© Triveni Enterprises, Lucknow (India)
ISSN : 0254-8704
http : //www.geocities.com/j_environ_biol/

J. Environ. Biol.
29(6), 827-830 (2008)
j_environ_biol@yahoo.com

Estimation of *Cochlodinium polykrikoides* growth potential using a dialysis membrane

Young Sik Lee*

Aquaculture Environment Institute, National Fisheries Research and Development Institute (NFRDI), 361 Yongun-ri Sanyang-up Tongyeong-city, Kyungnam - 650-943, Korea

(Received: April 06, 2007; Revised received: September 22, 2007; Accepted: October 05, 2007)

Abstract: *We developed a test to measure the growth potential of C. polykrikoides using a dialysis membrane and artificial seawater. Nitrite nitrogen and inorganic phosphorus in the medium were almost completely removed when the medium was dialyzed against artificial seawater for five or more 6-hour cycles using a dialysis membrane (Spectrum's Spectra/Por® 7 Membrane) with a molecular-weight cut-off of 50,000, regardless of the presence of C. polykrikoides. The phytoplankton grew well even after dialysis. To estimate the growth potential of C. polykrikoides, a minimum initial concentration of >100 cells/ml is required. Methods using short-term starvation culturing of C. polykrikoides to measure growth potential were determined to be ineffective; instead, controlled tests using artificial seawater are recommended. The dialysis membrane used in this study can also be employed to measure the algal growth potential of other phytoplankton species.*

Key words: *Algal growth potential, Artificial seawater, Cochlodinium polykrikoides, Dialysis membrane*
PDF of full length paper is available with author ***(*leeys@momaf.go.kr)***

Introduction

Algal growth potential (AGP) is frequently determined using an algal assay procedure to obtain information regarding limiting nutrients and the mechanisms of phytoplankton growth. Originally, measurements of AGP in seawater, as developed by the National Environmental Pollution Research Institute of Japan, used single strains of phytoplankton, such as *Thalassiosira pseudonana*, *Dunaliella tertiolecta*, *Hornellia* sp, *Heterosigma* sp, and *Skeletonema costatum* (Sudo *et al*., 1981; Mahoney, 1989). However, since 1980, mostly indigenous phytoplankton assemblages have been used, particularly for identifying nutrients that limit phytoplankton growth (Caraco *et al*., 1987; Harrison *et al*., 1990; Fisher *et al*., 1992). In 1996, the classification of indigenous phytoplankton by size using an AGP assay was reported (Lee *et al*., 1996).

In Korea, red tide caused by *Cochlodinium polykrikoides* occurs every year around the southern and eastern coastal areas resulting in enormous economic damage (Kim *et al*., 1997; Kim, 1998). No effective countermeasures have been developed, and the damage can only be reduced by measures such as scattering of loess (Bae *et al*., 1998). Therefore, identifying the mechanisms of *C. polykrikoides*-induced red tide is important for preventing outbreaks of this species. Although several lines of research are being pursued (Kim *et al*., 1999; Mathivanan *et al*., 2007; Yang *et al*., 2000), detailed mechanisms have not yet been elucidated.

One approach to determining the mechanisms of *C. polykrikoides* red tide is to use an AGP test for *C. polykrikoides*. However, *C. polykrikoides* concentrations in marine water are very low, except during the red tide period. Even then, however, the alga is found only in limited marine areas. Therefore, cultured *C. polykrikoides* have been used to conduct laboratory AGP tests. In these tests, suspensions of phytoplankton in artificial seawater are repeatedly centrifuged to remove nutrients, such as nitrogen, phosphorus, and vitamins. However, during or after centrifugation of *C. polykrikoides*, many problems occur, such as poor pelleting of the cells or damage to the cells to the extent that they are unable to grow. An alternative method is washing the cells with artificial seawater using filter paper. In this case, even if the filter paper is of an appropriate pore size, the quantity of *C. polykrikoides* will gradually diminish because some of the cells pass through the filter paper each time. Furthermore, filtering is very time-consuming. To circumvent these problems, we developed an AGP test for *C. polykrikoides* using a dialysis membrane and artificial seawater.

Materials and Methods

A Spectrum's Spectra/Por®7 dialysis membrane with a molecular-weight cut-off (MWCO) of 50,000 was used in this study. The single-cell size of *C. polykrikoides* varies according to the culture conditions, but it is typically between 28 and 48 μm in length, and between 17 and 31μm in width. Thus, a membrane with a MWCO of 50,000 will not allow passage of *C. polykrikoides* cells.

The *C. polykrikoides* strain used in this study was isolated during the summer of 2002 from the South Sea of Korea. The medium for maintenance of the stock culture and all experiments consisted of Na_2SiO_3-excluded f/2 medium and surface seawater from the Kuroshio Current region (Guillard and Ryther, 1962).

To determine the dilution level of the medium during dialysis, concentrated stock solutions of $NaNO_2$ and NaH_2PO_4 were added to the stock culture medium. The nitrogen source contained in the f/2 medium is sodium nitrate. However, we used sodium nitrite because sample volume for sodium nitrite determination needs much than sodium nitrate.

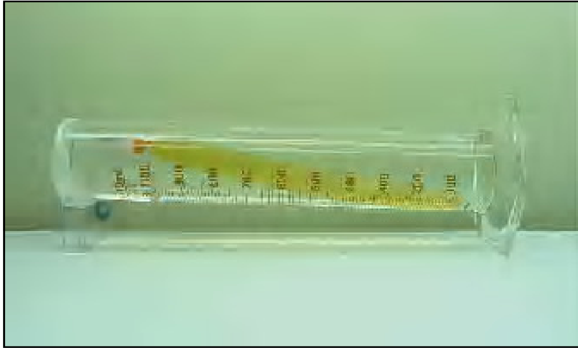

Fig. 1: Schematic diagram of medium washing method with artificial seawater using a dialysis membrane

After sealing the bottom of the dialysis membrane, 100 ml of medium mixed with nitrite nitrogen and inorganic phosphorus were added, and the top of the membrane was sealed (Fig. 1). The dialysis membrane was inserted into a 1 liter measuring cylinder containing 900 ml of sterilized artificial seawater (Sudo *et al*., 1981), and the cylinder was then sealed with Parafilm. After allowing the cylinder to settle for some time, the medium inside the dialysis membrane was extracted, and fresh artificial seawater was added to the cylinder. The volume ratio between the medium and artificial seawater was 1:9. The concentrations of nitrite nitrogen and inorganic phosphorus in the extracted medium were determined using methods described by Parsons *et al*. (1984). To determine the effect on *C. polykrikoides* of washing out nitrite nitrogen and inorganic phosphorus from the medium, *C. polykrikoides* was added to the dialysis membrane along with stock culture medium, and the above test was repeated.

C. polykrikoides was then cultured to determine the growth potential of the cells after they had been washed with artificial seawater in the dialysis membrane. Washing was carried out by dialyzing a cell suspension against artificial seawater for five 6 hr cycles. The cells were cultured by adding 5 ml of f/2 medium into a 15 ml test tube, followed by the washed *C. polykrikoides* cells. The initial concentrations of the inoculate were 20, 50, 100, 200, and 500 cells/ml. Culturing conditions were 23±2°C, 140±10 µmol m^{-2} sec^{-1} brightness, and a 12:12 hr cycle of light and darkness. Growth was monitored using a PHYTO-PAM chl fluorometer (Schreiber *et al*., 2002).

To determine whether a starvation culture, which limits the effects of the medium, was needed to accurately measure the growth of *C. polykrikoides*, washed cells were cultured again after starvation culture. Five ml of *C. polykrikoides* were washed with artificial seawater for five 6 hr cycles and then transferred to a 15 ml test tube. Starvation culture was carried out for 0, 2, 4 and 6 days under the same culture conditions as described above. Subsequently, f/2 medium elements, with the exception of Na_2SiO_3, were added to the test tubes and the cells were cultured further for various times. Growth of starvation cultures was monitored as described above.

To determine whether washing method using dialysis membrane can be employ to measure the AGP test of other phytoplankton species, *Heterosigma akashiwo* and *Prorocentrum minimum*, which are frequently bloom forming plankton in coastal water of Korea, were cultured after five cycles of 6 hr rinses with artificial seawater in the dialysis membrane.

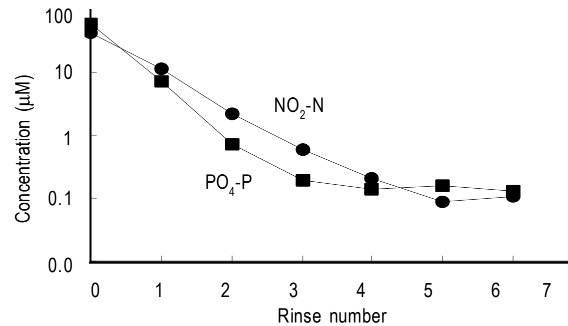

Fig. 2: Changes in concentrations of NO_2-N and PO_4-P after rinsing with artificial seawater. The exposure period and concentration of *C. polykrikoides* were 12 hr and 0 cells/ml, respectively

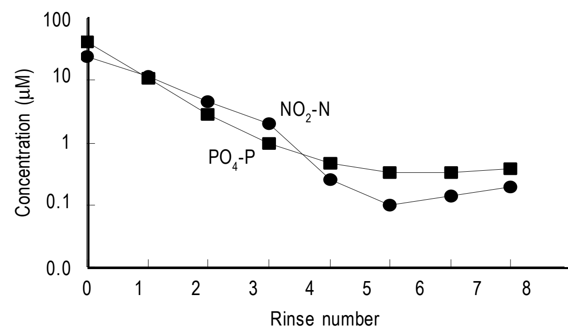

Fig. 3: Changes in concentrations of NO_2-N and PO_4-P after rinsing with artificial seawater. The exposure period was 6 hr and *C. polykrikoides* was added at a concentration of 1,500 cells/ml

Results and Discussion

Washing of nitrogen and phosphorus: Figure 2 shows the changes in concentration of nitrite nitrogen and inorganic phosphorus in the medium as a function of the increased number of 12 hr washings with artificial seawater using a dialysis membrane. The initial concentrations of nitrite nitrogen and inorganic phosphorus were 59.02 and 41.81 µM, respectively. After the first washing with artificial seawater, these values rapidly decreased to 7.45 and 11.37 µM, respectively. As the number of washings increased, the concentrations of both components decreased further. After the fifth washing, the concentrations of nitrite nitrogen and inorganic phosphorus were 0.23 and 0.08 µM, respectively.

Figure 3 shows the results of dialysis in the presence of 1,500 *C. polykrikoides* cells/ml and 6 hr washings. Similar to the results shown in Fig. 2, the concentrations of nitrite nitrogen and inorganic phosphorus rapidly decreased to similar levels with an increased number of washings and thus were not affected by the presence of *C. polykrikoides*. Since the background nitrite nitrogen and inorganic phosphorus concentrations in the artificial seawater

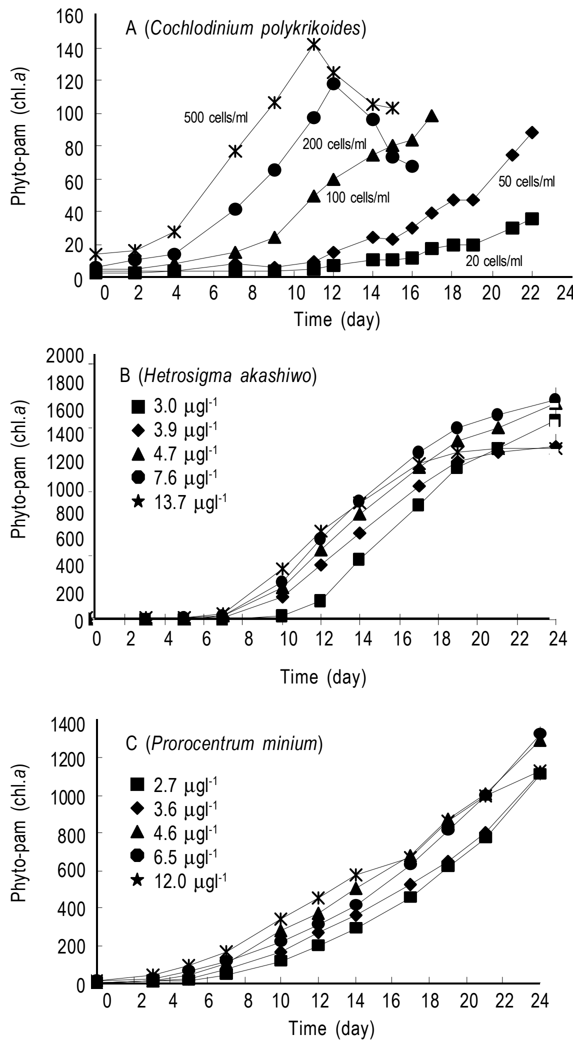

Fig. 4: Growth curve of *C. polykrikoides, Hetrosigma akashiwo* and *Prorocentrum minimum* at different initial concentration after rinsing with artificial seawater using a dialysis membrane (five cycles of 6 hr rinses). All treatments were done in triplicate (n=3)

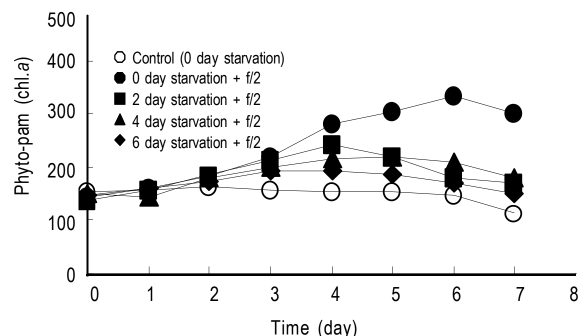

Fig. 5: Growth curve of *C. polykrikoides* in f/2 medium (minus Na_2SiO_3) after different starvation periods. Starvation was preceded by five 6 hr cycles of washing with artificial seawater. All treatments were done in triplicate (n=3)

were about 0.3 and 0.1 µM, respectively, further removal of these components from the medium was not possible. However, the concentrations of medium components that are not present in the artificial seawater should decrease even further following repeated dialysis. Thus, it can be expected that almost every nutrient will be removed from the medium after at least five 6 hr cycles of washing with artificial seawater.

Growth and initial concentrations for measuring AGP after rinsing with artificial seawater: *C. polykrikoides* was washed with artificial seawater using a dialysis membrane for five 6 hr cycles and then inoculated at different concentrations into f/2 medium. The results of this experiment are shown in Fig. 4 A. Although the rate of growth differed, *C. polykrikoides* grew even after it had been washed with artificial seawater.

In the AGP test, if the initial phytoplankton concentration is high, a maximum growth yield can be obtained relatively quickly, whereas with an inoculate containing a low concentration of cells, almost double the amount of time is required (Fig. 4 A). In this study, more than 16 days were required to clearly measure growth when the initial concentration of *C. polykrikoides* was 50 cells/ml or lower, but fewer than 9 days were required when a concentration of 100 cells/ml or higher was used. Therefore, it seems that a minimum initial *C. polykrikoides* concentration of 100 cells/ml is required. This is similar to the initial concentration used in a kinetics test of *Gymnodinium mikimotoi* (Yamaguchi and Itakura, 1999).

Figure 4 B, C show the growth curve of *Heterosigma akashiwo* and *Prorocentrum minimum* at different initial concentration after rinsing with artificial seawater using a dialysis membrane for five cycles of 6 hr rinses. Although the growth rate differed, *Heterosigma akashiwo* and *Prorocentrum minimum* grew well even after they had been washed with artificial seawater for five cycles of 6 hr rinses.

Starvation culture: After *C. polykrikoides* was washed with artificial seawater using a dialysis membrane for five 6 hr cycles, it was starvation-cultured and then cultured further in f/2 medium. The results of this experiment are shown in Fig. 5. In the control, which was not shifted to f/2 medium or subjected to starvation culture, there was almost no change in the yield of *C. polykrikoides*. However, when f/2 medium was added, the maximum concentration of non-starved cells after 6 days was nearly twice as high as the initial concentration. Cells that had been subjected to starvation culture for 2, 4, or 6 days also grew when shifted to f/2 medium.

Goldman *et al*. (1979) reported that phytoplankton take up and store an excess of nutrients from rich medium. Therefore, cells are subjected to starvation culture in order to minimize the influence of the medium. However, during starvation culture, when phytoplankton growth is limited by the limiting nutrient, the stored nutrients will remain unused by the cells. In this study, even after 6 days of starvation, the addition of f/2 medium promoted growth of the *C. polykrikoides*, suggesting that the excessive nutrients dissolved in

seawater of the Kuroshio current region that were taken up and stored by *C. polykrikoides* were not removed by starvation culturing. Therefore, it seems that the short-term starvation culturing of *C. polykrikoides* does not allow accurate AGP testing; instead, controlled tests using artificial seawater are may provide more valid results.

Acknowledgments

This work is funded by a grant from the National Fisheries Research and Development Institute (RP-05-ME-2).

References

Bae, H.M., H.G. Choi, W.C. Lee and S.J. Yoon: Control of the red tide by yellow loess dispersion. Proceedings of the Korea-China joint symposium on harmful algal blooms hosted during 5-7, December 1997 by the National Fisheries Research and Development Institute and the Chinese Academy of Fisheries Science, Pusan, Korea. pp.53-60 (1998.)

Caraco, N., A. Tamse, O. Boutros and I. Valiela: Nutrient limitation of phytoplankton growth in brackish coastal ponds. *Can. J. Fish. Aquat. Sci*., **44**, 473-476 (1987).

Fisher, T.R., E.R. Peele, J.W. Ammerman and L. Harding: Nutrient limitation of phytoplankton in Chesapeake Bay. *Mar. Ecol. Prog. Ser*., **82**, 51-63 (1992).

Goldman, J.C., J.J. McCarthy and D.G. Peavey: Growth rate influence on the chemical composition of phytoplankton in oceanic waters. *Nature,* **279**, 210 - 215 (1979).

Guillard, R.R.L. and J.H. Ryther: Studies of marine planktonic diatoms. 1. *Cyclotella nama* (Hustedt) and *Detonula confervacea* (Cleve). *Can. J. Microbiol*., **8**, 229 - 239 (1962).

Harrison, P.J., M.H. Hu, Y.P. Yang and X. Lu: Phosphate limitation in estuarine and coastal waters of China. *J. Exp. Mar. Biol. Ecol*., **140**, 79-87 (1990).

Kim, H.G.: Harmful algal blooms in Korea coastal waters focused on three fish-killing dinoflagellates. Proceedings of the Korea-China joint symposium on harmful algal blooms hosted during 5-7, December 1997 by the National Fisheries Research and Development Institute and the Chinese Academy of Fisheries Science, Pusan, Korea. pp. 1-20 (1998).

Kim, H.G., S.G. Lee, K.H. An, S.H. Youn, P.Y. Lee, C.K. Lee, E.S. Cho, J.B. Kim, H.G. Choi and P.J. Kim: Recent red tides in Korean coastal waters. National Fisheries Research and Development Institute, Republic of Korea (1997).

Kim, H.G., W.J. Choi, Y.G. Jung, C.S. Jung, J.S. Park, K.H. An and C.I. Baek: Initiation of *Cochlodinium polykrikoides* blooms and its environmental characteristics around the Narodo Island in the western part of the South Sea of Korea. *Bull. Nat. Fish. Res. Dev. Inst. Korea*, **54**, 119-129 (1999).

Lee, Y.S., T. Seiki, T. Mukai, K. Takimoto and M. Okada: Limiting nutrients of phytoplankton community in Hiroshima Bay, Japan. *Water Res*., **30**, 1490-1494 (1996).

Mahoney, J.B.: Algal assay of relative abundance of phytoplankton nutrients in northeast United States coastal and shelf waters. *Water Res*., **23**, 603-615 (1989).

Mathivanan, V., P. Vijayan, S. Sabhanayakam and O. Jeyachitra: An assessment of plankton population of cauvery river with reference to pollition. *J. Environ. Biol.,* **28**, 523-526 (2007).

Parsons, T.R., Y. Maita and C.M. Lalli: A Manual of Chemical and Biological Methods for Seawater Analysis. Pergamon Press, New York (1984).

Schreiber, U., R. Gademann, P. Bird, P.J. Ralph, A.W.D. Larkum and M. Kuhl: Apparent light requirement for activation of photosynthesis upon rehydration of desiccated beachrock microbial mats. *J. Phycol*., **38**, 125-134 (2002).

Sudo, R., S. Tai, O. Yagi, M. Okada, M. Hosomi and A.N. Yamane: Determination of Algal Growth Potential by Algal Assay Procedure (Comprehensive studies on the eutrophication of fresh-water areas). No. 26, Research Report from the National Institute for Environmental Studies (1981).

Yamaguchi, M. and S. Itakura: Nutrition and growth kinetics in nitrogen- or phosphorus-limited cultures of the noxious red tide dinoflagellate *Gymnodinium mikimotoi*. *Fisheries Sci.,* **65**, 367-373 (1999).

Yang, J.S., H.Y. Choi, H.J. Jeong, J.Y. Jeong and J.K. Park: The outbreak of red tides in the coastal waters off Kohung, Chonnam, Korea: 1. Physical and chemical characteristics in 1997. *J. Korean Soc. Oceanog.*, **5**, 16-26 (2000).