

Andreas Oeltjen
Jürgen Marquardt
Erhard Rhiel*

Geomicrobiology, ICBM,
Carl-von-Ossietzky-University
Oldenburg, Oldenburg, Germany

Differential circadian expression of genes *fcp2* and *fcp6* in *Cyclotella cryptica*

Summary. The steady-state mRNA concentrations of two *fcp* genes encoding fucoxanthin chlorophyll *a/c* light-harvesting polypeptides of the centric diatom *Cyclotella cryptica* were investigated over a 4-day period by RNA dot-blotting experiments. Before and during the first day of the experiment, the cultures were grown under a 12-h light/12-h dark regime. On the following 3 days, the algae were kept in darkness. On the first day, the steady-state mRNA concentration of *fcp2* followed a diurnal pattern, with a maximum occurring around noon, approximately 6 h after the onset of light. The gene *fcp6* also had a diurnal pattern on the first day. Its maximum, however, occurred immediately after the onset of light. During the subsequent incubation period in darkness, the diurnal pattern of expression of both *fcp* genes continued, thus demonstrating that their steady-state mRNA concentrations oscillated in a circadian manner. [Int Microbiol 2004; 7(2):127–131]

Key words: *Cyclotella* · Bacillariophyceae · fucoxanthin–chlorophyll *a/c* · steady-state mRNA concentrations · circadian/diurnal rhythms

Received 26 January 2004
Accepted 26 April 2004

*Corresponding author:
Erhard Rhiel
Geomikrobiologie, ICBM
Carl-von-Ossietzky-Universität Oldenburg
P.O.B. 2503
D-26129 Oldenburg, Germany
Tel. +49-4417983389. Fax +49-4417983384
E-mail: erhard.rhiel@uni-oldenburg.de

Introduction

The main function of light-harvesting complexes (LHCs) of photosynthetic eukaryotes is to absorb light energy and transfer it to the reaction centers of the two photosystems [6,7,10]. The synthesis of LHC subunits and the regulation of the expression of *Lhc* genes encoding LHC subunits have been intensively studied in green plants, and it has been found that the quantity and quality of light influence the expression of *Lhc* genes. *Lhc* gene activity is linked to a biological clock and oscillates in a circadian manner [cf. 15]. Investigations of factors affecting the expression of *fcp* genes, encoding fucoxanthin chlorophyll *a/c* binding proteins (Fcps), which represent the LHCs of chromophytic algae, are limited and mainly address effects caused by light conditions. Thus, the *fcp* mRNA levels of the brown alga *Macrocystis pyrifera* and of *Cyclotella cryptica* were shown to be influenced by light quality and quantity [1,13]. In *C. cryptica*, the transcript levels of the genes *fcp6*, *fcp7* and *fcp12* increased under high-intensity light conditions, whereas those of *fcp1*, *fcp2*, *fcp3*

and *fcp4* increased in low-intensity light [13]. Little is known about circadian/diurnal expression patterns of the genes encoding LHC proteins in chromophytic algae. Expression of *cac* (encoding a chlorophyll *a/c* binding protein) of the chryso-phyte *Giraudyopsis stellifer* did not show a circadian pattern [14], whereas for *fcp2* of *C. cryptica* a diurnal expression pattern could be demonstrated [13]. The transcript levels of this gene increased after the onset of light, reached a maximum approximately 6 h after the beginning of the light phase, and decreased afterwards. A similar accumulation of *fcp* mRNA upon illumination was found in the diatom *Thalassiosira weissflogii* [12], but whether the transcript level subsequently decreased was not investigated. Even in the case of *C. cryptica*, it is uncertain whether fluctuation of *fcp2* mRNA is a singular event caused by the onset of light or the result of a circadian oscillation controlled by an internal clock. This question is the subject of the current work. The relative abundancies of *fcp2* and *fcp6* mRNAs were monitored over 4 days. These two genes were chosen as representatives of *fcp* genes expressed under low or high photon fluence rates, respectively.

Materials and methods

Cultures and growth conditions. *Cyclotella cryptica* was obtained from the Stammsammlung für Algenkulturen (Göttingen, Germany) (SAG, strain no. 1070-1) and grown in *Cyclotella* medium [18] as described previously [13]. Cultures used for the experiments were gassed with air using an aquarium pump and grown at a constant temperature of 18°C in a light/dark regimen of 12 h/12 h. A light intensity of approximately 250 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ was provided by Osram L30W/20 and Osram L30W/25 white fluorescence light tubes. This rather high photon fluence rate was chosen in order to examine the expression pattern of *fcp6*, which has been found to be induced by high light intensity [13]. The expression of *fcp2*, which shows higher steady-state mRNA concentrations under low-intensity white light, can also be investigated under these conditions. Photon flux rates were measured with an Almemo 2290-2 measuring device (Ahlborn Mess- und Regeltechnik, Holzkirchen, Germany) equipped with a DK-PHAR.2.002 S2000TDC615VP Underwater Quantum Sensor (Deka Sensor Technology, Teltow, Germany). The cultures were exposed to light from 8:00 to 20:00 and darkness from 20:00 to 8:00 the following day. This rhythm was continued for 5 to 7 days before the beginning of the sampling period. Samples were taken at 2:00, 7:00, 9:00, 14:00, 19:00 and 21:00. At the end of the light phase of the first day, the light/darkness rhythm was stopped and *Cyclotella* was cultivated in continuous darkness for the following 3 days. The sampling schedule was maintained. No samples were taken at 2:00 on the first day or at 21:00 on the last day. For sampling, 150 ml of culture were harvested by centrifugation in a Sorvall RC5B refrigerating centrifuge using an A6/14 rotor. The cell pellets were either immediately processed for RNA isolation or kept frozen at -70°C.

RNA isolation and RNA dot blots. Total RNA was isolated according to the method of Sambrook et al. [16] as described previously [3]. The amount and purity were checked spectroscopically, and the quality was monitored by formaldehyde agarose gel electrophoresis. The isolated RNA was stored at -70°C. For RNA dot-blotting experiments, either 2 or 5 μg of total RNA from the different RNA samples were dissolved in 400 μl formaldehyde buffer [DEPC-treated H_2O :20 \times SSC (3 M sodium chloride, 300 mM sodium citrate pH 7.0):formaldehyde, in a ratio of 5:3:2], heated to 68°C for 10 min, immediately chilled on ice and spotted onto dry nylon membranes (Biodyne Plus Nylon Membrane, Pall Gelman Laboratory, USA) using a Minifold I Dot-Blot System (Schleicher & Schuell, Dassel, Germany). The RNA was fixed by baking the membranes at 80°C for 2 h.

Probe syntheses. cDNA clones harboring *fcp2* and *fcp6* from *C. cryptica* were used as templates and the *fcp* genes were amplified and labeled with the PCR DIG probe synthesis kit (Boehringer Mannheim, Germany) using gene-specific primers [5]. For normalization, a 750-bp fragment of the

18S rDNA gene of *C. cryptica* was amplified from chromosomal DNA and labeled with the PCR DIG probe synthesis kit [13]. All probes were heated to 95°C for 10 min and dissolved in DIG Easy Hyb hybridization solution (Boehringer Mannheim) prior to use.

Hybridization, signal quantification and statistical analyses. Hybridization experiments were carried out as described in [5]. Stringency washes were performed twice at 65°C in 0.5 \times SSC containing 0.1% SDS. The hybridization conditions were the same for the different gene probes. All solutions were treated with DEPC and autoclaved prior to use. For signal detection, the anti-digoxigenin-alkaline phosphatase-conjugate and CSPD (DIG Luminescent Detection Kit, Boehringer Mannheim) were used according to the manufacturer's recommendations. X-ray films (Lumi Film, Boehringer Mannheim) of the hybridizations using the *fcp* probes were exposed for either 3 or 16 h. The exposure time was shortened to 15–30 min for hybridizations using the 18S rDNA probe. Images of the of X-ray films were recorded and stored as digitalized files using a Herolab EASY CCD Camera (type 429h), a Herolab RH-3 illumination chamber and a personal computer equipped with EASY Analysis System software (Herolab, Wiesloch, Germany). The intensities of the hybridization signals of the RNA dot-blot were calculated using the EASY Image Plus software.

The results described below were derived from two independent experiments using fresh cultures of *C. cryptica*. In both experiments, three to four RNA dot blots were set up for each of the RNA fractions and then probed with the different gene probes. Thus, the signal intensities of at least six to eight dot blots were evaluated for each of the time points in each experiment. The 14:00 value of day 1 was set to 100%. Statistical analyses (t-test) were done using the Sigma Plot 3.0 software package (Jandel Scientific Software, Erkrath, Germany). *p* values < 0.05 were considered as significant.).

Results

In order to answer the question whether the steady-state *fcp2* mRNA concentration followed a circadian rhythm, aliquots of *C. cryptica* cultures were withdrawn over a 96-h period comprising a light/dark cycle on the first day and then complete darkness for the remaining 3 days. RNA dot-blot analyses showed that the intensity of the hybridization signals, i.e. the steady-state *fcp2* mRNA concentration, varied over the 4 days, whereas the intensity of the hybridization signals for 18S rDNA transcript remained almost the same. The results of one experiment are shown in Fig. 1 (upper and lower rows);

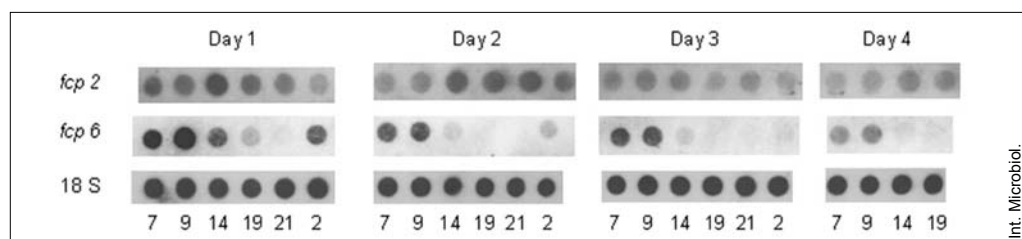


Fig. 1. Circadian expression pattern of the genes *fcp2* and *fcp6*. *C. cryptica* was grown under white light of 250 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ in a 12 h light:12 h dark regime. The light phase was from 8:00 to 20:00. RNA was isolated from 150 mL aliquots of the culture and equal amounts of RNA were dotblotted and hybridized with the *fcp2* (*fcp2*, upper row), the *fcp6* (*fcp6*, middle row) or the 18S rDNA gene probe (18S, lower row). As the 18S rDNA signal intensity stayed almost constant its transcript levels were used to normalize the *fcp* signals. The days and hours of sample withdrawal are indicated above and below the dot blots.

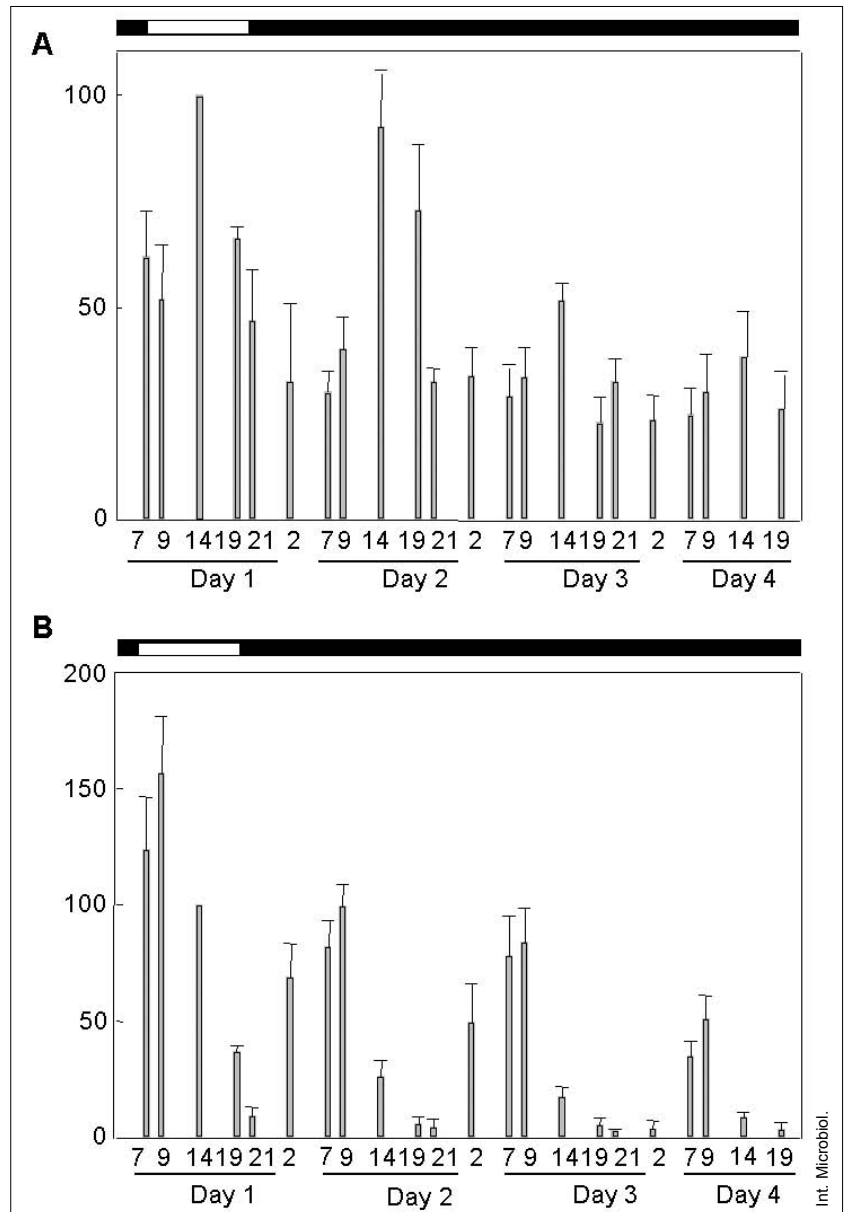


Fig. 2. Bar graphs representing a compilation of the expression patterns of the genes (A) *fcp2* and (B) *fcp6*. The mean values (\pm standard deviations) of one of the two experiments outlined in Material and methods are given. Abscissa: time of day; ordinate: steady-state *fcp* mRNA concentrations (*fcp* mRNA signal intensities normalized to the 18S rRNA signal intensities). The 14:00 value of day 1 was set to 100%. Dark horizontal bars indicate incubation in darkness; open horizontal bars indicate incubation in the light.

the second experiment yielded the same results. The steady-state *fcp2* mRNA concentration increased with the onset of light, reached a maximum approximately 6 h after the beginning of the light phase and decreased thereafter.

The averaged results obtained for *fcp2* and normalized to the 18S rDNA signal intensity of one of the two experiments are compiled in the bar histogram of Fig. 2A. During the first day of sampling, oscillations of the steady-state mRNA concentrations were clearly visible and the relative steady-state mRNA concentration of *fcp2* increased approximately twofold from 7:00 towards noon, peaked at 14:00 and declined during the night to values below those measured at 7:00 on the first day. The differences between the values

measured at 7:00 and 14:00 and those measured at 14:00 and 21:00 were statistically significant ($p < 0.01$). When cell samples were withdrawn at the same times during the following 3 days in complete darkness, the transcript level showed the same oscillating pattern, again with minima prior to the onset of light and in the late evening and maxima occurring around 14:00. During the 3 days in darkness, the amplitudes, i.e. the values measured at 14:00, decreased continuously. Analyses of t-tests confirmed that, with one only exception, the increases from 7:00 to 14:00 and the decreases from 14:00 to 21:00 were statistically significant (p values < 0.022). The second day (first day in darkness), the transition from 14:00 to 19:00 was less significant (p value: 0.08) for both experiments.

Furthermore, the steady-state mRNA abundancies of *fcp6* were also investigated since this gene belongs to those *fcp* genes whose transcript levels increase under high-intensity light conditions [13]. Both experiments yielded the same results, which are summarized in Fig. 1 (middle row) and Fig. 2B, respectively. During the first day, a rhythmic pattern in the steady-state mRNA concentration of *fcp6* that deviated from the one measured for *fcp2* was observed. The *fcp6* transcript level was already rather high at the end of the dark phase and peaked immediately after the onset of light at 9:00. The increase from 7:00 to 9:00 was less pronounced and not significant (p value: 0.16). Later on, the relative amount of *fcp6* mRNA declined and, in the late evening and early night, rather low values were found compared to those registered at 2:00 the following day (9:00–21:00: $p < 0.001$). Over the following 3 days in darkness, the oscillation pattern continued. Similar to the results obtained for *fcp2*, the amplitudes decreased continuously. Analyses of the t-tests again confirmed a statistical significance for the increases detected early in the morning and the decreases measured in the afternoon, with $p < 0.001$.

Discussion

Diurnally and/or circadian oscillating steady-state mRNA levels are well known for the *Lhc* genes of green plants, including angiosperms and green algae. Generally, the transcript levels increase after the onset of illumination, reach a maximum around noon and decrease in the afternoon. The oscillations in mRNA accumulations continue when the plants are transferred from light-dark growth conditions to either constant illumination or darkness. Light-dark and dark-light transitions are believed to synchronize the endogenous rhythm, thus rephasing the oscillation pattern each new day [summarized in 15].

There is scant information available regarding the diurnal/circadian expression of genes encoding LHC polypeptides in chromophytic algae. In the chrysophyte *Giraudyopsis stellerifer*, *cac* was light-induced at the transcriptional level but did not show circadian expression, as no rhythmicity was observed when the alga was transferred from light/dark cycles to continuous darkness [14]. The steady-state *fcp* mRNA concentrations of the centric diatom *Thalassiosira weissflogii* increased after illumination and reached their highest amounts 6 to 8 h after the onset of light [12]. This finding fits well with our results on *fcp2* of *C. cryptica*, as a diurnal rhythm could be clearly demonstrated on the first day of the experiments. As this expression pattern was maintained during the subsequent incubation period in continuous darkness,

we concluded that expression of *fcp2* of *C. cryptica*, which is believed to encode a typical light-harvesting polypeptide attached to the two photosystems [3,4], is controlled by a circadian clock, similar to what has been found in higher plants.

The temporal expression pattern of *fcp6* of *C. cryptica* deviated from that registered for *fcp2*. After the first day, steady-state mRNA levels increased as early as 2:00, during the dark phase, and there was only a relatively small increase from 7:00 to 9:00, although light was switched on at 8:00. Furthermore, the oscillation pattern continued in complete darkness. This finding shows that *fcp6* is also controlled by a circadian clock rather than by an increase of the photon fluence rate.

Phylogenetic analysis revealed that Fcp6, the protein encoded by *fcp6*, shares the highest homology to Fcp of the haptophyte *Isochrysis galbana* and to the light-inducible and *cab*-related proteins LI818r-3 and LI818 of *Chlamydomonas reinhardtii* and *Chlamydomonas eugametos* [4]. No information is available about the expression pattern of the gene encoding Fcp of *Isochrysis galbana*, whereas it is known that *LI818r-3* mRNA levels abruptly increase upon illumination. This is similar to our results on *fcp6*. An oscillation of the *LI818r-3* transcript level, although with reduced amplitudes, was observed even when the *Chlamydomonas* cells were kept in darkness at the onset of the normal light period [17]. In contrast, the mRNA levels of the genes *lhca1*1* and *cabII-1* of *Chlamydomonas reinhardtii*, which encode typical light-harvesting polypeptides of photosystem I and photosystem II, reached maxima 6 to 8 hours after the onset of the light period. Currently, the exact functions of LI818r-3 and LI818 are not known, but their possible roles as substitutes for LHC polypeptides and in protecting reaction centers against photodamage have been discussed. As the highest level of *fcp6* mRNA of *C. cryptica* occurs under high-intensity white light, too, and as it shows a temporary expression pattern similar to that registered for *LI818* mRNA [13, current investigation], its gene product might play a similar role in photoprotection. Expression of the gene in the early morning hours may act to insure that the protein is already present at the onset of light under high-light conditions.

The differential daytime accumulation of *fcp2* and *fcp6* mRNA might be controlled by the promoter sequences of these genes. Daytime-specific promoter motifs are known from higher plant *lhc* genes. [8,9,11, and references cited therein]. At present, genomic DNA sequence data for *fcp* genes and their 5'- and 3'-flanking regions are available only for *fcpA*, *fcpB*, *fcpC* and *fcpD* of *Phaeodactylum tricorutum* [2]. A survey of the entire 5564-bp DNA fragment for the higher-plant promoter motifs did not produce any information. Therefore, it may be that *Phaeodactylum tricorutum*,

and thus diatoms in general, use different motifs than higher plants. Further studies on the 5'-flanking regions of low- and high-light-inducible *fcp* genes of *C. cryptica* are needed to gain further insight into this topic.

Acknowledgements. The authors wish to express their gratitude to the Deutsche Forschungsgemeinschaft (Rh 20/3-3) for financial support.

References

1. Apt KE, Clendennen SK, Powers DA, Grossman AR (1995) The gene family encoding the fucoxanthin chlorophyll proteins from the brown alga *Macrocystis pyrifera*. *Mol Gen Genet* 246:455–464
2. Bhaya D, Grossman AR (1993) Characterization of gene clusters encoding the fucoxanthin chlorophyll proteins of the diatom *Phaeodactylum tricorutum*. *Nucleic Acids Res* 21:4458–4466
3. Eppard M, Rhiel E (1998) The genes encoding light-harvesting subunits of *Cyclotella cryptica* (Bacillariophyceae) constitute a complex and heterogeneous family. *Mol Gen Genet* 260:335–345
4. Eppard M, Krumbein WE, von Haeseler A, Rhiel E (2000) Characterization of *fcp4* and *fcp12*, two additional genes encoding light-harvesting proteins of *Cyclotella cryptica* (Bacillariophyceae) and phylogenetic analysis of this complex gene family. *Plant Biology* 2:283–289
5. Eppard M, Rhiel E (2000) Investigations on gene copy number, introns and chromosomal arrangement of genes encoding the fucoxanthin chlorophyll *a/c*-binding proteins of the centric diatom *Cyclotella cryptica*. *Protist* 151:27–39
6. Green BR, Durnford DG (1996) The chlorophyll-carotenoid proteins of oxygenic photosynthesis. *Annu Rev Plant Physiol Plant Mol Biol* 47:685–714
7. Grossman AR, Bhaya D, Apt KE, Kehoe DM (1995) Light-harvesting complexes in oxygenic photosynthesis: diversity, control, and evolution. *Annu Rev Genetics* 29:231–288
8. Harmer SL, Hogenesch JB, Straume M, Chang HS, Han B, Zhu T, Wang X, Kreps JA, Kay SA (2002) Orchestrated transcription of key pathways in *Arabidopsis* by the circadian clock. *Science* 290:2110–2113
9. Hoffrogge R, Mikschofsky H, Piechulla B (2003) Surface plasmon resonance spectroscopy (SPR) interaction studies of the circadian-controlled tomato LHCa4*1 (CAB11) protein with its promoter. *Chronobiol Int* 20:543–558
10. Jansson S (1994) The light-harvesting chlorophyll *a/b*-binding proteins. *Biochim Biophys Acta* 1184:1–19
11. Kellmann J-W, Merforth N, Wiese M, Pichersky E, Piechulla B (1993) Concerted circadian oscillations in transcript levels of nineteen *Lha/b (cab)* genes in *Lycopersicon esculentum* (tomato). *Mol Gen Genet* 237:439–448
12. Leblanc C, Falcitatore A, Watanabe M, Bowler C (1999) Semi-quantitative RT-PCR analysis of photoregulated gene expression in marine diatoms. *Plant Mol Biol* 40:1031–1044
13. Oeltjen A, Krumbein WE, Rhiel E (2002) Investigations on transcript sizes, steady-state mRNA concentrations and diurnal expression of genes encoding fucoxanthin chlorophyll *a/c* light harvesting polypeptides in the centric diatom *Cyclotella cryptica*. *Plant Biology* 4:250–257
14. Passaquet C, Lichtlé C (1995) Molecular study of a light-harvesting apoprotein of *Giraudyopsis stellifer* (Chrysoophyceae). *Plant Mol Biol* 29:135–148
15. Piechulla B (1999) Circadian expression of the light-harvesting complex protein genes in plants. *Chronobiol Int* 16:115–128
16. Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning: a laboratory manual. Cold Spring Harbour Laboratory Press, 2nd ed., Cold Spring Harbour, New York
17. Savard F, Richard C, Guertin M (1996) The *Chlamydomonas reinhardtii* LI818 gene represents a distant relative of the *cab/III* genes that is regulated during the cell cycle and in response to illumination. *Plant Mol Biol* 32:461–473
18. Werner D (1969) Silicoborate als erste nicht C-haltige Wachstumsfaktoren. *Arch. Mikrobiol.* 65:258–274

Expresión circadiana diferenciada de los genes *fcp2* y *fcp6* en *Cyclotella cryptica*

Resumen. A lo largo de períodos de cuatro días, se estudiaron las concentraciones de mRNA en el estado estacionario correspondientes a dos genes *fcp* que codifican los polipéptidos captadores de luz de las clorofilas *a* y *c* y de la fucoxantina de la diatomea céntrica *Cyclotella cryptica*. Se hicieron experimentos de hibridación puntual de RNA. Antes del experimento y durante el primer día del mismo, el cultivo se realizó en régimen de alternancia luz-oscuridad de 12 horas y los tres días siguientes las algas se mantuvieron en la oscuridad. El primer día, la concentración de mRNA del gen *fcp2* en el estado estacionario seguía un modelo diurno, con un máximo alrededor del mediodía, unas seis horas después de iniciarse la fase iluminada. El gen *fcp6* también siguió un modelo diurno el primer día, si bien alcanzaba el valor máximo justo al principio de la fase iluminada. Durante el período subsiguiente de incubación en la oscuridad se mantuvo el modelo diurno de ambos genes, lo que demuestra que sus concentraciones de mRNA en el estado estacionario oscilaban de manera circadiana. [*Int Microbiol* 2004; 7(2):127–131]

Palabras clave: *Cyclotella* · bacilariofíceas · fucoxantina-clorofila *a/c* · concentración de mRNA en el estado estacionario · ritmos circadianos/diurnos

Expressão circadiana diferenciada dos genes *fcp2* e *fcp6* em *Cyclotella cryptica*

Resumo. Foram estudadas, num período de quatro dias, as concentrações de mRNA no estado estacionário de dois genes *fcp* que codificam os polipeptídeos captadores de luz das clorofilas *a* e *c* da fucoxantina da diatomacea céntrica *Cyclotella cryptica*. Foram feitos estudos mediante experimentos de hibridação pontual de RNA. Antes do experimento e durante o primeiro dia, o cultivo foi realizado em regime de alternância luz-escuridão de 12 horas e nos três dias seguintes as algas foram mantidas no escuro. No primeiro dia, a concentração de mRNA do gen *fcp2* seguiu um modelo diurno, com um máximo em torno do meio-dia, mais ou menos seis horas depois de ter início a fase iluminada. O gen *fcp6* também seguiu um modelo diurno no primeiro dia e alcançou o valor máximo no início da fase iluminada. Durante o período subsequente de incubação no escuro, o modelo diurno de expressão de ambos os genes se mantiveram, o que demonstra que suas concentrações de mRNA no estado estacionário oscilavam de maneira circadiana. [*Int Microbiol* 2004; 7(2):127–131]

Palavras chave: *Cyclotella* · bacilariofíceas · fucoxantina-clorofila *a/c* · concentração de mRNA no estado estacionário · ritmos circadianos/diurnos

