

Utilization of various nitrogen, phosphorus, and selenium compounds by *Cochlodinium polykrikoides*

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Abstract: The ability of *Cochlodinium polykrikoides* to use various nitrogen, phosphorus, and selenium compounds as a nutrient source was examined in batch culture experiments to obtain biological information on the mechanism of *C. polykrikoides* bloom formation. *C. polykrikoides* grew using a variety of organic or inorganic nitrogen sources except for L-proline. Maximum yields of *C. polykrikoides* were obtained by gradually increasing ammonium from 5 to 20 μM , but the yield was inhibited by addition of more than 50 μM . Growth was observed in media containing various phosphorus sources, such as phosphate and 11 different organic compounds. Organic nitrogen and phosphorus seem to play an important role in the dominance of phytoplankton species and mass growth of *C. polykrikoides*. The ability to use a variety of organic nutrients may allow *C. polykrikoides* to grow to a high density in spite of inorganic nitrogen and phosphorus depletion. *C. polykrikoides* grew in the presence of selenite, selenate, and Se-(methyl) selenocysteine hydrochloride. However, growth yield was inhibited by addition of more than 500 μM selenate and 1000 μM Se-(methyl) selenocysteine hydrochloride. Therefore, much of the oil effluents seen in 2005 may have a temporary inhibitory action on *C. polykrikoides* growth but can be expected to have a positive effect in the long term.

Key words: *Cochlodinium polykrikoides*, Growth kinetics, Oil effluent, Organic nitrogen and phosphorus, Phytoplankton bloom, Selenium
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Introduction

In 1995, large dinoflagellate *Cochlodinium polykrikoides* blooms first appeared around the South Sea of Korea. The *C. polykrikoides* blooms develop initially in comparatively clean offshore water with almost no contamination from the land, and they show a strong tendency to remain in offshore water after their development in the South Sea of Korea. These blooms cause enormous damage to aquacultured fish and a large budget is spent every year towards their prevention.

Large *C. polykrikoides* blooms now occur every year, and many studies have investigated the relationship between their occurrence and environmental factors such as temperature, salinity, light, and inorganic nutrients to elucidate the mechanisms involved in *C. polykrikoides* blooms and to predict the time and place of their outbreak (Lee *et al.*, 2001; Lee, 2006; Lee and Lee, 2006). However, a precise explanation of the mechanisms involved in *C. polykrikoides* blooms is currently lacking. Therefore, more detailed studies on nutrient availability and *C. polykrikoides* growth kinetics are necessary to determine why blooms occur in clean offshore water.

Nitrogen and phosphorus are important nutrients for the growth of phytoplankton (Mathivanan *et al.*, 2007; Park and Shin, 2007). Recent studies have indicated that *Gymnodinium mikimotoi* and *Gymnodinium catenatum* are able to grow using dissolved organic nitrogen and phosphorus compounds as nutrient sources (Yamaguchi and Itakura, 1999; Yamamoto *et al.*, 2004). According to Yamaguchi and Itakura (1999), selenium is also needed for growth in *G. mikimotoi*. In September 1995, large *C. polykrikoides* blooms

occurred off the Korean coast after the oil tanker Sea Prince ran aground near Sorido, Yosu in July of that year (Lee *et al.*, 2001). Crude petroleum, which contains a large quantity of selenium (Lemly, 2004), leaked from the stranded vessel and would have been expected to be involved in a *C. polykrikoides* bloom. However, little is known about the utilization of organic nitrogen and phosphorus by *C. polykrikoides*, and the effect of selenium compounds on its growth.

In this study, the ability of *C. polykrikoides* to use various nitrogen, phosphorus, and selenium compounds as a nutrient source was examined in batch culture experiments to obtain biological information on the mechanism of *C. polykrikoides* bloom formation.

Materials and Methods

Strain and culture conditions: The strain of *C. polykrikoides* used in this experiment was isolated from the coastal seawater off Narodo in the South Sea of Korea during summer 2002. The isolates were rinsed repeatedly with sterile seawater. This procedure was repeated until axenic conditions were obtained and confirmed by 4',6-diamidino-2-phenylindole (DAPI) staining. Stock cultures were maintained with f/2 medium using surface seawater taken from between the South Sea of Korea and Jeju because *C. polykrikoides* could not be cultured with coastal seawater from the South Sea of Korea, except during the bloom period. The surface seawater between the South Sea and Jeju was collected in October 2003 and had background DIN and DIP concentrations of 2.8 and 0.20 μM , respectively. The following experiments were conducted using the same surface seawater. The culture was performed at $23 \pm 2^\circ\text{C}$ with fluorescent illumination of $140 \pm 10 \mu\text{mol m}^{-2} \text{sec}^{-1}$ on a 12 hr light : 12 hr dark cycle (lights on at 08:00 and off at 20:00 hr). The



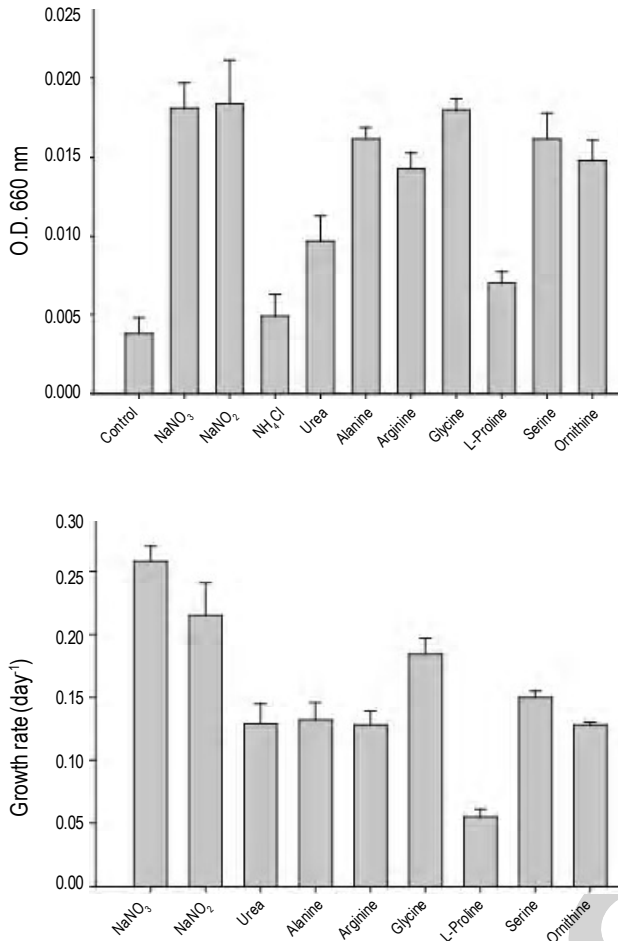


Fig. 1: Maximum growth yield and growth rate of *C. polykrikoides* by spike of variety nitrogen compounds (concentrations of added N compounds are adjusted to 88.2 μ M, which were 10% of F/2 medium). Control means no nitrogen addition. Error bars indicate standard deviation ($n=3$)

culture vessels were 50-ml sterilized polystyrene flasks (Nunclon Delta, Naperville, IL, USA) containing 20 ml f/2 medium.

Utilization of nitrogen, phosphorus, and selenium: The utilization of various nitrogen, phosphorus, and selenium compounds for growth of *C. polykrikoides* was tested in batch culture experiments. Ten nitrogen sources (NaNO₂, NaNO₃, NH₄Cl, urea, alanine, arginine, glycine, L-proline, serine, and ornithine), 12 phosphorus sources (NaH₂PO₄, ATP, ADP, AMP, GMP, UMP, D-fructose phosphate, glucose 1-phosphate, p-nitrophenyl phosphate, triphosphosphate, sodium pyrophosphate, b-glycerophosphate), and 3 selenium sources [selenite (Na₂SeO₃), selenate (Na₂SeO₄), Se-(methyl) selenocysteine hydrochloride] were selected, all of which are easily soluble in distilled water at room temperature. These nitrogen, phosphorus, and selenium compounds were added to 50-ml sterilized polystyrene flasks (Nunclon) containing 20 ml autoclaved nitrogen-, phosphorus-, and selenium-limited f/2 medium, respectively,

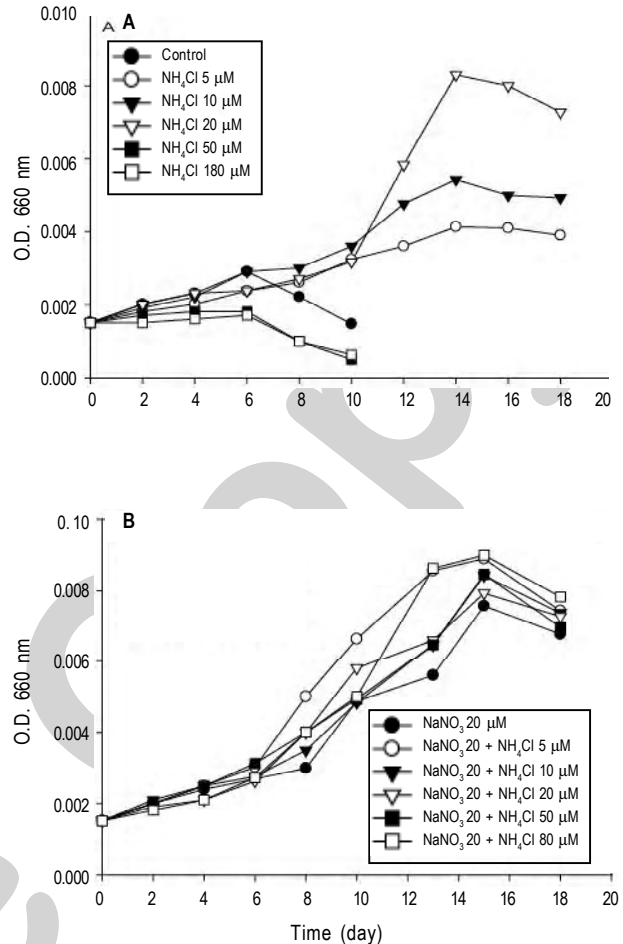


Fig. 2: Growth response of *C. polykrikoides* by spike of variety concentration of ammonium nitrogen (A) and combination of ammonium nitrogen and nitrate nitrogen (B). Control means no nitrogen addition ($n=3$)

after sterilization by filtration through a 0.2- μ m disposable syringe filter (Nalgene, 25-mm). The concentrations of added nitrogen and phosphorus compounds were adjusted to 88.2 and 3.6 μ M, respectively, which were 10% of the f/2 medium concentration. The effect of various ammonium concentrations in combination with 20 μ M nitrate on *C. polykrikoides* growth was also investigated.

Before *C. polykrikoides* inoculation, the cells were cultured for 1 week in 500-ml sterilized polystyrene flasks (Nunclon) containing nitrogen- and phosphorus-depleted f/2 medium. The concentration of inoculum was adjusted to 200 cells ml⁻¹ for all nitrogen, phosphorus, and selenium compounds. Incubation was performed under the same conditions as the stock cultures and growth was monitored every 2 days by optical density (O.D.) using a spectrophotometer (Uvikon 930: 660 nm; Kontron, Munich, Germany) or a PHYTO-PAM chl. *a* fluorometer (Heinz Walz GmbH, Effeltrich, Germany) (Schreiber *et al.*, 2002). The growth rate was calculated by least

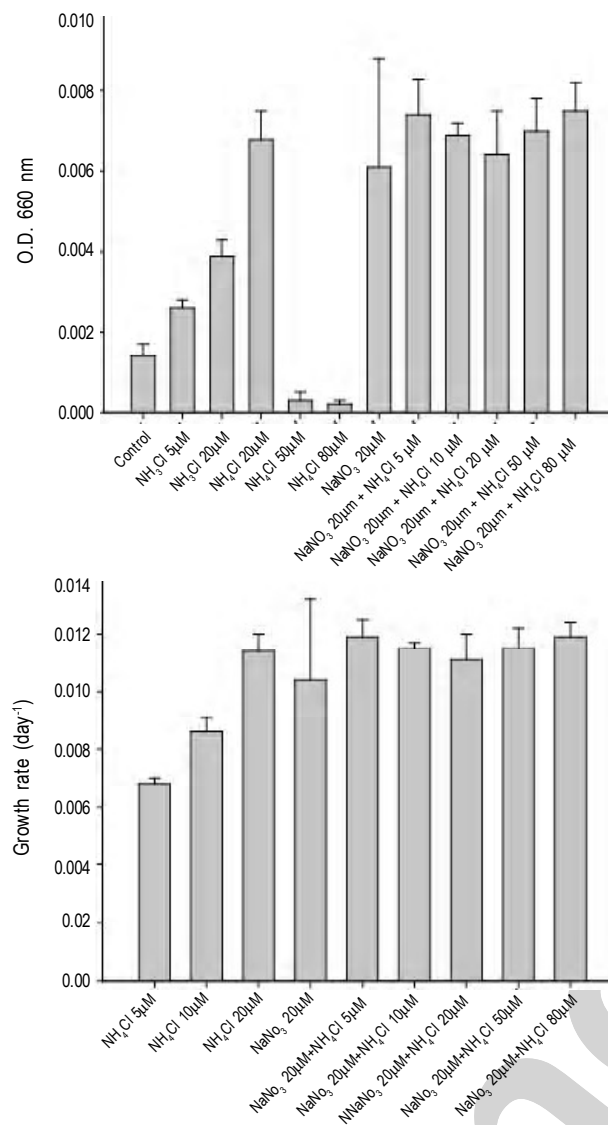


Fig. 3: Maximum growth yield and growth rate of *C. polykrikoides* by spike of variety concentration of nitrate nitrogen and ammonium nitrogen. Control means no nitrogen addition. Error bars indicate standard deviation (n=3)

squares regression analysis of the natural logarithm of fluorescence on given days using data from the exponential portions of the growth curves. Statistical analysis was performed using the SPSS Windows Program (10.1; SPSS Inc., Chicago, IL, USA), and tests were determined to be significant at the $p < 0.005$ level.

Results and Discussion

Nitrogen and phosphorus compounds: Fig. 1 shows the maximum growth yield and growth rate of *C. polykrikoides* using various nitrogen compounds. Maximum yields of *C. polykrikoides* in media with addition of nitrogen sources, except for ammonium ($p = 0.996$) and L-proline ($p = 0.314$), were significantly higher than in the control ($p < 0.002$). The lowest yield of *C. polykrikoides* was observed in the presence of ammonium. Growth rates were calculated

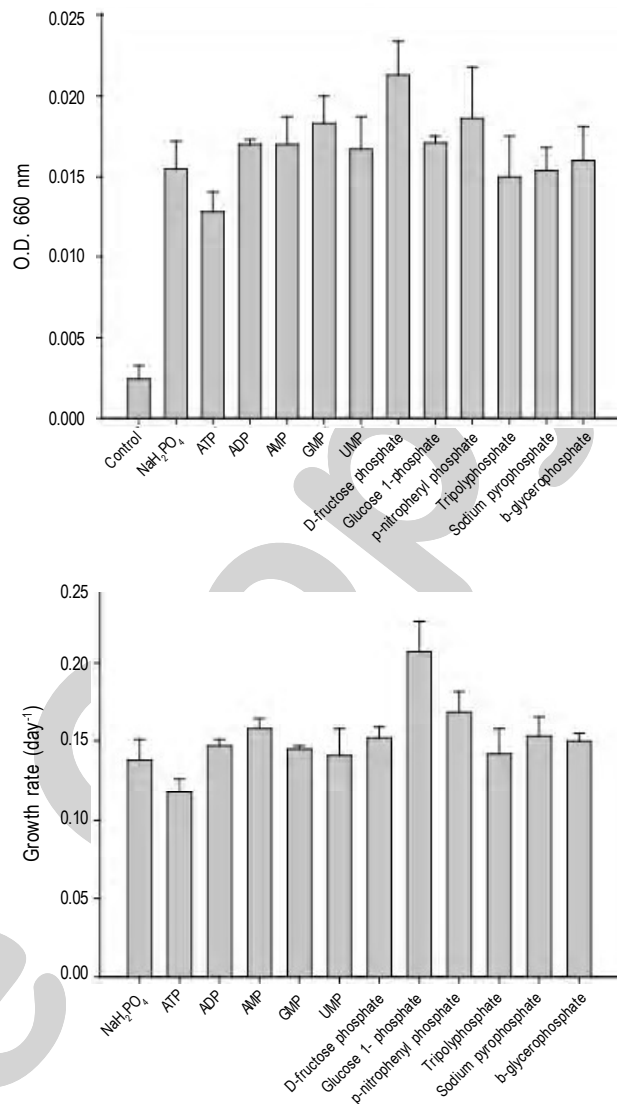


Fig. 4: Maximum growth yield and growth rate of *C. polykrikoides* by spike of variety phosphorus compounds (concentrations of added P compounds are adjusted to 3.6 μM , which were 10% of F/2 medium). Control means no phosphorus addition. Error bars indicate standard deviation (n=3)

for the nitrogen sources that supported fairly good growth of *C. polykrikoides*. The growth rate ranged from 0.055 to 0.258 day^{-1} . The highest growth rate was observed with addition of nitrate and the lowest with addition of L-proline. *C. polykrikoides* was able to grow in the presence of all the nitrogen sources that were tested at a concentration of 88.2 μM , regardless of whether they were organic or inorganic, with the exception of ammonium and L-proline. *Alexandrium catenella* can use urea and glutamine as an organic nitrogen source among 21 organic compounds (Matsuda *et al.*, 1999; Collos *et al.*, 2004). Only urea, glutamine, and tryptophan from among 21 organic nitrogen sources support the growth of *G. mikimotoi* (Yamaguchi and Itakura, 1999). *Heterocapsa circularisquama* cannot utilize urea and uric acid as a nitrogen source and uses nitrate, nitrite and ammonium (Yamaguchi *et al.*, 2001).



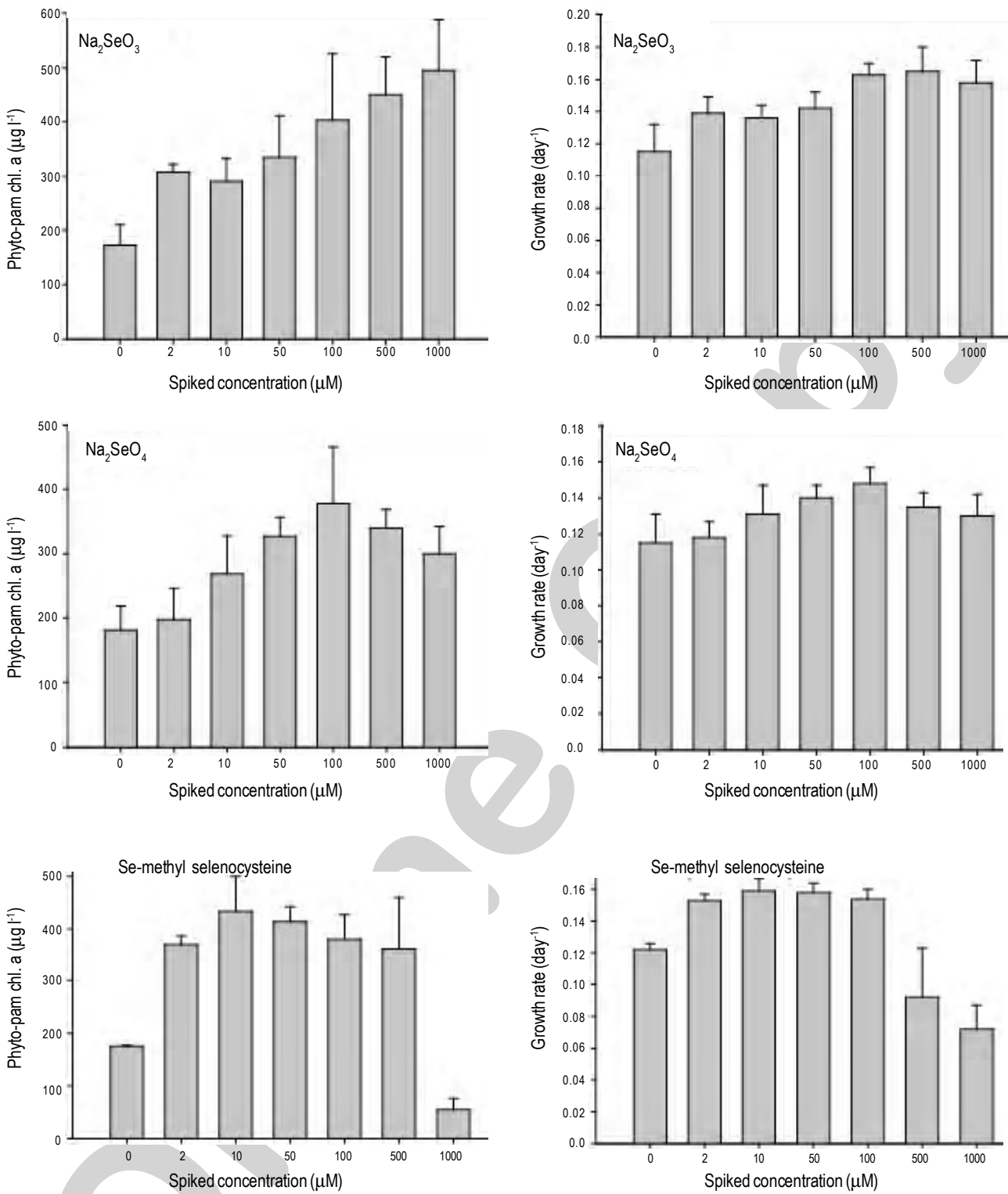


Fig. 5: Maximum growth yield and growth rate of *C. polykrikoides* by spike of variety selenium concentrations and compounds. Error bars indicate standard deviation (n=3)



Therefore, *C. polykrikoides* seems to utilize a greater variety of organic nitrogen sources than the other phytoplankton mentioned above.

Figure 2 shows growth responses of *C. polykrikoides* by spike of variety concentration of ammonium nitrogen and in combination with 20 μM nitrate nitrogen. The maximum growth yield were observed at about incubation time of 15 days except in control and above 50 μM of ammonium additions. Matsuda *et al.* (1999) showed that there was a time lag of 9 days before growth started in addition of 250 μM of ammonium and 3 days at lower ammonium concentrations. Moreover, a time lag of 3 days was also observed in additions of nitrate. However, in present study, no time lag was observed by additions of ammonium nitrogen and nitrate nitrogen.

Figure 3 also shows the maximum growth yield and growth rate of *C. polykrikoides* for various ammonium concentrations in combination with 20 μM nitrate. The maximum yield of *C. polykrikoides* was obtained by gradually increasing the concentration of ammonium from 5 to 20 μM (control and 5 μM $\text{NH}_4\text{-N}$, $p = 0.007$; control and 10 μM $\text{NH}_4\text{-N}$, $p < 0.0001$), but the yield with more than 50 μM ammonium was lower than in the control. The yields in combination with 20 μM nitrate were higher than the control. No inhibit effect of *C. polykrikoides* growth was observed in additions of 50 to 80 μM of ammonium concentration in combination with 20 μM of nitrate. It seems that *C. polykrikoides* vitality is increased by addition of nitrate, although the explanation is not clear.

In the case of *G. mikimotoi*, the yield or growth rate following addition of 250 μM ammonium was lower than that for nitrate and nitrite, but there was no inhibitory action (Yamaguchi and Itakura, 1999). Matsuda *et al.* (1999) reported that *A. catenella* can use ammonium at a concentration of less than 251.6 μM as a nitrogen source but does not grows by adding 501.6 μM ammonium. Yamaguchi *et al.* (2001) reported that 250 μM ammonium is a good nitrogen source for the growth of *H. circularisquama*. Watanabe *et al.* (1982) showed that growth of *Heterosigma akashiwo* is slightly inhibited by 2 mM ammonium. Therefore, *C. polykrikoides* seems to be more inhibited by ammonium than *A. catenella*, *G. mikimotoi*, *H. circularisquama* or *H. akashiwo*.

Figure 4 shows the maximum growth yield and growth rate of *C. polykrikoides* following addition of various phosphorus compounds. Maximum yield of *C. polykrikoides* in the presence of phosphorus sources was significantly higher than in the control ($p < 0.0001$), and the highest value was observed with D-fructose phosphate. The growth rate ranged from 0.118 to 0.208 day^{-1} and the highest rate was observed with glucose 1-phosphate.

Alexandrium catenella, *G. mikimotoi*, *G. catenatum* and *H. circularisquama* grow similarly in various dissolved organic phosphorus compounds and $\text{PO}_4\text{-P}$ (Matsuda *et al.*, 1999; Yamaguchi and Itakura, 1999; Yamaguchi *et al.*, 2001; Oh *et al.*, 2002). However, *Skeletonema costatum*, *Chattonella antique*, and *Chattonella marina* use only five to seven kinds of organic compounds

as a phosphorus source (Matsuda *et al.*, 1999). Therefore, phosphorus utilization by *C. polykrikoides* is similar to that of *A. catenella*, *G. mikimotoi*, *G. catenatum*, and *H. circularisquama*.

From the above results, it seems that organic nitrogen and phosphorus play an important role in the dominance of phytoplankton species and mass growth of *C. polykrikoides*. High-density growth of *C. polykrikoides* has been observed off the coasts of Korea and Japan (Lee, 2006), where inorganic nitrogen and phosphorus concentrations are lower than in estuaries and inland seas (Lee *et al.*, 2001). Therefore, the ability to use a variety of organic nutrients may allow *C. polykrikoides* to grow to a high density in spite of inorganic nitrogen and phosphorus depletion in these surface seawaters.

Little is known about the organic nitrogen and phosphorus concentrations in offshore waters, where *C. polykrikoides* grows on a large scale. According to Lee and Lee (2006), high-density *C. polykrikoides* blooms were observed after typhoon or heavy rainfall. Therefore, it is necessary to study the apparent spatial and temporal variations in organic nitrogen and phosphorus concentrations to understand and control the mass growth of *C. polykrikoides* in waters off the coast of Korea, especially after heavy rainfall events.

Selenium compounds: Figure 5 shows the maximum growth yield and growth rate of *C. polykrikoides* following addition of various concentrations of selenium compounds. Maximum yield of *C. polykrikoides* was obtained when the concentration of selenite, as an inorganic selenium source, was gradually increased up to 1000 μM (0 and 2 μM , $p = 0.653$; 0 and 1000 μM , $p = 0.019$). However, the yield of *C. polykrikoides* gradually increased following addition of selenate up to a concentration of 100 μM (0 and 100 μM , $p = 0.007$) and decreased at a concentration of above 500 μM . With regard to Se-(methyl) selenocysteine hydrochloride, the yield following addition of less than 500 μM was higher than that for 0 μM (0 and 500 μM , $p = 0.006$), but the yield following addition of 1000 μM was lower than that for 0 μM . The growth rates with selenite, selenate, and Se-(methyl) selenocysteine hydrochloride were 0.115–0.165, 0.115–0.148 and 0.072–0.159 day^{-1} , respectively. The growth rate gradually increased as the concentration of selenite was increased from 0 to 1000 μM , and the rate after addition of selenate was higher than in the control for all spiked concentrations. However, the growth rate in the presence of Se-(methyl) selenocysteine hydrochloride below 100 μM was higher than in the control, and the rate for more than 500 μM was lower than that for 0 μM .

Selenium has no effect on the growth of some phytoplankton such as *Chaetoceros gracilis*, *Chaetoceros simplex*, *Gymnodinium sanguineum* and *Gymnodinium simplex* (Harrison *et al.*, 1988). However, selenite is required for growth of some phytoplankton such as *Chaetoceros debilis*, *S. costatum* and *Thalassiosira rotula* (Harrison *et al.*, 1988). The selenite present in the open ocean at a concentration of about 0.05 μM , and the concentration is approximately an order of magnitude greater in coastal bays (Brand, 1986; Cospser *et al.*, 1993). Growth yield of *Chattonella verruculosa*



was increased when selenite was added at a concentration of 10^{-4} to $10 \mu\text{M}$ (Imai *et al.*, 1996). Thus, selenium seems to be an essential microelement for the growth of *C. polykrikoides* and phytoplankton such as *C. debilis*, *S. costatum*, *T. rotula* and *C. verruculosa*. Riedel *et al.* (1996) reported that selenate is generally more toxic to phytoplankton than selenite. In the present study on *C. polykrikoides* growth, there was no inhibition after addition of $1000 \mu\text{M}$ selenite, but some inhibition was observed with more than $500 \mu\text{M}$ selenate, as reported by Riedel *et al.* (1996). With regard to Se-(methyl) selenocysteine hydrochloride, *C. polykrikoides* growth yield was also inhibited by addition of $1000 \mu\text{M}$ and the growth rate was inhibited by addition of more than $500 \mu\text{M}$.

In conclusion, selenium seems to be an important trace metal on *C. polykrikoides* growth. And it is difficult to know for certain whether selenium compounds such as selenite, selenate and Se-(methyl) selenocysteine hydrochloride function as trigger elements for mass growth of *C. polykrikoides* in Korea, but these compounds seem to have a positive effect on *C. polykrikoides* growth. Therefore, much of the oil effluents seen in 1995 may have a temporary inhibitory action on *C. polykrikoides* growth, but they can be expected to have a positive effect in the long term.

According to Lee and Lee (2006), no *C. polykrikoides* cell division was observed by addition of a large quantity of iron. In this study, *C. polykrikoides* growth was also inhibited at high concentration of selenate and Se (methyl) selenocysteine. These results imply that the concentrations of trace metal seem to be an important factor for the mass growth of *C. polykrikoides*. Therefore, it is important to measure the spatial and temporal variations in selenium and iron concentrations to understand and control of *C. polykrikoides* blooms formations.

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