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

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1. Introduction

In 1995, large *Cochlodinium polykrikoides* blooms first appeared around the South Sea of Korea (http://www.nfrdi.re.kr). 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 toward 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. 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. 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.

2. Materials and methods

2.1. Strain and culture conditions

The strainof *C. polykrikoides* used in this experiment was isolated from the coastal seawater off Narodo in the South Sea of Korea during summer2002. 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 Jejudo 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 Jejudo was collected in October 2003 and had background DIN and DIP concentrations of 2.8 and 0.199 uM, respectively. The following experiments were conducted using the same surface seawater. The culture was performed at 23 ± 2℃ with fluorescent illumination of 140 ± 10 μmol m^2 sec^1 on a 12-h light 12-h dark cycle (lights on at 08:00 and off at 20:00 h). The culture vessels were 50-ml sterilized polystyrene flasks (Nunclon Delta, Naperville, IL, USA) containing 20 ml f/2 medium.

2.2. Utilization of nitrogen, phosphorus, and selenium

The utilization of various nitrogen, phosphorus, and selenium compounds for growthof C. polykrikoides was tested in batch culture experiments. Ten nitrogen sources ($NaNO_2$, $NaNO_3$, NH_4Cl, urea, alanine, arginine, glycine, L-proline, serine, and ornithine), 12 phosphorus sources (NaH_2PO_4, ATP, ADP, AMP, GMP, UMP, D-fructose phosphate, glucose 1-phosphate, p-nitrophenyl phosphate, tripolyphosphate, sodium pyrophosphate, b-glycerophosphate), and 3 selenium sources [selenite (Na_2SeO_3), selenate (Na_2SeO_4), 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, after sterilization by filtration through a 0.2-um disposable syringe filter. The concentrations of added nitrogen and phosphorus compounds were adjusted to 88.2 and 3.6 uM, respectively, which were 10% of the f/2 medium concentration. The effect of various ammonium concentrations in combination with 20 μM nitrate on *C.polykrikoides* propagation 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 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 (HeinzWalz GmbH, Effeltrich, Germany) (Schreiber et al., 2002). The growth rate was calculated by least 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.

3. Results and discussion

3.1. Nitrogen and phosphorus compounds

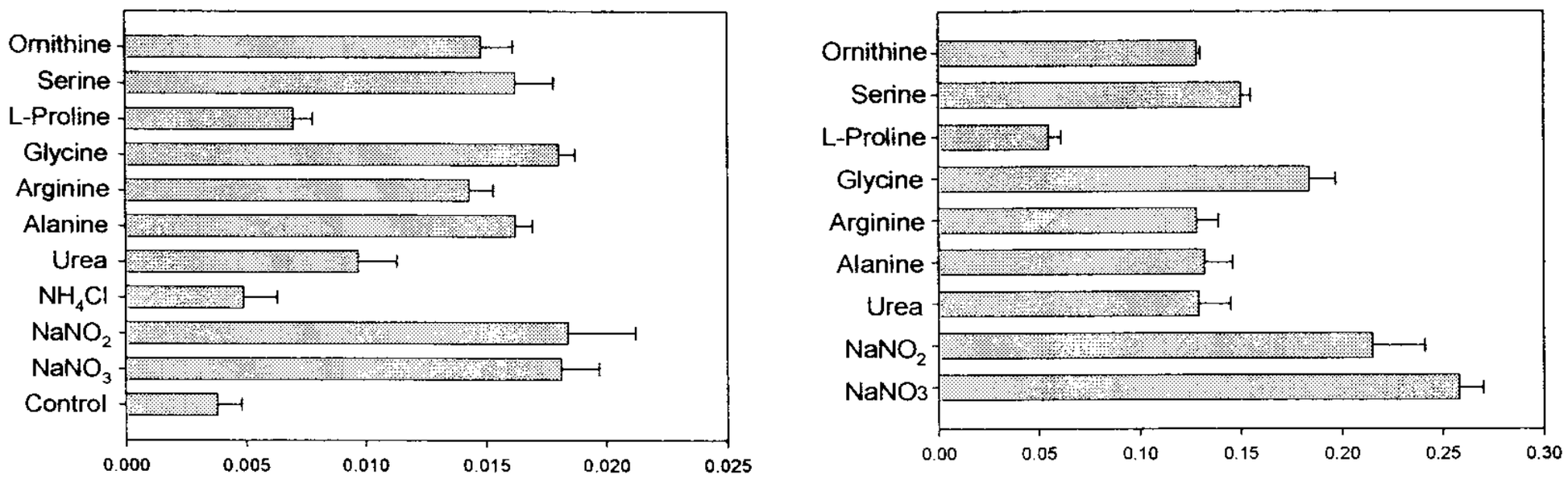

Fig. 1. Maximum fluorescence and growth rate *C. polykrikoides* by spike of variety nitrogen compounds (Concentrations of added N compounds are adjusted to 88.2uM, which were 10% of F/2 medium.). Control means no nitrogen addition. Error bars indicate standard deviation (n=3)

Figure 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 controls ($P < 0.002$). The lowest yield of *C. polykrikoides* was observed in the presence of ammonium. Growth rates were calculated for the nitrogen sources that supported fairly good propagation of *C. polykrikoides*. The growth rate ranged from 0.055 to 0.258 day1. 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 only glutamine as an organic nitrogen source among 21 organic compounds (Matsuda et al., 1999). 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). Therefore, *C. polykrikoides* seems to utilize a greater variety of organic nitrogen sources than the other phytoplankton mentioned above.

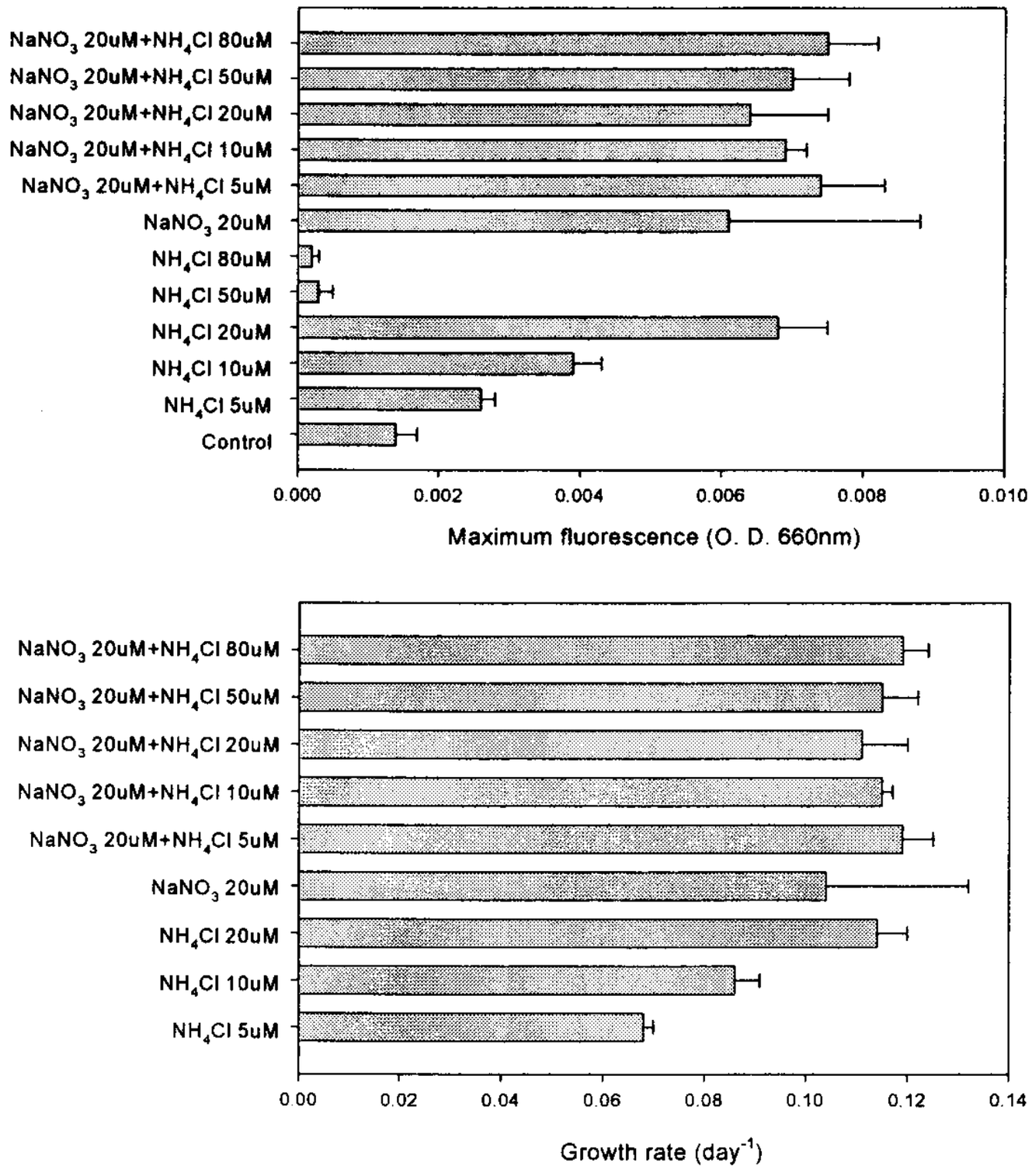

Fig. 2. Maximun fluorescence and growth rate of *C. polykrikoides* by spike of variety concentration of nitrate nitrogen and ammonium nitrogen. Control means no nitrogen addition. Error bars indicate standard deviation (n=3)

Figure 2 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 NH_4N, P = 0.015; control and 10 μM NH_4N, P = 0.000), but the yield with more than 50 μM ammonium was lower than in the controls. The yields in combination with 20 μM nitrate were higher than the control. No inhibit effect of *C. polykrikoides* propagation 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 andItakura, 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 propagate 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. circularusquama,* or *H. akashiwo.*

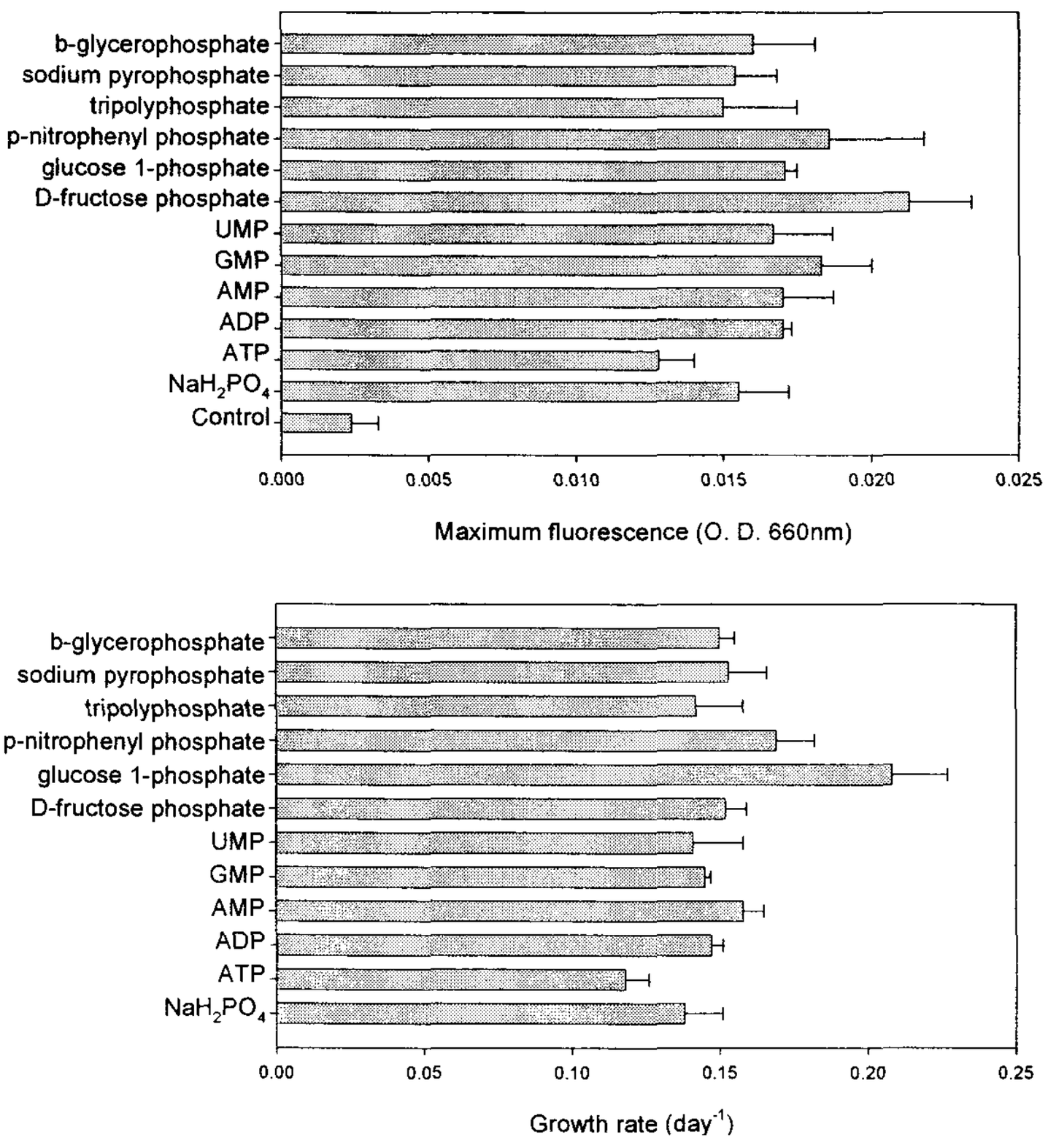

Fig. 3. Maximun fluorescence and growth rate of *C. polykrikoides* by spike of variety phosphorus compounds (Concentrations of added P compounds are adjusted to 3.6uM, which were 10% of F/2 medium.). Control means no phosphorus addition. Error bars indicate standard deviation (n=3)

Figure 3 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 controls ($P < 0.000$), 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* propagated

similarly in various dissolved organic phosphorus compounds and PO4P (Matsuda et al., 1999; Oh et al., 2002; Yamaguchi andItakura, 1999; Yamaguchi et al., 2001). 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 propagation of *C. polykrikoides*. High-density propagation of *C. polykrikoides* has been observed off the coasts of Korea and Japan, where inorganic nitrogen and phosphorus concentrations are lower than in estuaries and inland seas (Kim, 2003; 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.

The organic nitrogen and phosphorus concentrations in eutrophic seawaters were measured by the level of inorganic nitrogen and phosphorus (Matsuda et al., 1999). However, little is known about the organic nitrogen and phosphorus concentrations in offshore waters, where *C. polykrikoides* propagates on a large scale. Therefore, it is necessary to study the apparent spatial and temporal variations in organic nitrogen and phosphorus concentrations to understand and control the occurrence and mass propagation of *C. polykrikoides* in waters off the coast of Korea.

3.2. Selenium compounds

Figure 4 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 0.1150.165, 0.1150.148, and 0.0720.159 dayl, 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 controls for all spiked concentrations. However, the growth rate in the presence of Se-(methyl) selenocysteine hydrochloride below 100 μM was higher than in the controls, and the rate for more than 500 μM was lower than that for 0 μM.

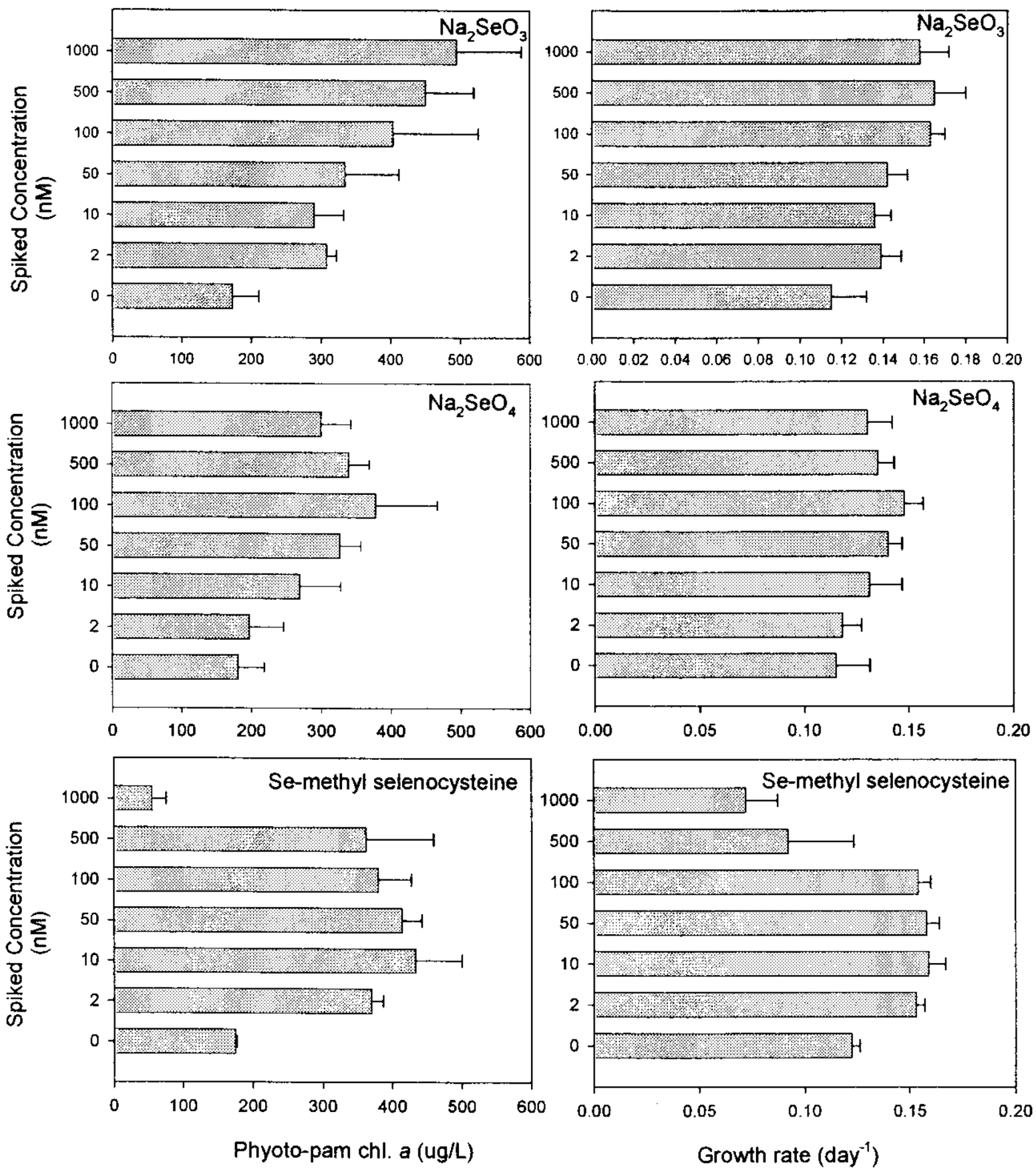

Fig. 4. Maximun growth yield and growth rate of *C. polykrikoides* by spike of variety selenium concentrations and compounds. Error bars indicate standard deviation (n=3)

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). Propagation yield of *Chattonella verruculosa* was increased when selenite was added at a concentration of 104 to 10 μM (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 μM selenite, but some inhibition was observed with more than 500 nM 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 nM and the growth rate was in-

hibited by addition of more than 500 nM.

In conclusion, 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 propagation 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 2005 may have a temporary inhibitory action on *C. polykrikoides* propagation, but they can be expected to have a positive effect in the long term.

4. Conclusions

C. polykrikoides was propagated using a variety of organic or inorganic nitrogen sources except for ammonium and 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 propagation 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* was propagated 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* propagation but can be expected to have a positive effect in the long term.

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