Application of DNA Content and Total Protein Concentration to Predict Blooms Caused by *Cochlodinium polykrikoides* (Dinophyceae) in Korean Coastal Waters

Eun Seob Cho* and Yong Kyu Choi

South Sea Fisheries Research Institute, Coastal Environment Management, Yeosu 556-823

Received October 29, 2003 / Accepted February 7, 2004

We applied nuclear DNA content stained with 4'-6'-diamidino-2-phenylindole (DAPI) and total protein concentration to predict the existence of *Cochlodinium polykrikoides* before huge blooms occurred, based on a short-term survey at sites in the South Sea. Fluctuations in environmental conditions and nutrients (nitrate, nitrite, and phosphate) were of a similar range, regardless of sampling sites or early and middle field observations. However, *C. polykrikoides* abundance was significantly different depending on the station, with a higher cell density of 34, 62, and 57 cells L^{-1} at Stn C2, C5, and C6, respectively than what was found in early August, 2000. In mid August, 2000, the highest cell density of 547 cells L^{-1} at Stn C3 was observed. The relationship between *C. polykrikoides* abundance, DAPI-stained DNA content, and total protein concentration was a positive correlation coefficient, in particular a higher positive correlation was exposed to even a smaller abundance of *C. polykrikoides*. These results suggest that DNA stained by DAPI and total protein concentration could play an important index in easily predicting the presence of *C. polykrikoides* before blooms.

Key words – *Cochlodinium polykrikoides*, image analysis system, nuclear DNA content, prediction, total protein concentration, red tide

Outbreaks of red tide in Korean coastal waters have significantly increased in frequency, severity, and duration over the last two decades. So far, 43 species have been identified as the causative marine microalgae of red tides. In Korean's coastal waters, *Cochlodinium polykrikoides* Margalef bloomed for the first time in 1982. It was associated with the massive mortality of caged young fish and is regarded as being a potentially ichthyotoxic dinoflagellate [11]. Since then, blooms caused by *C. polykrikoides* have occurred continuously in Korean coastal waters [11]. *C. polyrikoides* mainly occurred along the coast of the South Sea from July to October, reaching a peak bloom in September [11]. In 1995, the main red tide organism, *C. polykrikoides*, caused a massive fish and shellfish die off. Furthermore, a massive oil spill caused by the tanker "Sea Prince" drew great public attention to Korea. The economic loss from this tragedy reached nearly 76.4 billion won[11]. To cope with serious economic and public health effects caused by *C. polykrikoides*, it is necessary to rapidly predict and monitor the abundance of *C. polykrikoides* before it blooms in Korean coastal waters. We have undertaken the task of discriminating between morphologically similar marine microalgae, in particular the dinoflagellates *C. polykrikoides*, *Gyrodinium impudicum* Fraga et Bravo, and *Gymnodinium catenatum* Graham, and also diatoms in the genus *Pseudo-nitzschia* by using fluorescent lectins and rDNA-targeted oligonucleotide probes [3,4,6-8]. These novel methods are now of increasing importance in routine monitoring programs. Moreover, it is possible to predict an outbreak of *C. polykrikoides* in Korean coastal waters based on the determination of growth rate. Meanwhile, Chang and Carpenter [2] developed a method to determine growth rate based on the diel cycle of DNA synthesis. Subsequently, Yamaguchi[17] suggested that 4'-6'-diamidino-2-phenylindole (DAPI) was a useful tool in determining growth rate based on the pattern of DNA synthesis. In our previous study, DAPI staining study with an image analysis system was demonstrated to be a practical method to monitor harmful algal bloom (HAB) species [5]. A question that arose from this study was whether nuclear DNA content stained with DAPI could be a potential tool to predict red tide in *C. polykrikoides* or not. Little is known about the variability in total protein concentration to monitor outbreaks of marine microalgae.

In this paper, we present data from a short-term survey conducted in early and middle August, 2000 before the first *C. polykrikoides* bloom. Fluctuations in *C. polykrikoides* density were determined in relation to environmental conditions (temperature, salinity, and chlorophyll *a*), nu-

*Corresponding author
Tel : 061-686-8942, Fax : 061-686-1588
E-mail : eun-5657@hanmail.net

trients (NO_3-N, NO_2-N, and PO_4-P), and total abundance of phytoplankton including *C. polykrikoides*. Nuclear DNA content stained by DAPI and total protein concentrations were also estimated. We did not attempt to clarify the relationship between DAPI-stained DNA content using an image analysis system, or the fluctuation of total protein concentration in conjuction with the abundance of *C. polykrioides*. However, we did examine the possibility of whether these methods could be a useful tool in predicting the blooms caused by *C. polykrikoides*.

Materials and Methods

Field survey

The study areas were located at the far east of Korea stretching from 35°1'E to 34°16'E latitude and sampling was done at Kadukdo (C1), Kojedo (C2), Namhaedo (C3), Yeosu (C4), Kohung (C5) and Kumundo (C6) coasts in early and middle August, 2000 (Fig. 1). Sea surface water temperature and salinity were measured with CTD (SBE19, Sea Bird). Concentration of dissolved nutrient (NO_3-N, NO_2-N and PO_4-P), which passed through Whatman GF/F glass fiber filters, was determined as the procedure of Strickland and Parsons [16]. Chlorophyll *a* was harvested on Whatman GF/F glass fiber filters and then the filters extracted in 90% acetone and measured on a UV-VIS spectrophotometer (DMS 80, Varian). *C. polykrikoides* was counted directly in the living state after concentration with 20 μm (pore size) sieve. All epiphytic phytoplankton cell

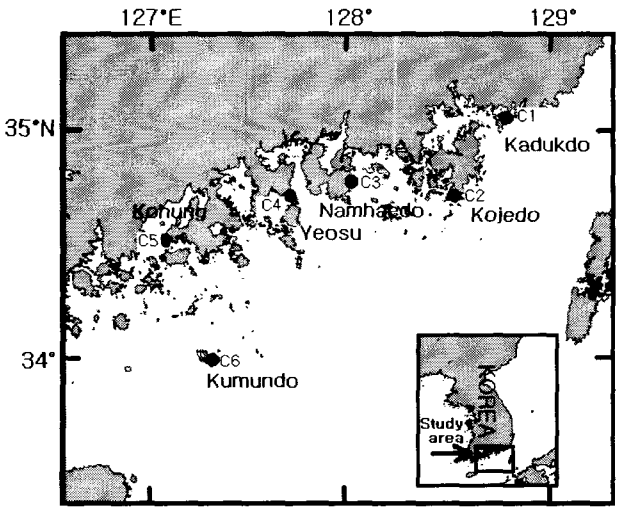

Fig. 1. Study area (inset) and sampling locations (●) on the south coast, Korea, in early and middle August, 2000 before initial bloom caused by *C. polykrikoides*.

abundance was encountered using Sedgwich-Rafter Count Chamber in samples preserved with Lugol's iodine solution under the light microscope.

Measurement of DNA content

Cells were fixed with 2% glutaraldehyde and 1 μg/mL DAPI in Tris buffer 0.5 μg/mL (10 mM Tris, 10 mM EDTA-2Na, 100 mM NaCl, 10 mM 2-mercapto-ethlamine hydrochloride, pH 7.4) was added. After staining, treated cells were examined under Olympus BX 40 epifluorescence microscope using UV (excitation, 330-385 nm; emission, >420 nm). For measurement of DNA content, the amount of light emitted from a particular region of fluorochrome-stained DNA was measured by image analysis system as described by Choi *et al.* [10]. Some microphotographs were recorded by a scanner (ScanJet ADF, Hewlett Packad) and directly processed with personal computer. Quantification of DNA content in selected 100 cells randomly was performed by use of image analysis software (Optimas 5.1 version for windows 3.1).

Measurement of total protein concentrations

Natural samples were harvested by 20 μm and directly frozen (-20℃) until required. Protein concentrations were determined using the bicinconinic acid (BCA) assay (Sigma) with bovine serum albumin (BSA) as a standard [12]. The experiments were repeated ten times with each sample. Data analysis and determination of statistical significance was carried out using Anova *t*-test.

Results

First field study

In early August, 2000, water temperature varied from 23.8 to 26℃, with a maximum (26℃) at Stn C3 and a minimum (23.8℃) at Stn C5. Salinity was in a similar range of 31.4 to 32.9 psu for all sites tested (Fig. 2a). Nitrate concentration was $\leq$8 μM at Stn C1, C3, C5, and C6 and $\geq$10 μM at Stn C2, and C4, whereas nitrite and phosphate were almost < 1 μM regardless of sampling sites (Fig. 2b). In contrast to environmental conditions and nutrients, *C. polykrikoides* population density showed a great deal of fluctuation depending on the station, with the highest abundance of 34, 62, and 57 cells L^{-1} at Stn C2, C5, and C6, respectively, and the lowest abundance of 5, and 6 cells L^{-1} at Stn C3, and C4, respectively (Fig. 2c). Chlorophyll *a*

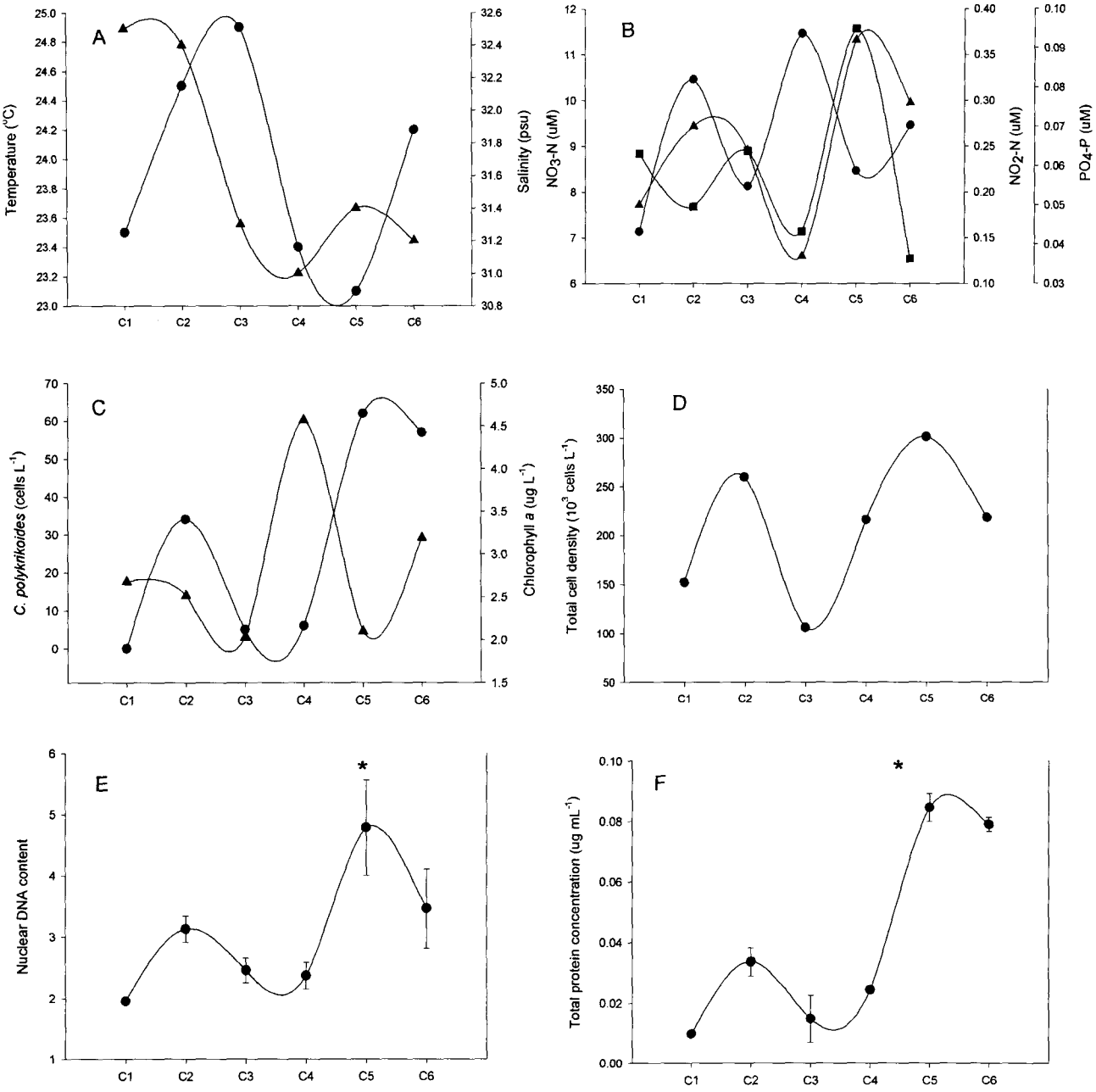

Fig. 2. Changes in environmental conditions, nutrients, total phytoplankton cell numbers, cell number of *C. polykrikoides*, relative content of DNA and total protein concentration at Kadukdo (C1), Kojedo (C2), Namhaedo (C3), Yeosu (C4), Kohung (C5) and Kumundo (C6) coasts in early August, 2000. A, Temperature (circle) and salinity (triangle); B, NO_3-N (circle), NO_2-N (square) and PO_4-P (triangle); C, The abundance of *C. polykrikoides* (circle) and chlorophyll *a* (triangle); D, Total cell number; E, DNA content; F, Total protein concentration. Data are means SE. Superscript means significantly different ($p<0.05$).

content varied from 0.12 to 0.37 μg L^{-1}, which had similar pattern to the environmental and nutrient conditions (Fig. 2c). However, the value of total phytoplankton cell numbers fluctuated, with a maximum value (301×10^3 cells L^{-1}) at Stn C5 and a minimum (106×10^3 cells L^{-1}) at Stn C3 (Fig. 2d). The relative DNA content (RD) of DAPI stained nuclei at each sampling site showed higher RD levels of 4.7 ($p<0.05$) and 3.4 at Stn C5 and C6, respectively (Fig. 2e). Marked fluctuations were observed in total concentrations, with higher levels of 0.08 μg mL^{-1} ($p<0.05$) at Stn C5 and 0.07 μg mL^{-1} at Stn C6 and lower levels of 0.03 μg mL^{-1} at Stn C1, C2, C3 and C4 (Fig. 2f). The relationship between the abundance of *C. polykrikoides* and RD was observed to be a high positive correlation; *C. polykrikoides*

cell density and total protein concentration was also a high value (Fig. 3a, b). As for the correlation coefficient between RD and total protein concentration, it was considered high (Fig. 3c).

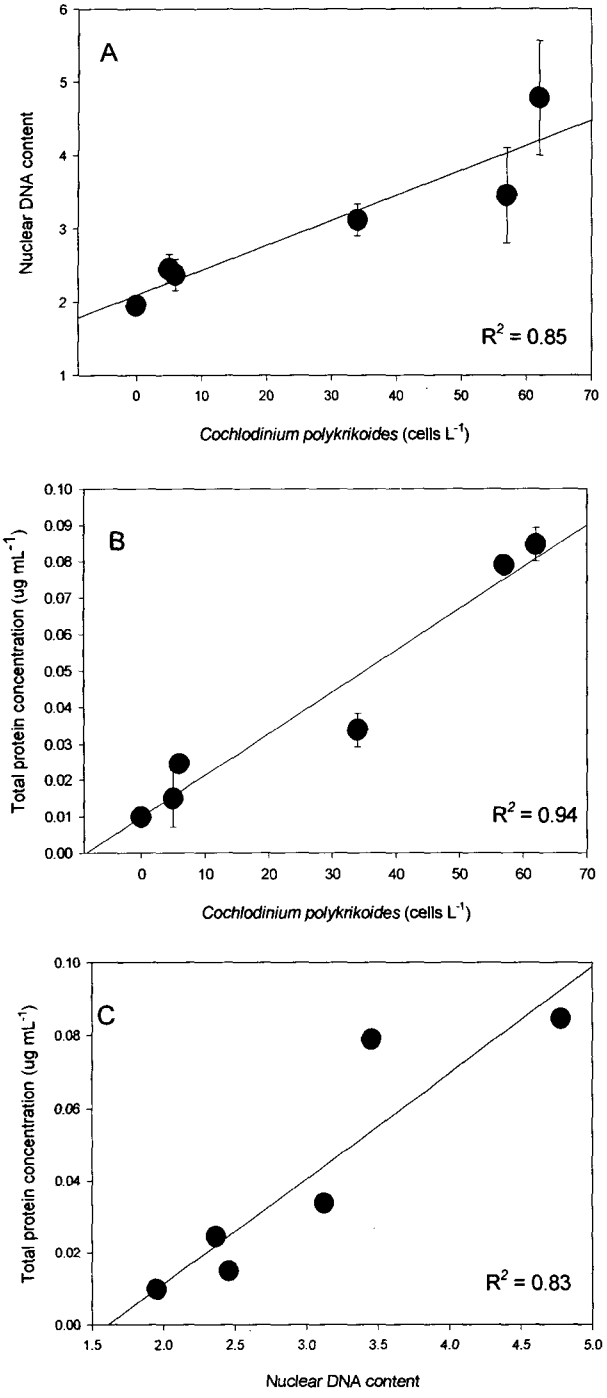

Fig. 3. The relationship between cell number of *C. polykrikoides* and DNA content (A), the abundance of *C. polykrikoides* and total protein concentrations (B), relatative DNA content and total protein concentration (C) in early August, 2000. Data are means ± S.E.

Second field study

During the second field observation in the middle of August, 2000, fluctuations in environmental factors (water temperature, and salinity) and nutrients (nitrate, nitrite, and phosphate) were similar to the First field study in early August, 2000 (Fig. 4a, b). However, the abundance of *C. polykrikoides* was significantly different (Fig. 4c). The cell density of *C. polykrikoides* during Second field study was measured to be ten times as much *C. polykrikoides* as during the First field study, with the highest cell number (547 cells L^{-1}) at Stn C3, and at Stn C1, C4, and C5 *C. polykrikoides* was not present. The phytoplankton population with a total number of cells L^{-1} reached a maximum of 314 $\times 10^3$ cells at Stn C3, while showing a minimum of 2.7 $\times$ 10^3 cells at Stn C3 (Fig. 4d). The higher abundance of *C. polykrikoides* that was recorded during the Second field study was attributed to a higher RD. Similar to RD patterns during the First field study, twice as much RD was observed during the Second field study as compared with the First field study, with a maximum RD of 9.12 (p<0.05) at Stn C3 (Fig. 4e). Total protein concentrations also fluctuated in the Second field study as seen in Fig. 2f, with a maximum of 0.07 μg mL^{-1} (p<0.05) at Stn C6, and a similar content as much as that of the First field study (Fig. 4f). Unlike Fig. 3, Fig. 5a, b showed a low positive correlation between *C. polykrikoides* cell number and RD, as well as between *C. polykrikoides* cell density and total protein concentration. The low correlation coefficient between RD and total protein concentration is also shown in Fig. 5c.

Discussion

Considering our present data, fluctuations in environmental properties (temperature and salinity) and nutrients (nitrate, nitrite, and phosphate), including chlorophyll *a* during the study period appeared to be regardless of sampling sites. In contrast, the abundance of *C. polykrikoides* was significantly different at various stations, indicating that these sources (environmental factors and nutrients) are not likely to be very useful bioindicators for quick prediction of blooms caused by *C. polykrikoides* (Fig. 2a, b, c and Fig. 4a, b, c). As an inductive factor for outbreaks of red tide, it is likely that temperature has been regarded as a more important contributor to the blooms than any other variable that was measured. Some reports have suggested that temperature is an important environmental variable

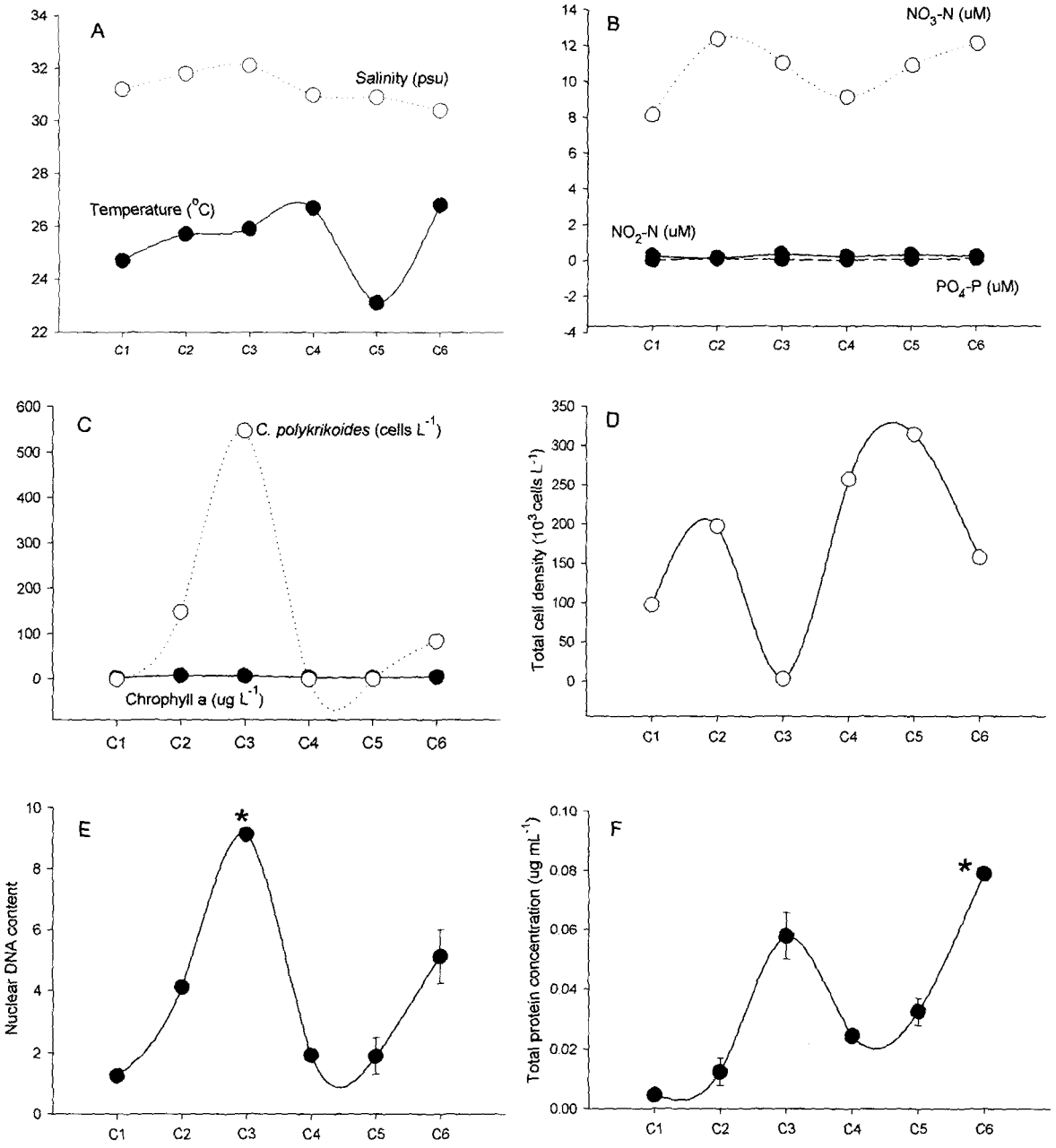

Fig. 4. Changes in environmental conditions, nutrients, total phytoplankton cell numbers, cell number of *C. polykrikoides*, relative content of DNA and total protein concentration at Kadukdo (C1), Kojedo (C2), Namhaedo (C3), Yeosu (C4), Kohung (C5) and Kumundo (C6) coasts in middle August, 2000. A, Temperature (circle) and salinity (triangle); B, NO_3-N (circle), NO_2-N (square) and PO_4-P (triangle); C, The abundance of *C. polykrikoides* (circle) and chlorophyll a (triangle); D, Total cell number; E, DNA content; F, Total protein concentration. Data are means S.E. Superscript means significantly different ($p<0.05$).

for understanding the physiological ecology of algae in nature, as it can not only affect key biological processes, but also photosynthesis, enzymatic activity and respiration [1,13-15]. Previously, we have found that growth in *C. polykrikoides* began at 15℃ and well into the range of 20-25 ℃ (unpublished data). During this study period, temperature was monitored at 23-26℃, which is associated with the optimal environmental conditions for *C. polykrikoides* growth. However, low density was recorded for *C. polykrikoides* in early August, 2000 ($\leq$60 cells L^{-1}), with 0.02% of total phytoplankton being the target species (Fig. 2c, d). This suggests that temperature is not an important extrinsic factor in the fluctuations noted in *C. polykrikoides* density.

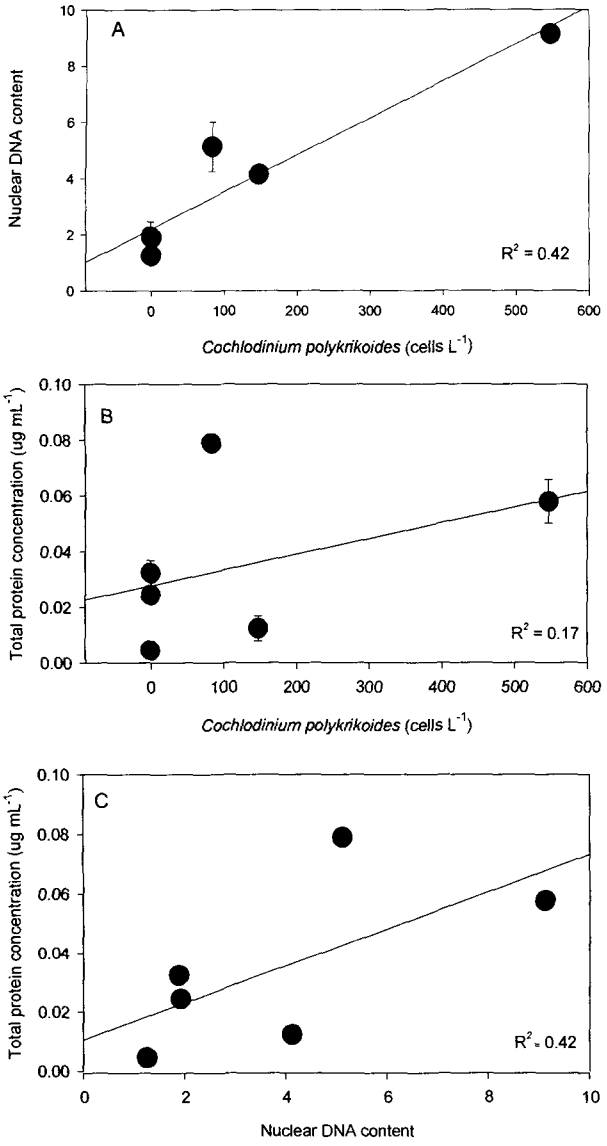

Fig. 5. The relationship between cell number of *C. polykrikoides* and DNA content (A), the abundance of *C. polykrikoides* and total protein concentrations B), relative DNA content and total protein concentration (C) in middle August, 2000. Data are means±S.E.

Unlike environmental conditions and nutrients (Fig. 2e, f), fluctuation in the DAPI-stained DNA content and total protein concentrations was similar to the density fluctuations of *C. polykrikoides*. Interestingly, the highest RD and total protein concentrations at Stn C5 were significantly different ($p<0.05$) compared to the results at Stn C1 (no occurrence of *C. polykrikoides* in early and middle August, 2000). In addition, a high positive correlation ($r^2>0.8$) was found between RD, total protein concentration, and *C. polykrikoides* density (Fig. 3a, b, c), persisting with even few cells (Fig. 2c). Therefore, it is thought that DAPI-stained DNA content and total protein concentration measurements are better bioindicators for predicting and monitoring the outbreaks of *C. polykrikoides* before a bloom than that of environmental factors and nutrients. Similar results during the First field study were also found during the Second field study: the higher the RD and total protein concentrations, the higher the abundance of *C. polykrikoides*. In particular, Stn C1 showed the lowest RD and total protein concentrations (Fig. 4c, e, f), as low as the First field study. Considering the First and Second field study, it can be confirmed that *C. polykrikoides* cell numbers fluctuated in a pattern attributable to the differences in RD and total protein concentrations, with a well defined maxima (4.7-9.12 in RD and 0.05-0.08 μg mL^{-1} in total protein concentration, 62-547 cells L^{-1} in *C. polykrikoides*) and minima (1.5-1.95 in RD and 0.01 μg mL^{-1} in total protein concentration, 0 cell L^{-1} in *C. polykrikoides*, Figs. 2, 4).

From our observations, a higher abundance of *C. polykrikoides* at Stn C2, C3, and C6 (Fig. 4) was correlated to a higher relative importance (%) of dinoflagellates (20-25%, data not shown) compared to a lower abundance of *C. polykrikoides* at Stn C2, C3, C4, C5, and C6 (Fig. 2). However, when diatoms were encountered, relative importance (%) was shown in reverse to dinoflagellates. Possibly, the lower correlation between RD and *C. polykrikoides* abundance (Fig. 5) could be associated with a higher constitution of dinoflagellates than that of Fig. 3. Accordingly, the composition of the phytoplankton's biomass was found to have more of an effect on the nuclear DNA content and the correlation coefficient than on total protein concentrations. Henceforth, diatoms may play an important role in higher correlations between RD and the presence of *C. polykrikoides*. Yamaguchi [17] suggested that the DAPI-DNA complex was determined by cell growth and cell cycle, depending on DAN synthesis. With this suggestion, higher correlation (Fig. 3) was attributed to higher physiological activity in diatoms, whereas lower correlation was associated with higher DNA synthesis in dinoflagellates (Fig. 5).

Measuring nuclear DNA content stained with DAPI using an image analysis system has major advantages such as the treatment is quick and HABs [5] are easily detected. In addition, protein concentrations also easily deal with HABs. In a previous study, we reported that fluorescent lectin probes could be used to discriminate *C. polykrikoides* from morphologically similar microalgae in natural samples before the first outbreaks of *C. polykrikoides*[9]. To quali-

tatively and quantitatively analyze *C. polykrikoides*, the methods in this study (DNA-DAPI and total protein concentrations), along with fluorescent lectin probes, may play an important role in developing more accusative and selective predictions on outbreaks of *C. polykrikoides* in Korean coastal waters.

Acknowledgements

We appreciate being the first to stimulate a new detection method by Prof. G. W. Kim, Kongju National University. We also thank to Dr. Kim Y.O., Biotechnology Division, National Fisheries Research and Devlopment Institute, for providing us with BSA and helpful comments. This work was supported by a grant-in-aid from the Maritime Affairs and Fisheries Ministry of Korea.

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초록 : total DNA 및 단백질 함량변화에 의한 *C. polykrikoides* 조기적조 예측 응용

조은섭 · 최용규
(국립수산과학원 남해수산연구소)

본 연구는 남해안에서 발생되는 유해성 *C. polykrikoides* 조기적조을 예측하기 위한 기법으로 DAPI로 염색시킨 DNA와 단백질 함량변화을 단기간으로 조사했다. 조사기간 중의 환경요인, 영양염 (질산, 아질산, 인산염) 농도는 조사지점이나 시기에 관계없이 거의 비슷하게 보였다. 그러나 *C. polykrikoides* 밀도는 조사지점에 따라 현저하게 다르게 나타났다. 2000년 8월 초순의 경우 C2, C5, C6에서 리터 당34, 62, 57 세포를 각각 출현했으며, 8월 중순에는 C3에서 최고 547 세포가 보였다. *C. polykrikoides* 출현밀도와 DNA 및 단백질 함량과는 양성적인 상관관계를 보였다. 특히 *C. polykrikoides* 세포밀도가 아주 낮을 경우에 높은 상관값을 나타내었다. 따라서 DNA 및 단백질 함량변화 기법은 *C. polykrikoides* 조기적조을 쉽게 예측 할 수 있는 중요한 도구도 이용될 수 있다.