limited medium. For the triplicate of the NO_3-limited medium, the $NaNO_3$ and NaH_2PO_4 concentrations were adjusted to 10 μM; the same adjustments were also made to the NH_4Cl and NaH_2PO_4 concentrations in the NH_4-limited medium. For the triplicate of the PO_4-limited medium, the $NaNO_3$ and NaH_2PO_4 concentrations were adjusted to 100 μM and 1.0 μM, respectively. As a control, $NaNO_3$ and NaH_2PO_4 concentrations were limited to 10 μM and 1.0 μM. Semi-continuous cultures were performed by removing 50 mL of the cell suspension and replacing it with the same amount of fresh medium to sustain the same concentration each day at around 09:00 a.m. to 10:00 a.m. Cell densities in the aliquots of suspension removed were determined microscopically on a daily basis. Each cell suspension was filtered through a Whatman GF/C filter and used for subsequent nutrient analysis.

RESULTS

Batch cultures of *Chattonella antiqua* with an addition of nitrogen and phosphorus sources result in maximum growth at day five, followed by decreases in cell density (Fig. 1). Nitrate and nitrite were preferred by *C. antiqua* as a sole nitrogen source, and ADP was preferred as the phosphorus source (Fig. 2). In the medium containing NH_4Cl, a continuous decrease of the cell density was observed.

When the cellular uptake of $NaNO_3$ or NaH_2PO_4 by *Chattonella antiqua* was traced (Fig. 3), the level of nitrogen was decreased by a function of time; 100 μM of nitrogen was consumed within five days. In contrast, the amount of phosphate in the culture remained similar to the initial amount with a slight decreasing tendency.

The maximum growth of *Chattonella antiqua* was found in a medium containing 50 μM of $NaNO_3$ and 8 μM of NaH_2PO_4 (SGR = 1.86). Half-saturation constants, Ks, were calculated as 4.94 μM for NO_3 and 0.79 μM for PO_4 (Fig. 4). The growth of *C. antiqua* was not within the function of the N:P ratio ($R^2 = 0.29$). With an N:P ratio as low as 10, the growth of the *C. antiqua* population was apparent, with a higher division rate. At a level above 50 μM of $NaNO_3$ or 8 μM of NaH_2PO_4, the SGRs were rather decreased.

The influx of nutrients is continuous in the open sea. Therefore, a study on the population growth of

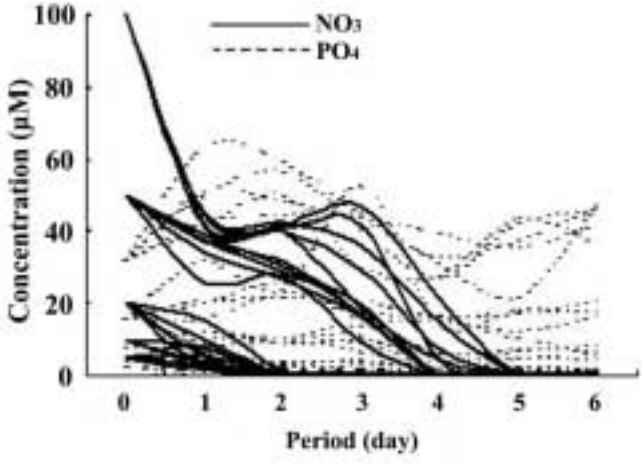

Fig. 3. Consumption of nitrate and phosphate by *Chattonella antiqua.*

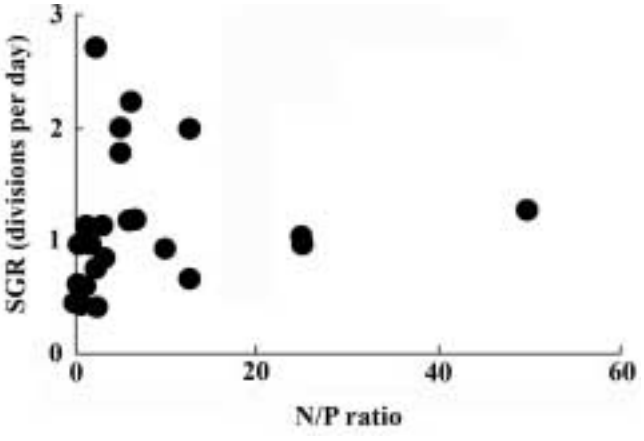

Fig. 4. Growth of *Chattonella antiqua* population as function of the N:P ratio.

Chattonella antiqua in such an open system was necessary. A depletion of NH_4 did not affect the cell density growth (Fig. 5). In both NH_4-limited medium and NO_3-limited medium, each containing 10 μM of NaH_2PO_4, the overall number of *C. antiqua* increased to reach a peak. Cell densities showed a decrease at day seven in both NO_3-limited medium and PO_4-limited medium. After the initial decrease, the density increased again in the NO_3-limited medium, but it continued to decrease in the PO_4-limited medium. In the control where the $NaNO_3$ and NaH_2PO_4 concentrations were limited to 10 μM and 1.0 μM, the cell density remained at a low level. A continuous supplement of phosphate might lead to a recovery of the cell population from a diminished density.

DISCUSSION

This study has shown that the Korean isolate of

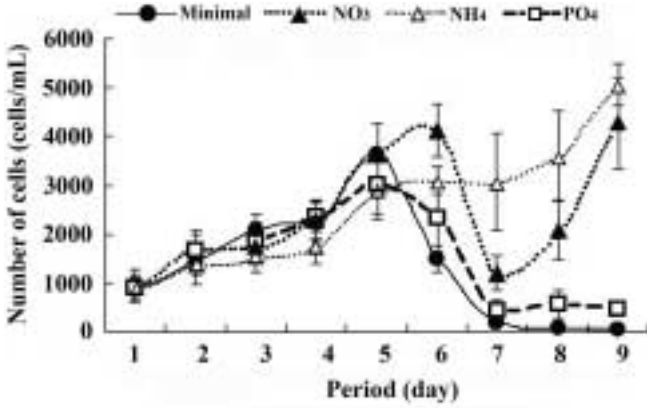

Fig. 5. Population growth of *Chattonella antiqua* in semi-continuous cultures.

Chattonella antiqua prefers nitrate as a nitrogen source and that ADP was the optimal phosphorous source among a wide variety of tested nutrients. In addition, phosphate was a limiting factor for the growth of the Korean isolate of *C. antiqua.* Such a preference for inorganic N sources and organic P sources has been also described in a wide variety of phytoflagellates (Nakamura 1985a, 1985b; Yamaguchi *et al.* 2001).

It is interesting that there was no significant decrease in the amount of phosphate detected in the *Chattonella antiqua* cultures from this study, despite the observation that the depletion of phosphate brought about a rapid decrease of the *C. antiqua* population in the semi-continuous culture. An intensive comparative study regarding the capability of utilization and excretion of phosphorus sources may be necessary (Yamamoto *et al.* 1999).

In general, phytoplanktons use both nitrate and ammonium as preferred nitrogen sources (Yamamoto *et al.* 2004). In our semi-continuous culture, a minimum amount of phosphate is required. It was found that ammonium was somewhat inhibitory for the Korean isolate of *Chattonella antiqua.*

An excess supply of nitrate would result in an increase of the *Chattonella antiqua* population and a bountiful phosphate amount beyond the minimum was not an additive required for cell density growth (Nakamura 1985b). It has been suggested that the ability to use organic and inorganic phosphorus sources may contribute to the successful bloom development of phytoplanktons (Oh *et al.* 2002; Yamamoto *et al.* 2004).

This observation is very interesting since the addition of ammonium (*ca.* 2 μM concentration) promoted the increase of cellular density (Nakamura and Watanabe 1983b), and it suppressed nitrate influx into cells, according to a previous study performed with Japanese isolates

(Nakamura 1985a). However, since controversy remains about the taxonomic assignment of *Chattonella* species (Imai 2000; Sako *et al.* 2000), a detailed comparative study with both Korean and Japanese isolates should be performed.

Diurnal vertical migrations are known to be important in the acquisition of nutrients in phytoplankton, with concurrent physiological and ultrastructural modifications (Seo and Fritz 2000a): floating in surface water during the day and sinking in the night for the uptake of nitrogen sources from below the thermocline (Smayda 1988).

The nightly uptake of nitrogen sources and the daytime photosynthesis are under a cross control, since the nitrogen starvation in phytoplankton could be characterized by a loss of cell protein and a sudden decline in photosynthetic function (Flynn and Hipkin 1999). Therefore, it is quite important to clarify temporal nutrient dynamics according to diurnal vertical migration to understand the occurrence and bloom development of *Chattonella antiqua*.

Above all, the application of these results to an explanation for *Chattonella* blooms should be made with caution, since only a little is known regarding the chemical properties of organic nitrogen and phosphorus compounds in the natural environment (Cembella *et al.* 1984).

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