Dependence of Sub-Cellular Activities of the Blooming and Harmful Dinoflagellate *Cochlodinium Polykrikoides* on Temperature

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Water temperature-dependent fluctuations of biochemical and molecular activities in the harmful dinoflagellate, *Cochlodinium polykrikoides* were studied. In terms of genomic DNA concentration, a similar value of 0.6 was observed at 12°C and 15°C. However, DNA significantly increased beyond 18°C (p<0.05), to a maximum of 1.8 at 24°C. DNA concentration significantly decreased to 0.6. The concentrations of RNA and total protein were likely at their highest values of 1.7 and 0.07 μg ml⁻¹ at 24°C, respectively. RNA and total protein concentrations began to increase at 18°C. Oxygen availability between lower and higher temperatures was significantly different and increased from 18°C according to light intensity, regardless of wavelengths (p<0.05). At 24°C, the highest value of the maximum electron transport rate (ETRmax), ranging from 537.9 (Ch 1) to 602.5 μmol electrons g⁻¹ Chl a s⁻¹ (Ch 4), was also apparent. Nitrate reductase (NR) and ATPase activities were at their highest values of 0.11 μmol NO₂⁻ μg⁻¹ Chl a h⁻¹ and 0.78 pmol 100 mg⁻¹ at 24°C, respectively. In an analysis of C:N, the concentration of C and N also significantly increased (p<0.05). Most of the measurements for the cellular activities at 27°C, however, were less than at 24°C. These results suggest that the sub-cellular activities of *C. polykrikoides* are sensitive to changes in water temperature. It may be desirable to estimate at 18°C the initiation of the massive blooming development of *C. polykrikoides*. In nature, it will be very difficult to maintain the massive blooms beyond 24°C because of a possibly significant decrease in molecular activity of *C. polykrikoides*.

**Key words**: Biochemical activities, blooming, *Cochlodinium polykrikoides*, DNA, RNA, water temperature

Introduction

The outbreaks of Harmful Algal Blooms (HABs) in Korea’s coastal waters have significantly increased in duration over the last two decades, with 43 species so far identified as causative marine microalgae [25]. *Cochlodinium polykrikoides* Margalef, a red tide phytoplankton in Korea, is an unarmored, chain-forming member of the dinoflagellate genus *Cochlodinium*, *C. polykrikoides* first bloomed in 1982 and has produced blooms annually in Korean coastal waters [25]. Moreover, *C. polykrikoides* was associated with considerable fish kills and economic losses for the Korean aquaculture industry [25].

*Cochlodinium* blooms compared with other red tides in Korea were that the rapid speed of their population development. Once the *Cochlodinium* blooms, it can spread to the entire coastal region of Korea within several weeks [25]. To provide a better understanding of why the first *Cochlodinium* bloomed, numerous studies have been undertaken regarding the intrusion of warm water current [18,19], offshore water [37], low salinity water [6], and biological interactions [16,19]. In addition, excystment of a resting cyst of *C. polykrikoides* [26] may play an important role in *Cochlodinium* blooms. Little is known about the precise factors causing the initial bloom.

The elucidation of the mechanism of *C. polykrikoides* based on temperature, salinity, and nutrients in the laboratory has studied [22,24,27]. Particular interest has been shown on the species-specific significance for key biological processes (e.g., enzymatic activity, photosynthesis, respiration, etc.) in response to changing natural environments. More recently, Kim et al. [22] suggested that temperature should have a greater influence on the growth of *C. polykrikoides* than salinity and irradiance. In this role, it is assumed that a desirable temperature may dramatically influence fluctuation in molecular and biochemical activities.

Most studies on bloom mechanisms and dynamics have concentrated on nutrient kinetics, assimilation, preferences,
and uptake according to hydrographic properties [1,4,6,11, 31,30,34]. In contrast, little is known about growth characteristics of phytoplankton assemblages based on molecular studies [e.g., cell cycle proteins [29], DNA content [32], and enzyme activity [11,12]]. Relatively little attention has also been given to assessing the degree of biochemical and molecular processes of C. polykrikoides associated with the fluctuation of temperature. This study attempts to provide an analysis of the fluctuations of biochemical and molecular activities in C. polykrikoides in relation to main environmental factors in the laboratory. Further, this study is an attempt to broaden the understanding of a cell biological magnitude to Cyclotella blooms’ process in nature.

Materials and Methods

Cultures

Cyclotella polykrikoides (GenBank accession A1208248) was isolated from red tide waters in Korea using a capillary pipette under a light microscope. The species was grown in f/2-Si medium [13] at 20°C under an intensity of 50 μmol m⁻² s⁻¹ from white fluorescent tubes (12L:12D light: dark cycle) and maintained in the South Sea Fisheries Research Institute.

Cultivation conditions

This experiment consisted of quadruplicate cultures, each 1 L of medium in 2.1 flask vessels. Each vessel was inoculated with approximately 500-700 cells ml⁻¹ in a multi-chamber room (Dongyang Industry, Incheon, Korea). The cell was grown under culture conditions designated as: 12°C, 15°C, 18°C, 21°C, 24°C, and 27°C, with a continuous light intensity of 100 μmol m⁻² s⁻¹ from white fluorescent tubes without variations of salinity, pH, and medium. Experiments were carried out for 29 days.

Measurements of DNA, RNA, and protein concentrations

Aliquot of C. polykrikoides was collected by centrifugation (250× g, 10 min) and preserved in -20°C until required. Genomic DNA was extracted from 0.05 g of wet weight of material using an AccuPrep Genomic DNA Extraction kit (Bioneer, Korea). Total RNA was determined by using a TRI Reagent kit (Molecular Research Center) after homogenizing the pellets. The RNA was frozen at -80°C until analyzed. Protein concentrations were determined using the bicinchoninic acid (BCA) assay (Sigma) with bovine serum albumin (BSA) as a standard. The experiments were repeated eight times with each sample. The quantifications of DNA, RNA, and protein concentration were measured by using a UV Spectrophotometer (GeneQuant pro, Pharmacia) based on absorption at 260 nm and 280 nm [28].

Measurement of photosynthesis

Chl fluorescence was measured with the PHYTO-PAM (Walz, Germany) equipped with a standard 10×10 mm quartz cuvette. For excitation of Chl fluorescence the PHYTO-PAM applies an array of four different types of light emitting diodes (LED) peaking at 470 nm, 555 nm, 620 nm, and 650 nm. The PHYTO-PAM was operated in conjunction with a notebook personal computer and PhytoWin software provided with the instrument. Control determined the filtrate of a sample that passed through a 0.45 μm filter retaining all microalgae. The parameters of the maximum electron transport rate (ETRmax) and initial slope alpha were provided for opening a window listing the Curve Fit Parameters for Chl-Ch4. The ETR is given in relative units as calculated by the fluorometer software. The relative units are proportional to μmol electrons m⁻² s⁻¹. ETRmax was calculated as:

$$\text{ETR (μmol electrons g}^{-1} \text{Chl a} \cdot \text{S}^{-1}) = \frac{\text{PAR}}{a \cdot \text{PAR}^2 + b \cdot \text{PAR} + c}$$

where, a, b, c is the least square deviation and PAR (μmol quanta m⁻² s⁻¹) is a scalar irradiance.

Nitrate reductase activity

Nitrate reductase (NR) activity was carried out as described in Joseph et al. [17]. Briefly, cells were filtered onto a 25 mm Whatman GF/C glass fiber filter and ground in extract buffer (200 mM phosphate buffer, pH 7.9, with 0.03% dithiothreitol (DTT), 0.3% polyvinyl pyrrolidone (PVP), 0.1% Triton X-100, 5 mM ethylene-diaminetetra-acetic acid (EDTA), and 3% BSA. After grinding, the homogenized suspension was centrifuged (300× g, 4 min). The reaction was run in a final volume of 1.0 mL containing 200 mM phosphate buffer, 0.2 mM NADH, 0.05 mM FAD, and 10 mM KNO3. The time zero reaction was stopped immediately by the addition of 550 mM zinc acetate.

Carbon and nitrogen analysis

The cells were harvested by centrifugation (250× g, 10
min) and dried using a freeze dryer (SFD5024, Samwon, Korea) under -70°C for 5 days. Dried samples were used to determine C and N contents. Dried cells were ground using mortars and pestles, and approximately 1-2 mg of samples was placed into a tine boat for determination of C and N contents using a CHN elemental analyzer (Flash EA1112).

**ATPase measurement**

ATPase was carried out using an ATP determination kit (Invitrogen) according to the manufacturer’s instructions.

**Statistics**

Data analysis was carried out using a one-way analysis of variance (ANOVA) to observe the treatment effects. Duncan’s multiple range test by SPSS ver. 10.0 software was used to evaluate the significant differences between groups. Differences were considered statistically significantly at \( p<0.05 \).

**Results**

When the cells were exposed to different temperatures, variations of concentrations in extracted genomic DNA, total RNA, and total protein based on the ratio of the absorption at 260 nm and 280 nm are shown in Fig. 1. The concentration of genomic DNA in lower temperatures of 12°C and 15°C had a similar value of 0.6, but doubled at a temperature of 18°C (Fig. 1a). Beyond 18°C, the concentration of genomic DNA continuously increased, with a maximum value of 1.8 at 24°C. However, the higher temperature of 27°C caused a marked decrease when compared to 18°C, 21°C, and 24°C, with a similar value of 0.7 at 12°C and 15°C. Based on these statistics, the concentration of genomic DNA between lower temperatures (12-15°C) and higher temperatures (18-24°C) was significantly different \( (p<0.05) \). As with the fluctuation of genomic DNA, the concentration of total RNA between lower and higher temperatures was certainly different (Fig. 1b). At 12°C, the lowest value was approximately 0.5, but this continuously increased after 15°C, with a peak value of 1.7 at 24°C. Total RNA exposed to 21°C and 24°C was significantly different \( (p<0.05) \) compared to lower temperatures. After 24°C, total RNA also considerably decreased. In contrast to genomic DNA, the concentration of total protein between lower and higher temperatures was not greatly different (Fig. 1c). Total protein began to increase after 15°C, but did not continuously increase at higher temperatures. At 24°C, this study observed the highest value of 0.07 μg mL\(^{-1}\), which was approximately ten times greater than 12°C \( (p<0.05) \). The ETRmax, initial alpha, and \( r^2 \) of rapid light curves of the cells using the PHYTO-PAM exposed to different temperatures in cultures were analyzed (Table 1). The ETRmax in C. polykrikoides exposed to lower temperatures ranged from 14.5 μmol electrons g\(^{-1}\) Chl a s\(^{-1}\) (Chl, 12°C) to 50.1 μmol electrons g\(^{-1}\) Chl a s\(^{-1}\) (Chl, 15°C), whereas higher temperatures were found...
Table 1. Mutiple comparison of the ETRmax initial slope alpha, and $r^2$ of rapid curves of Cochlodinium polykrikoides using PHYTO-PAM for 29 days after inoculation

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>ETRmax (µmol electrons g$^{-1}$ Chl a s$^{-1}$)</th>
<th>Alpha</th>
<th>R$^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ch1</td>
<td>Ch2</td>
<td>Ch3</td>
</tr>
<tr>
<td>12</td>
<td>14.5±3.52$^b$</td>
<td>27.9±7.99$^a$</td>
<td>22.3±12.14$^a$</td>
</tr>
<tr>
<td>15</td>
<td>27.9±12.39$^a$</td>
<td>25.7±9.05$^b$</td>
<td>49.5±19.88$^b$</td>
</tr>
<tr>
<td>18</td>
<td>254.3±29.34$^a$</td>
<td>273.5±30.81$^b$</td>
<td>219.1±71.23$^b$</td>
</tr>
<tr>
<td>20</td>
<td>345.7±75.19$^a$</td>
<td>299.9±101.52$^b$</td>
<td>307.8±91.32$^b$</td>
</tr>
<tr>
<td>24</td>
<td>537.9±121.38$^a$</td>
<td>555.7±133.41$^b$</td>
<td>599.3±107.15$^b$</td>
</tr>
<tr>
<td>27</td>
<td>59.8±35.64$^a$</td>
<td>73.4±27.92$^b$</td>
<td>105.2±33.78$^b$</td>
</tr>
</tbody>
</table>

. C. polykrikoides was grown under the light intensity of 100 µmol m$^{-2}$ s$^{-1}$ in L:D cycle of 12:12. The PHYTO-PAM uses four different wavelengths, which peak at 470 nm (Ch1), 535 nm (Ch2), 620 nm (Ch3), and 650 nm (Ch4). Values are mean±S.D. (n=10). Means sharing the same letter are not significantly different (p<0.05, Duncan test). Ch, channel.

to yield extremely higher values of the ETRmax than lower temperatures (p<0.05). At 24°C, this study discovered the highest value of the ETRmax, ranging from 537.9 µmol electrons g$^{-1}$ Chl a s$^{-1}$ (Ch 1) to 602.5 µmol electrons g$^{-1}$ Chl a s$^{-1}$ (Ch 4) which were not shown to be significantly different at Ch 1-Ch 4. However, at 27°C, the ETRmax considerably decreased regardless of different channels in comparison to 24°C. Subsequently, the values of the initial slope alpha and $r^2$ were significantly different between lower and higher temperatures (p<0.05), except at 27°C. In this role, Fig. 2 shows the fluctuations of the ETR in C. polykrikoides cultured at different temperatures according to light intensity. The yield of the ETR between lower and higher temperatures according to light intensity was remarkably different. Lower temperatures attained a lower value of ETR below 100 channels of devices, although the cells were exposed to higher light intensity. In contrast to lower temperatures, higher temperatures had a remarkably high value of ETR at 470 nm, 535 nm, 620 nm, and 650 nm according to light intensity. Like photosynthesis, NR activity was consistently increased corresponding to higher temperatures, with a maximum value of 0.11 µmol NO$_3^{-}$ µg$^{-1}$ Chl a h$^{-1}$ at 24°C (Fig. 3). At 27°C, NR activity was extremely less in comparison to 24°C. The comparison of NR activity between the lower temperature and higher temperature was significantly different (p<0.05). Fig. 4 shows the variations of C and N in the cells exposed to different temperatures. Higher temperatures were associated with significantly higher concentrations of C and N (p<0.05) than the lower temperature of 12°C, with more than ten times the concentration level. Fig. 5 shows the effect of different water temperatures on the fluctuation of ATPase in C. polykrikoides. When the cells cultured at 12°C, the concentration of ATPase was estimated at 0.08 pmol 100 mg$^{-1}$. At 15°C, the concentration of ATPase tripled in
Fig. 3. Effect of *Cochlodinium polykrikoides* cultured at different water temperature on NR activity. The error bar represents mean±S.D. (n=8). The superscript means significantly different (p<0.05).

Fig. 4. Effect of *Cochlodinium polykrikoides* cultured at different water temperature on C (A) and N (B) concentrations. C and N were analyzed by CHN analyzer. The error bar represents mean±S.D. (n=3). The superscript means significantly different (p<0.05).

Fig. 5. Effect of *Cochlodinium polykrikoides* cultured at different water temperature on ATPase concentrations. The error bar represents mean±S.D. (n=8). The superscript means significantly different (p<0.05).

**Discussion**

It is understood that a gene is the fundamental unit of information storage and includes both coding sequence and transcriptional regulatory sequences. In this role, the concentrations of DNA and RNA in phytoplankton have been used to act as indicators of the biomass structure and metabolic state of cells associated with protein synthesis and enzyme production [14,15,33]. Although regulation of gene expression in phytoplankton may be detected under different conditions, the productions of DNA and RNA in *C. polykrikoides* incubated at 12°C are significantly reduced under the same conditions (Fig. 1).

Moreover, other metabolic activities and molecular movements (e.g., photosynthesis, NR activity, ATPase, and the concentrations of C and N) in *C. polykrikoides* exposed to 12°C are also much lower (Figs. 2-5). In Korea, there have been no reported occurrences of *C. polykrikoides* in natural waters at 12°C [25]. Kim [21] suggested that the critical water temperature for *C. polykrikoides* was below 13°C. The present data agrees with Kim’s suggestion [21]. Kim [21] suggested that biological processes on the blooms of *C. polykrikoides* depending on water temperature occur in four phases: initiation (13-15°C), development (15-20°C), maintenance (20-30°C), and decline (13-15°C). In the present study, *C. polykrikoides* cultured at 15°C was found to have small fluctuations in DNA and electron transport movement for photosynthesis that were similar to the results from 12°C. However, NR activity and the concentration of C and N were significantly higher than at 12°C. It is suggested that a temperature of 15°C is not sufficient.
to initiate the blooming development of *C. polykrikoides* based on biochemical and molecular characteristics. This is mainly because photosynthesis provides essential molecular materials for growth in *C. polykrikoides*. Even if *C. polykrikoides* is incubated to an optimal temperature, progress in growth does not occur without lighting. Consequently, the minimum water temperature for the outbreak of *C. polykrikoides* is more desirable at 18°C than 15°C.

Kim et al. [24] suggested that *C. polykrikoides* had a high ecological efficiency for growth because of increasing capture and availability of organic nitrogen sources even under a small amount when being dissolved in water. It is assumed that the higher NR activity cultured at 15°C contributed to high nitrogen efficiency. Outbreaks of *C. polykrikoides* occur in the open sea where there is a little amount of nitrogen. Numerous researchers have reported the occurrence of blooms in the open sea over a period of several months [35,36]. Because higher concentrations of RNA, protein, and ATPase at 15°C are shown in the present study, *C. polykrikoides* possibly requires them to quickly increase nitrogen uptake. The initiation and cessation of nitrate assimilation appeared to be closely related to the appearance and disappearance of the bloom [23,7,9,10]. Consequently, nitrate assimilation may be an important clue in determining bloom control.

Recently, Kim et al. [20] found a hyaline cyst of *C. polykrikoides*. Subsequently, Kim et al. (2004) [22] suggested that vegetative cells of *C. polykrikoides* could survive even in winter, at a water temperature above 14°C. For the present study, this suggestion may be a positive signal because of high nitrate assimilation at 15°C, although photobiological activity is very low.

Nevertheless, establishment of a temperature of 18°C for the initial bloom development of *C. polykrikoides* in nature is desirable because one of the factors from the present study is related to photosynthesis. This study indicates that a temperature of 18°C plays an important role in the initial water temperature for the blooms in nature. However, there have been no reported outbreaks of *C. polykrikoides* at 18°C in nature [25]. Once established, *C. polykrikoides* change from solitary to multiple-chain cells under optimal conditions. When the seawater temperature is approximately at 18°C in nature, it is possible most cells have more chains of two or four cells than one cell.

Kim et al. [24] and Kim et al. [22] suggested that the maximum specific growth rate in *C. polykrikoides* is estimated at 25°C. The present study revealed that the highest concentrations and activities in *C. polykrikoides* based on biochemical and molecular analyses were at 24°C, which was in agreement with previous studies. In nature, if the water temperature is at or near 24°C, massive blooms of *C. polykrikoides* will occur. In Japan, *C. polykrikoides* has different physiological features that are associated with a different water temperature for the occurrence of the blooms [22]. However, Cho et al. [5] reported that Korean strains of *C. polykrikoides* had identical nucleotide sequences. On the basis of molecular characteristics, massive blooms of *C. polykrikoides* will possibly occur in all Korean waters under 24°C over a geographical barrier, but oceanographic conditions attempt to control the blooms. Interestingly, most cell-mediated movements remarkably decreased after 24°C as shown in the present study. It will be very difficult to maintain the massive blooms of *C. polykrikoides* in temperature higher than 24°C.

Acknowledgements

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References


초록: 수온에 따른 유해성 *Cocchlinium polykrikoides* 적조생물의 세포생리 변화

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본 연구는 유해성 *Cocchlinium polykrikoides* 적조생물을 대상으로 수온 변화에 따른 세포 생화학적 및 생리 활성도를 측정했다. Genomic DNA 함량은 12℃ 및 15℃에서 거의 비슷한 0.6을 보였으나, 18℃부터 급격히 높아져서 24℃ 최고 1.8를 나타내었다. RNA와 total protein도 24℃에 가장 높은 1.7과 0.07 μg ml⁻¹으로 나타났다. 광합 성량도 수온에 따른 큰 변화를 보였다. 빛의 파장에 관계없이 18℃ 이상에서 현저히 높은 값을 보였다. 24℃ ETRmax Chl-Ch4까지의 범위는 537.9에서 602.5 μmol electrons g⁻¹ Chl a s⁻¹ 나타났다. Nitrate reductase와 ATPase 효소 활성도는 24℃에서 각각 0.11 μmol NO₃⁻ μg⁻¹ Chl a h⁻¹, 0.78 pmol 100 mg⁻¹ 나타났다. CHN 분석에서 수도수온에 따라 C, H, N의 함량이 현저하게 상이하였다. 27℃ 대비 24℃에 비하여 대부분의 세포생리물질이 낮게 보였다. 따라서 *C. polykrikoides*는 수온 변화에 대하여 세포내시물질의 함량이 많은 차이를 보일 수 있어서 조기 적조 발생 조건은 18℃로 추측된다. 본 실험의 결과로 24℃ 이상이 되면 *C. polykrikoides* 대변식은 세포 내 생리물질의 현저한 저하로 형성되기 어려울 것으로 보인다.