

# Development of Economical Fertilizer-Based Media for Mass Culturing of *Nannochloropsis oceanica*

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## Abstract

This study was conducted to develop economical agricultural fertilizer media for the mass culturing of *Nannochloropsis oceanica*. Specific growth rates of *N. oceanica* cultured with differing concentrations of commercial compounds, urea fertilizers, and trace elements (Zn, Cu, Co, Mo) were compared with the growth rate in f/2 medium. Among the various added trace elements,  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$  was most effective for high growth of *N. oceanica*. The main nitrogen source in the agricultural fertilizers was ammonium, which was unsuitable for the growth of *N. oceanica*. Thus, the fertilizer at a lower concentration infused with  $\text{NaNO}_3$  as a nitrogen source was more effective than fertilizer at higher concentrations. In this study, the growth of *N. oceanica* cultured with an agricultural fertilizer medium composed of compound fertilizer (41.7 mg/L), urea fertilizer (34.4 mg/L),  $\text{NaNO}_3$  (150 mg/L), and  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$  (0.0588 mg/L) was similar to that of *N. oceanica* cultured in f/2 medium.

**Key words:** Agricultural fertilizer, Economical media, f/2 media, *Nannochloropsis oceanica*

## Introduction

In the past, aquaculture was the main industry using microalgae commercially. Today, various businesses and industries, including those involved in supplementary health products, cosmetics, medicine, and bio-energy, are making extensive use of microalgae (Borowitzka and Borowitzka, 1988).

The advantages of using microalgae as commercial bio-materials include eco-friendly culture methods that allow for continuous reproduction and wide-ranging uses without pollutants. However, weaknesses include the sudden death of microalgae, which often leads to high costs and low productivity (Chisti, 2007).

The cost of microalgae used as live food in artificial seed production of shellfish is nearly 30% of the total cost of seed production (Borowitzka, 1997). However, if stable and economical microalgae production can be developed, microalgae will likely become one of the most important materials in bio-industry.

For mass production of microalgae, reagents used for in-

door culture would be inappropriate because of their high cost. Instead, more economical resources, such as agricultural fertilizers, are frequently used (López-Ruiz et al., 1995; Valenzuela-Espinoza et al., 2002; Pacheco-Vega and Sánchez-Saavedra, 2009). Using agricultural fertilizers only, however, leads to the problem of lower cell growth rates than in common media such as f/2 (Guillard and Ryther, 1962).

*Nannochloropsis oceanica* is commonly used to culture rotifers for marine fishes (Cabrera et al., 2005; Kobayashi et al., 2005; Ferreira et al., 2009) and to create "green water" for nursery tanks (Cabrera and Hur, 2001) because they are nutritious and easy to mass-produce. Additionally, their high contents of vitamins (Brown et al., 1997), lipids (Patil et al., 2007; Seychelles et al., 2009), highly unsaturated fatty acids (Sukenic et al., 1993; Zittelli et al., 1999; Hu and Gao, 2003), protein (Volkman et al., 1993), and natural pigment (Lubián et al., 2000) distinguish *N. oceanica* as a prospective microalgal species to be further researched and developed for the marine

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bio-industry (Becker, 1981; Harting et al., 1988; Cha et al., 2010). In this study, we sought to develop economical media that could effectively replace f/2 for the mass production of *N. oceanica*.

## Materials and Methods

The agricultural fertilizers used in this study were as follows: urea fertilizer containing 46% nitrogen and compound fertilizer (Nam-Hae Chemicals Inc., Yeosu, Korea) containing 22% nitrogen, 12% phosphorus, 17% potassium, and 3% magnesium. The amount of the fertilizers used in this study followed the Schreiber medium standard (Schreiber, 1927) that consists of  $\text{NaNO}_3$  (100 mg/L) and  $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$  (20 mg/L). Because 1 L of filtered seawater with 166.7 mg of compound fertilizer and 137.6 mg of urea fertilizer equals the concentrations of nitrate and phosphate in Schreiber medium, this standard was set as 1.0 times the basic fertilizer medium. The fertilizers were ground, dissolved in warm water, and filtered immediately before use.

The *N. oceanica* (KMMCC-13) used in this study were obtained from the Korea Marine Microalgae Culture Center (KMMCC) at Pukyong National University, South Korea. To culture *N. oceanica*, the following steps were conducted. First, 100 mL of autoclaved fertilizer media and 10 mL of culture stock were put into a 250 mL Erlenmeyer flask. Standing cultures were then kept at 25°C under continuous lighting of 100  $\mu\text{mol m}^{-2} \text{s}^{-1}$  and 15 psu. The culture was conducted in triplicate. Cell density was assessed twice daily at the same times using a hemocytometer, and the daily specific growth rate (SGR) was measured by the Guillard method (1973):  $\text{SGR} = 3.322 \times \log(\text{N}_2/\text{N}_1)/(t_2-t_1)$ , where  $t_2$  and  $t_1$  are culture days after inoculation, and  $\text{N}_2$  and  $\text{N}_1$  are the cell density at  $t_2$ , and  $t_1$ , respectively.

### Culturing *N. oceanica* with differing concentrations of fertilizers and addition of trace elements

To find the optimal concentrations of fertilizers, cell growth was observed for 5 days in the following conditions: 1. f/2 medium as a control group, 2. fertilizer medium 1.0 times (166.7 mg/L of compound fertilizer and 137.6 mg/L of urea fertilizer), 3. fertilizer media 1.25 times (208.4 mg/L of compound fertilizer and 172.0 mg/L of urea fertilizer), and 4. fertilizer 1.5 times (250.1 mg/L of compound fertilizer, 206.4 mg/L of urea fertilizer).

For trace elements, those used in f/2 medium, such as  $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$  (0.110 mg/L),  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$  (0.0196 mg/L),  $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$  (0.044 mg/L), and  $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$  (0.012 mg/L), were added to the fertilizer medium at varying concentrations: 0.5, 1.0, 1.5, and 2.0 times. The growth rates in these media were observed for 7 days and compared with the growth rate in f/2 medium.

### Effects of the addition of $\text{NaNO}_3$

The concentration of the previous fertilizer medium was reduced to 0.5, 0.25, and 0.17 times. Then,  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$  (0.0588 mg/L) at three times the concentration in f/2 medium and  $\text{NaNO}_3$  (150 mg/L) at the same concentration as in f/2 medium were added. This experiment involved eight groups, and daily growth in each of the following groups was measured for 7 days: 1) f/2 medium; 2) fertilizer medium 1.25 times; 3) fertilizer medium 0.5 times (compound fertilizer 83.4 mg/L + urea fertilizer 68.8 mg/L +  $\text{NaNO}_3$ ); 4) group 3 +  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ ; 5) fertilizer medium 0.25 times (compound fertilizer 41.7 mg/L + urea fertilizer 34.4 mg/L +  $\text{NaNO}_3$ ); 6) group 5 +  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ ; 7) fertilizer medium 0.17 times (compound fertilizer 28.3 mg/L + urea fertilizer 23.4 mg/L +  $\text{NaNO}_3$ ); and 8) group 7 +  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ .

### Growth comparison with laboratory and industrial reagents

To develop an economical fertilizer medium for the mass production of *N. oceanica*, the  $\text{NaNO}_3$  and  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$  reagents were examined separately with laboratory reagents ( $\text{NaNO}_3$ , Samchun Pure Chemical Co., Ltd., Pyeongtaek, Korea;  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ , Shimakyu's Pure Chemicals, Osaka, Japan) and industrial reagents ( $\text{NaNO}_3$ , Rifa Ind. Co. Ltd., Ludwigshafen, Germany;  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ , Young Poong Inc., Seoul, Korea). The *N. oceanica* were cultured for 7 days and their growth was measured using the methods above.

### Statistics analyses

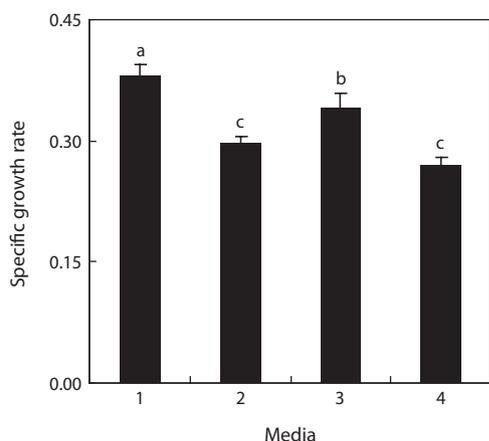
Results were analyzed by one-way analysis of variance, and Duncan's multiple range test (Duncan, 1955) was used to detect significant differences at the level of  $P < 0.05$ . SPSS ver. 17 (SPSS Inc., Chicago, IL, USA) was used for all analyses.

## Results

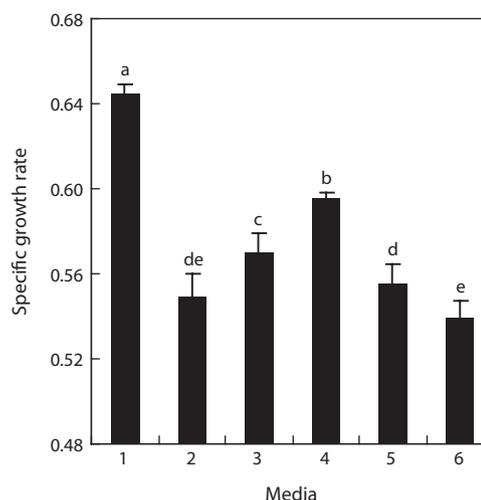
### Growth according to concentration of fertilizers and addition of trace elements

The growths of *N. oceanica* cultured in f/2 and agricultural fertilizer media for 5 days are shown in Fig. 1. The f/2 medium, as the control group, showed the significantly highest SGR of 0.3815. In fertilizer medium 1.25 times, the growth rate of 0.3407 was significantly lower than that in f/2 medium. However, this growth rate was significantly higher than those of the other experimental groups ( $P < 0.05$ ).

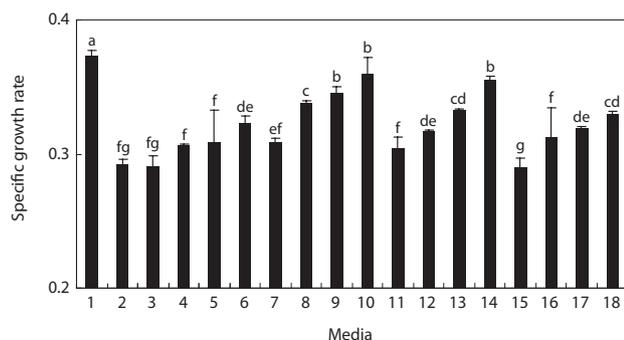
The results of the 7-day cultures of *N. oceanica* in fertilizer medium 1.25 times plus trace elements,  $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ ,  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ ,  $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ , and  $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$  at con-



**Fig. 1.** Specific growth rate of *Nannochloropsis oceanica* cultured with agricultural fertilizer media (1, f/2; 2, compound fertilizer 166.7 mg/L + urea fertilizer 137.6 mg/L; 3, compound fertilizer 208.4 mg/L + urea fertilizer 172.0 mg/L; 4, compound fertilizer 250.1 mg/L + urea fertilizer 206.4 mg/L).



**Fig. 3.** Specific growth rate of *Nannochloropsis oceanica* cultured with agricultural fertilizer media added with different concentrations of  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$  [1, f/2; 2, compound fertilizer 208.4 mg/L + urea fertilizer 172.0 mg/L; 3, 2 +  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$  (0.0392 mg/L); 4, 2 +  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$  (0.0588 mg/L); 5, 2 +  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$  (0.0784 mg/L); 6, 2 +  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$  (0.098 mg/L)].



**Fig. 2.** Specific growth rate of *Nannochloropsis oceanica* cultured with agricultural fertilizer media added with different concentrations of trace elements [1, f/2; 2, compound fertilizer 208.4 mg/L + urea fertilizer 172.0 mg/L; 3, 2 +  $\text{CoCl}_2$  (0.055 mg/L); 4, 2 +  $\text{CoCl}_2$  (0.110 mg/L); 5, 2 +  $\text{CoCl}_2$  (0.165 mg/L); 6, 2 +  $\text{CoCl}_2$  (0.220 mg/L); 7, 2 +  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$  (0.0098 mg/L); 8, 2 +  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$  (0.0196 mg/L); 9, 2 +  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$  (0.0294 mg/L); 10, 2 +  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$  (0.0392 mg/L); 11, 2 +  $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$  (0.022 mg/L); 12, 2 +  $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$  (0.044 mg/L); 13, 2 +  $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$  (0.066 mg/L); 14, 2 +  $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$  (0.088 mg/L); 15, 2 +  $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$  (0.006 mg/L); 16, 2 +  $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$  (0.012 mg/L); 17, 2 +  $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$  (0.018 mg/L); 18, 2 +  $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$  (0.024 mg/L)].

centrations of 0.5-2.0 times were as follows. Higher contents of trace elements produced higher growth rates of *N. oceanica* (Fig. 2). The fertilizer medium 1.25 times and the media infused with 0.5 times  $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$  (0.055 mg/L) and  $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$  (0.006 mg/L) showed the lowest growth rates in the range of 0.2903-0.2930.

The growth rates of the experimental groups infused with  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$  and  $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$  were 0.3096-0.3598 and 0.3042-0.3559, respectively. These growth rates were relatively high compared with those for other media infused with  $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$  or  $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$ , which showed rates of

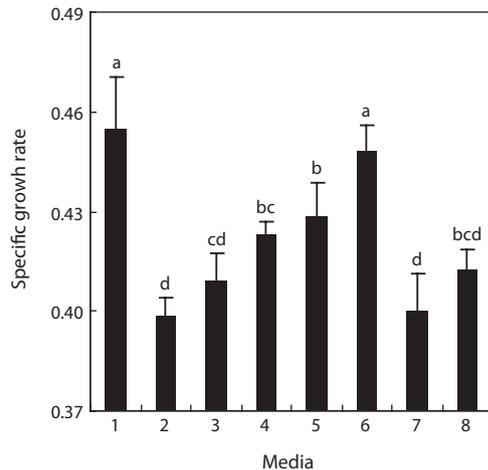
0.2903-0.3231.

In the experimental groups infused with either 1.5 (0.0294 mg/L) or 2 times (0.0392 mg/L)  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$  and 2 times  $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$  (0.088 mg/L), the growth rates of 0.3457-0.3598 were still significantly lower than the rate of 0.3726 for the control group in f/2 medium. However, the former groups showed higher growth rates than the rest of the experimental groups ( $P < 0.05$ ).

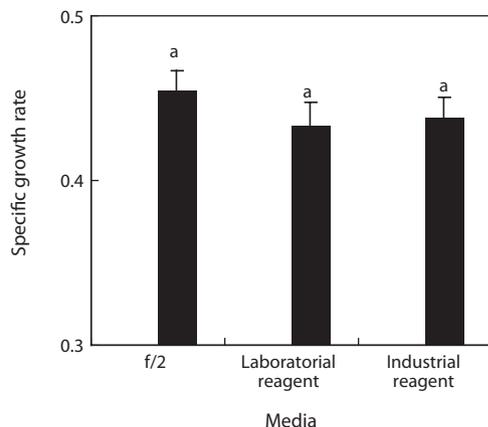
To test the exact concentration of  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$  for infusion, 2-, 3-, 4-, and 5-fold increased concentrations (0.0392-0.098 mg/L) of  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$  were added to fertilizer medium 1.25 times and cultures were grown for 8 days. The results indicated the significantly highest growth rate of 0.6446 in the control f/2 group ( $P < 0.05$ ) (Fig. 3). When three-fold  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$  was added, the growth rate was 0.5955. Although this growth rate was lower than that in f/2 medium, it was significantly higher than the other fertilizer media ( $P < 0.05$ ). For more than three-fold  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$  infusion, as the concentration of copper was increased, the growth rate decreased significantly ( $P < 0.05$ ).

### Growth according to addition of $\text{NaNO}_3$

On the basis of the findings in this study, growth differences in f/2 and fertilizer media seemed to be correlated with the high content of ammonia and low content of nitrate. With the infusion of  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$  (0.0588 mg/L) and  $\text{NaNO}_3$  (150 mg/L), the amounts of fertilizers were reduced by 0.5 times (compound fertilizer, 83.4 mg/L; urea fertilizer, 68.8 mg/L), 0.25 times (compound fertilizer, 41.7 mg/L; urea fertilizer, 34.4 mg/L), and 0.17 times (compound fertilizer, 28.3 mg/L;



**Fig. 4.** Specific growth rate of *Nannochloropsis oceanica* cultured with low concentration of agricultural fertilizer media added with  $\text{NaNO}_3$  (150 mg/L) and  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$  (0.0588 mg/L) [1, f/2; 2, compound fertilizer 208.4 mg/L + urea fertilizer 172.0 mg/L; 3, compound fertilizer 83.4 mg/L + urea fertilizer 68.8 mg/L +  $\text{NaNO}_3$ ; 4, 3 +  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ ; 5, compound fertilizer 41.7 mg/L + urea fertilizer 34.4 mg/L +  $\text{NaNO}_3$ ; 6, 5 +  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ ; 7, compound fertilizer 28.3 mg/L + urea fertilizer 23.4 mg/L +  $\text{NaNO}_3$ ; 8, 7 +  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ ].



**Fig. 5.** Specific growth rate of *Nannochloropsis oceanica* cultured with agricultural fertilizer media (compound fertilizer 41.7 mg/L + urea fertilizer 34.4 mg/L) added with laboratory or industrial reagent of  $\text{NaNO}_3$  (150 mg/L) and  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$  (0.0588 mg/L).

urea fertilizer, 23.4 mg/L) the level in Schreiber culture medium. As a result (Fig. 4), the growth rate of 0.3985 in fertilizer medium 1.25 times was significantly the lowest rate ( $P < 0.05$ ). Fertilizer medium 0.25 times infused with  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$  (0.0588 mg/L) and  $\text{NaNO}_3$  (150 mg/L) showed a high growth rate of 0.4481, about 96% of that in f/2 medium. In fact, this result was not significantly different from that in f/2 medium ( $P < 0.05$ ).

### Growth rate comparison between laboratory and industrial reagents

From the previously mentioned fertilizer media 0.25 times (compound fertilizer, 41.7 mg/L + urea fertilizer 34.4 mg/L) infused with  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$  0.0588 mg/L and  $\text{NaNO}_3$  150 mg/L,  $\text{NaNO}_3$  and  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$  were assessed separately with laboratory and industrial reagents. The growth rates of *N. oceanica* in fertilizer media with laboratory and industrial reagents were 0.4331 and 0.4383, respectively (Fig. 5). These results indicated no significant difference compared with the growth rate (0.4549) in f/2 medium ( $P < 0.05$ ).

## Discussion

Culture media for microalgae should be economical, allow for high growth rates, satisfy the needs of the microalgae, and be easy to prepare. f/2 medium, the most commonly used medium for small-scale indoor culture, is costly and difficult to set up for outdoor mass culture (Fabregas et al., 1985). Thus, agricultural fertilizers are commonly used as a replacement for f/2 culture medium (Fabregas et al., 1987; Bae, 2004; Pacheco-Vega and Sánchez-Saavedra, 2009). However, cell growth rates in such fertilizer-based media have not yet reached that in f/2 culture medium. The slower rate is attributable to the presence of nitrogen and phosphorus, major components in fertilizer cultures (Ukeles, 1980; González-Rodríguez and Maestrini, 1984; Bae, 2004). Lack of trace elements and vitamins necessary for the growth of microalgae are also reasons for lower growth rates (Stein, 1973).

Our aim was to develop media using economical and convenient agricultural fertilizers to replace f/2 medium for outdoor mass culture of *N. oceanica*, which has recently been in the commercial spotlight (Cha et al., 2010). Most common culture media for microalgae are based on Schreiber medium, containing  $\text{NaNO}_3$  (100 mg/L) and  $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$  (20 mg/L) (Schreiber, 1927). Thus, we tested for optimal concentrations of agricultural urea and compound fertilizers in relation to these nutrients.

From the second and third days after inoculation, the growth stage of the cells was in log phase in f/2 media. On the other hand, fertilizer media showed a longer lasting lag phase, and the lower level of the ultimate highest cell density was problematic. The following factors are believed to have caused such results: urea fertilizer, consisting of 46% nitrogen, and compound fertilizer consisting of 22% nitrogen, 12% phosphorus, 17% potassium, and 3% magnesium. Other causes may have been the lack of essential trace elements for the growth of *N. oceanica* and a nitrogen source consisting mostly of ammonia.

After adding the four trace elements (Co, Cu, Zn, Mo) used in f/2 medium to each fertilizer medium, growth rates of *N. oceanica* were observed. When  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$  (0.0588 mg/L) three times the concentration in f/2 medium was added, the *N. oceanica* growth rate was 80% of that in f/2 medium. Cu is essential for the growth of microalgae especially for pho-

tosynthesis and enzymatic reactions (Andrade et al., 2004). However, Okauchi et al. (2008) claimed that zinc and cobalt had greater influence than copper on the growth of *N. oculata* (see Guillard, 1973). In this study, however, copper was the most effective factor in the growth of *Nannochloropsis*. In Bae's (2004) study, the concentration of Cu added to f/2 medium was increased 2 to 80 times, and the growth rate of *N. oceanica* began to decrease above 5 times concentration ( $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$  0.0196 mg/L).

González-Rodríguez and Maestrini (1984) used 12 kinds of fertilizers for 16 microalgal species with Conway medium as a control (Walne, 1966). The result of their research, which was similar to our result, showed extremely low growth rates of *Nannochloris oculata*, *Isochrysis galbana*, *Chlamydomonas palla*, and *Chaetoceros* sp. However, for *Phaeodactylum tricorutum*, *Skeletonema costatum*, *Tetraselmis striata*, and *Thalassiosira pseudonana*, growth rates were similar to or higher than that in Conway medium, the control group. Such differences in growth rates could be due to the nitrogen source, requiring differing media for each kind of microalgae.

Bae (2004) cultured *N. oceanica* with agricultural fertilizer for 16 days and then analyzed the water. The results showed that the concentration of  $\text{NH}_4\text{-N}$  (10.0 ppm) in agricultural fertilizer was approximately 154 times higher than that in f/2 culture medium (0.065 ppm); in addition, the concentration of  $\text{PO}_4\text{-P}$  was nine times higher. These results imply that the growth of microalgae depends on their sensitivity to the ammonium concentration (1 mg atom N/L) (Kaplan et al., 1986). If the concentration is higher than 0.5 mg atom N/L, the growth of microalgae exposed to high intensities of light and pH is likely to decrease (Admiraal, 1977; Kalpan et al., 1986).

In this study, the growth of microalgae in the fertilizer media was slower than that in f/2 medium in the early stages of the experiment. The low level of the highest cell density was also believed to be due to the high ammonium content.

Thus, to reduce the concentration of ammonia in the fertilizer media, the amounts of fertilizers were reduced to 25% of Schreiber's nitrate and phosphate concentrations. The growth of *N. oceanica* in fertilizer media infused with  $\text{NaNO}_3$  (150 mg/L) and  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$  (0.0588 mg/L) and that in f/2 medium showed no significant difference ( $P < 0.05$ ). Moreover, in the case of using  $\text{NaNO}_3$  and  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ , which are less costly than industrial fertilizers, the growth rate of *N. oceanica* showed no significant difference compared with that in f/2 medium ( $P < 0.05$ ).

In conclusion, for 1 ton of filtered seawater, an optimal medium for the mass culturing of *N. oceanica* can be achieved with the following materials: urea fertilizer containing 22% nitrogen (34.4 g), compound fertilizer containing 22% nitrogen, 12% phosphorus, 17% potassium, and 3% magnesium (41.7 g), and industrial reagent grade  $\text{NaNO}_3$  (150 g) and  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$  (0.0588 g).

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