

Diel cycling of DNA staining and *nifH* gene regulation in the unicellular cyanobacterium *Crocospaera watsonii* strain WH 8501 (Cyanophyta)

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Summary

***Crocospaera watsonii* WH 8501 is a marine unicellular cyanobacterium that fixes nitrogen primarily during the dark phase of a light–dark (LD) cycle. Circadian clocks modulate gene transcription and cellular activity in many, if not all, cyanobacteria. A model for circadian control has been proposed in cyanobacteria, called the oscilloid model, which is based on topological changes of nucleoid DNA which in turn regulates gene transcription. In this study, the marine unicellular diazotrophic cyanobacteria *C. watsonii* WH 8501 and *Cyanothece* sp. ATCC 51142 were found to have daily fluctuations in DNA staining using Hoechst 33342 and SYBR I Green fluorescent dyes. Up to 20-fold decreases in DNA fluorescence of Hoechst-stained cells were observed during the dark phase when cultures were grown with a 12:12 LD cycle or under continuous light (LL). The variation in DNA staining was consistent with changes in DNA topology proposed in the oscilloid model. The abundance of *nifH* transcripts in *C. watsonii* WH 8501 was rhythmic under LD and LL cycles, consistent with a circadian rhythm. Cycles of DNA fluorescence and photosynthetic efficiency were disrupted when cultures were shifted into an early dark phase; however, *nifH* transcripts predictably increased in abundance following the premature transition from light to dark-**

ness. Thus, *nifH* gene expression in *C. watsonii* WH 8501 appears to be influenced by both circadian and environmental factors.

Introduction

Cyanobacteria are oxygenic phototrophic prokaryotes that are ubiquitous in the biosphere (Whitton and Potts, 2000). They play important roles in marine environments, and can be responsible for up to 50% of the primary production in surface waters (Chisholm *et al.*, 1988). They are particularly important to carbon flux in microbial food webs. Some cyanobacteria also fix atmospheric N₂ into biologically available nitrogen (N) (Bergman *et al.*, 1997; Gallon, 2001). In the marine environment, N₂ fixation was believed to be primarily due to filamentous non-heterocystous species (*Trichodesmium*) (Capone *et al.*, 1997) until the relatively recent discovery of N₂-fixing unicellular cyanobacteria in the open ocean (Zehr *et al.*, 1998; 2001). Since then, unicellular cyanobacteria, including *Crocospaera watsonii* WH 8501, have been shown to be quantitatively important in N₂ fixation in tropical and subtropical oceans (Zehr *et al.*, 2001; Falcón *et al.*, 2004; Mazard *et al.*, 2004; Montoya *et al.*, 2004; Campbell *et al.*, 2005; Church *et al.*, 2005; Foster *et al.*, 2007).

Cyanobacteria have several adaptations to separate nitrogen fixation from photosynthetic oxygen evolution, including temporal segregation of these metabolic processes and spatial separation via heterocyst formation (Griffiths *et al.*, 1987; Fay, 1992; Bergman *et al.*, 1997; Berman-frank *et al.*, 2003). Temporal or spatial separation is necessary because nitrogenase, the N₂-fixing enzyme, is a multi-component metalloprotein that can be irreversibly damaged by oxygen (Postgate, 1998). A number of unicellular N₂-fixing genera, including *Cyanothece* spp., are phylogenetically related to *C. watsonii*. However, *C. watsonii* WH 8501, some related strains (Webb *et al.*, 2009) and a few uncultivated unicellular cyanobacteria are the only known open ocean planktonic unicellular N₂-fixing cyanobacteria. *Crocospaera watsonii* WH 8501, like most unicellular N₂-fixing cyanobacteria, fixes N₂ during the dark period of the daily light–dark (LD) cycle. The diazotrophic unicellular cyanobacteria *Cyanothece*

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and *Gloeotheca* also fix N_2 in the dark (Gallon *et al.*, 1988; Reddy *et al.*, 1993; Chow and Tabita, 1994; Colón-López *et al.*, 1997; Taniuchi *et al.*, 2008). Respiration during this dark period presumably provides energy and decreases oxygen concentrations which facilitates the oxygen-sensitive nitrogen fixation process (Fay, 1992). In *Cyanotheca* sp. ATCC 51142 carbon is fixed via photosynthesis and stored as glucose polymers during the light period and is subsequently respired during the dark phase (Schneegurt *et al.*, 1994).

Daily cycles of gene expression and metabolism in eukaryotes and cyanobacteria are controlled by an endogenous timing mechanism called the circadian clock, which is controlled through the KaiABC proteins (Kondo and Ishiura, 2000). The *kai* genes are found in most cyanobacteria, including the unicellular nitrogen-fixing cyanobacteria *C. watsonii* WH 8501 and *Cyanotheca* sp. ATCC 51142. The circadian clock was shown to confer a reproductive fitness advantage in *Synechococcus elongatus* (Ouyang *et al.*, 1998; Dodd *et al.*, 2005). Metabolic processes with demonstrated circadian clock control include carbon metabolism, photosynthesis, nitrogen fixation and oxygen evolution (Chen *et al.*, 1998; Colón-López and Sherman, 1998; Yen *et al.*, 2004; Kucho *et al.*, 2005). The coordination of these processes with prevailing photoperiods (light/dark regimes) presumably allows cells to optimize metabolism for growth and utilization of energy.

A conceptual model of circadian clock function, called the 'oscilloid model', proposes that the phosphorylation of the KaiABC protein complex facilitates circadian rhythms in gene expression through fluctuations in supercoiling of cyanobacterial chromosomes, leading to cyclical changes in global cellular DNA topology (Mori and Johnson, 2001). Changes in DNA topology can be altered by DNA gyrases, DNA topoisomerases, other DNA-binding proteins, and the transcription activity of RNA polymerase (Pettijohn and Pfenninger, 1980; Menzel and Gellert, 1983; Liu and Wang, 1987; Dorman *et al.*, 1988; Wu *et al.*, 1988; Tupper *et al.*, 1994; Petrusenko *et al.*, 2006). Supercoiling and topology of bacterial DNA *in vivo* are affected by many physiological factors including oxygen level and the cellular ATP to ADP ratio (Yamamoto and Droffner, 1985; Hsieh *et al.*, 1991; Van workum *et al.*, 1996). Two recent studies of *S. elongatus* PCC 7942 showed that DNA topology changes over a 24 h cycle and may be related to the phosphorylation state of the KaiABC complex (Smith and Williams, 2006; Woelfle *et al.*, 2007). Those studies and the presence of *kai* genes in most cyanobacterial genomes suggest that other cyanobacteria may also use circadian fluctuations in DNA topology as a mechanism to control cellular processes.

The transcription of genes involved in nitrogen fixation has been shown to be sensitive to the supercoiling state of DNA in several different bacteria including

Rhodospseudomonas capsulata, *Klebsiella pneumoniae*, *Enterobacter cloacae*, *Sinorhizobium meliloti* and the cyanobacterium *Gloeotheca (Nageli)* sp. ATCC 27152 (Kranz and Haselkorn, 1986; Dimri and Das, 1988; Page and Gallon, 1992; Hu *et al.*, 2000; Liu *et al.*, 2005). Supercoiling of DNA is a topological change that can be assayed by changes in fluorescence using fluorescent DNA stains (Lebaron and Joux, 1994; Prospero *et al.*, 1994).

The objective of this study was to determine if there is a cycle of DNA staining that may reflect DNA topological changes and control of gene expression in the marine diazotrophic cyanobacterium *C. watsonii* WH 8501. We used a combination of flow cytometry (FCM), fluorescence DNA staining, quantitative reverse transcription polymerase chain reaction (qRT-PCR) and pulse-amplitude modulation (PAM) fluorometry to examine the cycles of DNA topological changes, *nifH* gene transcript abundance, and PSII photosynthetic efficiency in *C. watsonii* WH 8501 to determine if there are links between DNA topological changes, nitrogen fixation and photosynthesis.

Results and discussion

Flow cytometry and DNA-binding dye fluorescence have been used to study cell cycles in the marine cyanobacteria *Synechococcus* and *Prochlorococcus* and results showed that there were cycles of fluorescence associated with DNA replication and cell division (Binder and Chisholm, 1990; Jacquet *et al.*, 2001). Studies using epifluorescence microscopy showed that *S. elongatus* PCC 7942 had diel changes in DNA fluorescence that appear to be linked to DNA topological changes that are regulated by the circadian clock (Mori *et al.*, 1996; Smith and Williams, 2006). We report here that the marine unicellular cyanobacteria *C. watsonii* WH 8501 and *Cyanotheca* sp. ATCC 51142 also have cycles of DNA-binding fluorescence that are not due simply to the cell cycle. These cycles may also be related to control of cellular processes such as DNA topology and compaction.

Patterns of DNA-binding dye fluorescence during 12:12 light–dark cycles

There were distinct daily patterns of fluorescence (*y*-axis in Fig. 1A–C) of Hoechst 33342-stained *C. watsonii* WH 8501 cells (shown in green in Fig. 1A–C) over a 12:12 h light–dark cycle. Unstained cells (red in Fig. 1A–C) had similar fluorescence characteristics over the light–dark cycle (*x*- and *y*-axes in Fig. 1A–C). In the light phase there was a distinct population of Hoechst 33342-stained cells, but during the dark period cell fluorescence varied and there was a general reduction in the average DNA-binding dye fluorescence per cell so that there was no longer a

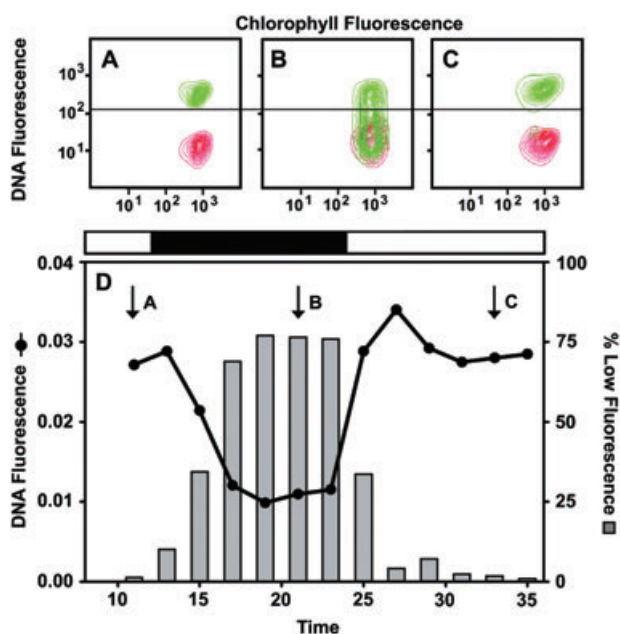


Fig. 1. Diurnal changes in DNA-binding dye fluorescence in Hoechst 33342-stained *C. watsonii* cells grown under a 12:12 light–dark cycle. Two-dimensional FCM plots of chlorophyll and DNA fluorescence (A, B and C). Arrows in (D) indicate time when samples were taken for data shown in (A), (B) and (C). Hoechst 33342-stained (green) and unstained (red) cells are shown. Line in (A), (B) and (C) were used for calculating the % low-fluorescence population. Cycles of normalized DNA-binding dye fluorescence (filled circles) and % low-fluorescence population (grey bars) for *C. watsonii* WH 8501 using Hoechst 33342 are shown in (D).

distinct population of dyed cells (Fig. 1B). Microscopic examination of culture samples and sorted cells confirmed the increase in numbers of low-fluorescence, poorly stained cells during the dark period (data not shown). Histograms of *C. watsonii* WH 8501 cells analysed by FCM showed that there were three separate populations in the dark: cells with a peak of fluorescence similar to that of DNA-stained light-phase cells, referred to here as the ‘high DNA-binding dye fluorescence’ (HIGH-DF) cells; cells with a peak in fluorescence similar to that of unstained cells, referred to as the ‘non-DNA-binding dye fluorescence’ (NON-DF) cells; and cells with a peak fluorescence between HIGH-DF and NON-DF cells referred to as ‘low DNA-binding dye fluorescence’ (LOW-DF) cells (red lines in Fig. 2E). During the light phase, at least 90% of the *C. watsonii* WH 8501 population was in the HIGH-DF population, which was 30 times more fluorescent than the LOW-DF cell population (Figs 1D and 2E). A large fraction of the cells had low DNA fluorescence during the dark phase, with some cells having similar fluorescence to unstained cells (Fig. 1D). Up to 75% of the *C. watsonii* WH 8501 cultures were in the LOW-DF population during the mid- to late portions of the dark phase compared with the light-phase population (Fig. 1D). The DNA fluorescence of some of the cells began to decrease within a few hours of the onset of the dark phase (13–16 h, Fig. 1D) and DNA fluorescence remained low throughout the dark phase.

DNA fluorescence increased sharply in some cells following the shift to the light phase. The DNA fluorescence increase in some cells during the first 3–5 h of the light phase was due to genome replication, which is followed by cell division (Fig. 1D) (Tuit *et al.*, 2006).

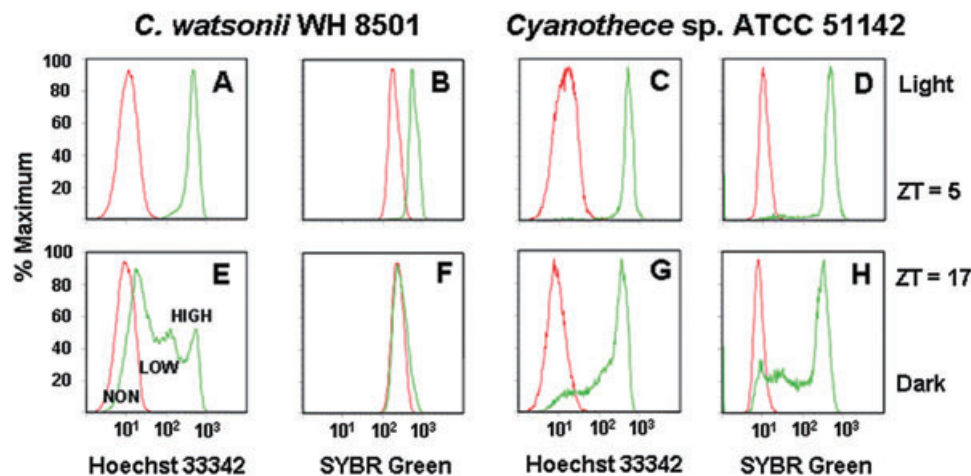


Fig. 2. Diel differences in DNA fluorescence of *C. watsonii* WH 8501 and *Cyanothece* sp. ATCC 51142 using Hoechst 33342 and SYBR Green DNA-binding dyes. Histograms show the fluorescence of *C. watsonii* WH 8501 (A, B, E and F) or *Cyanothece* sp. ATCC 51142 (C, D, G and H) using Hoechst 33342 (A, C, E and G) or SYBR Green (B, D, F and H) 5 h into the light period (A, B, C and D) or 5 h into the dark period (E, F, G and H). Each histogram shows the fluorescence values for samples that either have been stained with a DNA-binding dye (green traces) or were run without staining (red traces). ZT = Zeitberger time, or hours following light on.

The daily pattern of DNA-binding dye fluorescence in *C. watsonii* WH 8501 indicates that there are changes in the properties of genomic DNA over a daily cycle. Changes in DNA supercoiling previously reported (Smith and Williams, 2006; Woelfle *et al.*, 2007) could result in the cycles of DNA-binding dye fluorescence we observed using FCM. The magnitude in change in fluorescence is much greater than the twofold change anticipated from replication of DNA during cell division. The simplest explanation for this change in DNA fluorescence is a change in DNA topology as previously described in *S. elongatus*. This phenomenon was assayed in the morphologically similar *Cyanothece* sp. ATCC 51142 to determine if the fluorescence patterns observed by FCM are generally found in unicellular cyanobacteria.

Comparison of DNA-binding dye fluorescence patterns in C. watsonii WH 8501 to those in Cyanothece sp. ATCC 51142

Mid-light-phase populations of *C. watsonii* WH 8501 had greater fluorescence than unstained cells using Hoechst and SYBR DNA stains, and the cells were distributed normally, consistent with a single population of cells (Fig. 2A and B). However, the majority of cells from mid-dark-phase cultures were in the LOW-DF (or NON-DF) populations when stained with either Hoechst and SYBR DNA-binding dyes (Fig. 2E and F). *Cyanothece* sp. strains ATCC 51142 also had diel differences in DNA staining using both Hoechst 33342 and SYBR Green (Fig. 2C, D, G and H). Similar results were found with *Cyanothece* sp. strain HCC 1134 (data not shown). As in *C. watsonii* WH 8501, *Cyanothece* sp. ATCC 51142 populations had LOW-DF, HIGH-DF and NON-DF cells during the dark period of a 12:12 light–dark cycle using Hoechst or SYBR DNA stains (Fig. 2C, D, G and H). The light-phase Hoechst- or SYBR-stained *Cyanothece* sp. ATCC 51142 HIGH-DF cell populations contained a majority of HIGH-DF cells, which resulted in an average fluorescence 50–60 times greater than dark-phase cells. The SYBR DNA stain had a smaller increase in fluorescence in *C. watsonii* WH 8501 compared with *Cyanothece* sp. ATCC 51142, because of the high background level of green autofluorescence in *C. watsonii* WH 8501. For this reason, LOW-DF populations of SYBR green-stained *C. watsonii* samples were not well resolved, but the SYBR green-stained *Cyanothece* sp. ATCC 51142 cells show that the DNA-staining phenomenon is not dye specific. The DNA staining was visualized in *Cyanothece* sp. ATCC 51142 using epifluorescence microscopy (Fig. 3). The intracellular staining clearly shows condensation of DNA over a daily cycle, even under constant light conditions (Fig. 3). The change in DNA-binding dye fluorescence over the light–dark cycle appears to be characteristic of

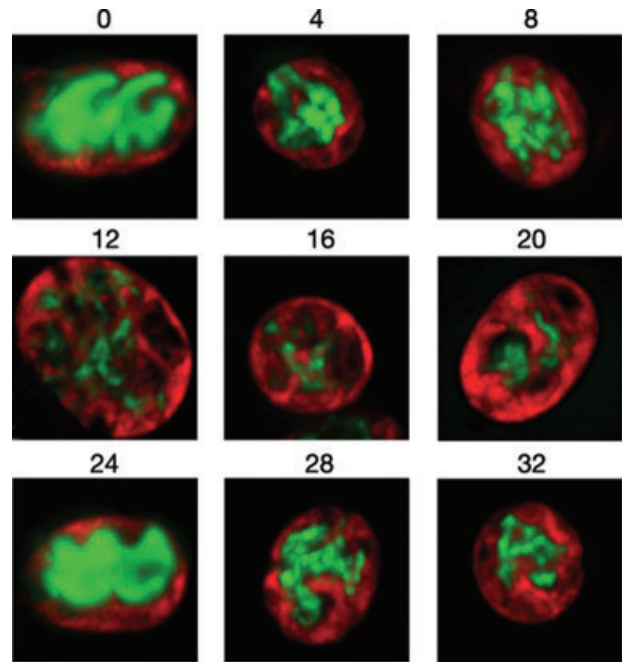


Fig. 3. Images of DAPI-stained *Cyanothece* sp. ATCC 51142. Cells were grown under constant light and sampled every 4 h over a 32 h period. Hour of sampling is indicated. Cells are approximately 5 μm across and outlined by their autofluorescence (red). DNA staining (green) shows a daily rhythm of chromosome compaction. Note the reduction in DNA-stained area at time points between 12 h and 20 h. This chromosome compaction is indicative of the compaction rhythm associated with cyanobacterial circadian clocks. See Smith and Williams (2006).

multiple strains of cyanobacteria. *Cyanothece* sp. ATCC 51142 and *C. watsonii* WH 8501 are both Group II unicellular cyanobacteria that fix N_2 . It is possible that the DNA compaction phenomenon is related to the physiological properties of Group II cyanobacteria, and could be involved in DNA replication and/or gene transcription regulation.

Effect of growth irradiance on DNA-binding dye fluorescence in C. watsonii WH 8501

Crocospaera watsonii WH 8501 cultures had little or no growth at irradiances between 6 and 20 $\mu\text{mol photon m}^{-2} \text{s}^{-1}$, while cultures grown at higher light levels ($> 80 \mu\text{mol photon m}^{-2} \text{s}^{-1}$) had an average growth rate of 0.32 day^{-1} (Fig. 4A). All 12:12 light–dark cultures, regardless of growth irradiance, had decreased DNA-binding dye fluorescence in cells during the dark phase (Fig. 4B). Cultures grown at lower light levels had more cells with low DNA-binding dye fluorescence (LOW-DF) earlier in the dark than cultures grown at higher light levels grown under 12:12 light–dark cycles. At 5 h into the dark phase, the low-light *C. watsonii* WH 8501 cultures grown at 6 and 20 $\mu\text{mol photon m}^{-2} \text{s}^{-1}$ had two to three times lower levels

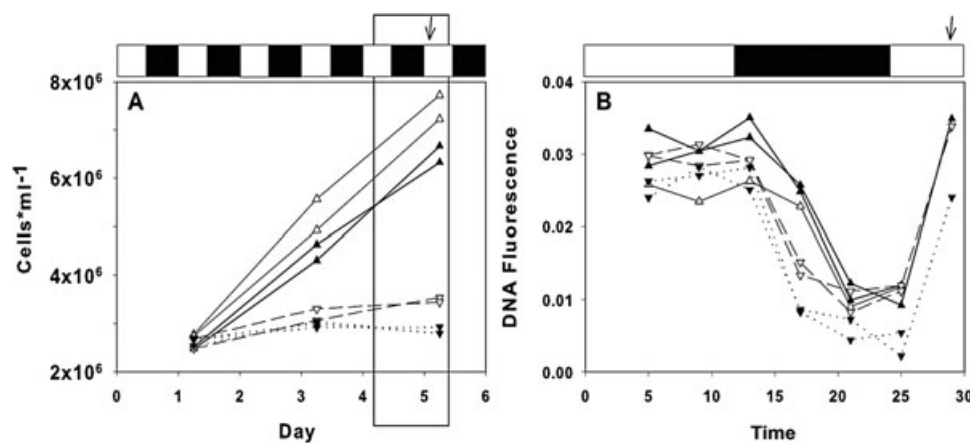


Fig. 4. Effect of light levels on growth rate and DNA stain fluorescence in *C. watsonii* WH 8501. Cell abundances (A) and normalized DNA fluorescence (B) in cultures (duplicates per treatment) grown at 6 (▼), 20 (▽), 90 (△) and 120 (▲) $\mu\text{mol photon m}^{-2} \text{s}^{-1}$. The box in (A) corresponds to sampling period for DNA stain fluorescence shown in (B). Arrow above bars is shown as reference point between (A) and (B). White and black bars above plots denote time in light or dark respectively.

of normalized (to bead fluorescence, see *Experimental procedures*) DNA-binding dye fluorescence than the high-light cultures. The lower irradiance cultures generally had lower DNA-binding dye fluorescence than the high-light cultures, even during illumination.

Shifts in timing of DNA fluorescence when cells are grown at lower irradiance suggest that there is a link between the DNA-binding dye fluorescence and growth rate. The changes in DNA fluorescence, which may reflect DNA topology change, may indicate that the topology changes are involved in chromosome duplication or in differentially regulating genes involved with growth, cell division or stress response. We investigated the relationship of shifts in DNA fluorescence to gene expression by examining the coincidental changes in gene expression of a well-known highly regulated gene, *nifH*, to determine if these shifts in DNA properties were reflected in gene regulation. We also examined how the timing of these changes coincided with photosynthetic parameters.

Effect of shifts in light–dark cycles on DNA-binding dye fluorescence, nifH gene transcription and photosynthetic efficiency

The cyclic pattern of DNA-binding dye fluorescence in *C. watsonii* WH 8501 cells continued under continuous light (LL 100) in a 12:12 light–dark entrained culture, with up to 50% of the *C. watsonii* WH 8501 cells in the LOW-DF population during the relative dark phase (the light phase that would have been the normal dark period) (see Fig. 5). The fact that the pattern of DNA-binding dye fluorescence continued even when incubated under LL suggests that the changes in DNA fluorescence may be related to cellular processes regulated by a circadian clock (Fig. 5).

Cultures grown under a 12:12 light–dark cycle at 60 (LD 60) and 100 (LD 100) $\mu\text{mol photon m}^{-2} \text{s}^{-1}$ had 70% and 55% of the LOW-DF population at the same time point, respectively (Fig. 5L and J), which shows that the pattern of DNA-binding dye fluorescence is related to light intensity.

However, when a 12:12 light–dark entrained culture (grown under 100 $\mu\text{mol photon m}^{-2} \text{s}^{-1}$ light irradiance) was switched to dark prior to the normal dark-phase cycle (early dark; eD), the percentage of cells in the LOW-DF population at 6 h into the entrained dark phase was less than 20% (Fig. 5K).

The shift of the light–dark cycle disrupted the cycle of DNA-binding fluorescence. If the topology change, or other factor causing a change in DNA-binding fluorescence was controlled by the circadian clock, it would be expected that the pattern would have continued even though the dark phase was started early. However, it is possible that the light to dark transition resets the clock for the circadian control of DNA property changes, rather than the dark to light transition.

The abundance of *nifH* transcripts increased shortly before the end of the light phase or entrained light phase in all cultures, achieving a maximum abundance within 6 h of the beginning of the dark phase (Fig. 5A–D). *nifH* transcripts were 84, 203, 439 and 5819 times more abundant during the dark phase compared with the light phase in the eD, LD 100, LL 100 and LD 60 cultures respectively (Fig. 5A–D). The diel magnitude of change of *nifH* expression in *C. watsonii* in this experiment was similar to that reported for natural populations of ‘Group B’ unicellular diazotrophic cyanobacteria (*C. watsonii* and close relatives) (Church *et al.*, 2005; Hewson *et al.*, 2007; Zehr *et al.*, 2007).

Photosynthetic efficiency, as measured by ΦPSII and F_v/F_m , was cyclic under LL and LD, increasing during the

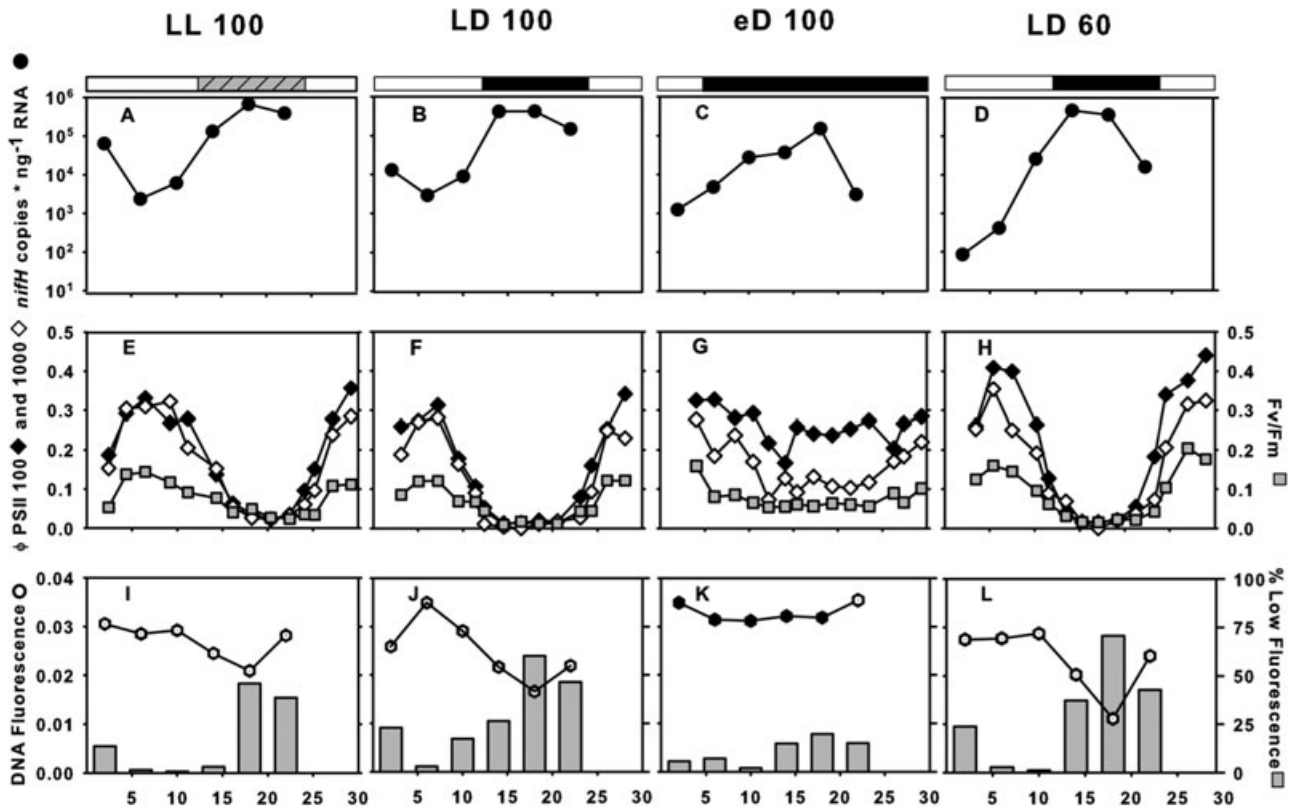


Fig. 5. Light period and intensity effects on *nifH* transcript abundance, photosynthetic efficiency and DNA stain fluorescence in *C. watsonii* WH 8501. *nifH* qRT-PCR results are shown in (A)–(D). PAM fluorometry Fv/Fm and Φ PSII results (at 100 and 1000 $\mu\text{mol photon m}^{-2} \text{s}^{-1}$, see *Experimental procedures*) are shown in (E) through (H). Hoechst 33 342 normalized DNA fluorescence and % low fluorescence population (I–L) as calculated in *Experimental procedures*. *Crocospaera watsonii* WH 8501 grown under 12:12 light–dark cycle at 100 or 60 $\mu\text{Einsteins}$ (B, F and J or D, H and L respectively), LL at 100 $\mu\text{mol photon m}^{-2} \text{s}^{-1}$ (A, E and I), or transferred into darkness 5 h after entering into the light (C, G and K, eD, early dark) (see *Experimental procedures*).

light phase and decreasing during the dark or subjective dark phase (Fig. 4E–H). However, cycling of photosynthetic efficiency in the early dark culture (eD) appeared to be disrupted by the premature dark phase, with cultures maintaining near-normal levels of Φ PSII at 100 $\mu\text{mol photon m}^{-2} \text{s}^{-1}$ during the entrained dark phase with a slight decrease and rebound at the transition to the entrained dark phase (Fig. 5G). The Fv/Fm and Φ PSII at 100 $\mu\text{mol photon m}^{-2} \text{s}^{-1}$ decreased when the eD culture was exposed to the early dark period, but the cyclic nature of Fv/Fm and Φ PSII disappeared (Fig. 5G). The effect of light–dark shifts on photosynthetic parameters was similar to that of changes in DNA-binding dye fluorescence. If DNA-binding dye fluorescence reflects DNA topological changes that are involved in the circadian rhythm, it would be expected that both of these patterns would be disrupted by the early dark shift.

Crocospaera watsonii WH 8501 and *Cyanotheca* sp. ATCC 51142 have been shown to temporally regulate metabolic processes, in particular nitrogen fixation (Sherman *et al.*, 1998; Tuit *et al.*, 2006; Toepel *et al.*, 2008). These cycles of gene transcription and metabolic

activity remain under LL and were seen in our experiments as cycles of *nifH* transcript abundance and photosynthetic efficiency respectively. However, *C. watsonii* exposed to premature darkness appeared to have a disrupted cycle for DNA fluorescence and photosynthetic efficiency, but this perturbation of light cycle did not appear to drastically alter the transcript abundance of *nifH* during the dark phase.

Woelfle and colleagues (2007) used CAGE (chloroquine agarose gel electrophoresis) to show that dark-phase plasmid DNA in *Synechococcus* sp. PCC 7942 shifted to a relaxed conformation when cultures were grown under 12:12 LD or LL conditions, which coincides with the period of low DNA fluorescence in *C. watsonii* and *Cyanotheca* sp. ATCC 51142. Woelfle and colleagues (2007) also saw that cells grown in darkness did not undergo diel fluctuations in plasmid DNA topology. This result is similar to the lack of change of DNA-staining fluorescence in the eD cells and suggests that the circadian rhythm in *C. watsonii* may be conditionally linked to the KaiC phosphorylation cycle as it is in *S. elongatus* PCC7942. Photosynthesis and nitrogen fixation

have been shown to be regulated independently and this may account for the different responses of *nifH* transcript abundance and photosynthetic efficiency to changes in the diel light cycle (Schneegurt *et al.*, 1994; Taniuchi *et al.*, 2008).

The results presented here support the conclusion that there are DNA changes in unicellular diazotrophic cyanobacteria that may be involved in daily regulation of genes, including regulation by a circadian clock. Reduced DNA fluorescence and increased chromosome compaction in *S. elongatus* PCC 7942 by onset of the dark period has been observed by fluorescence microscopy (Smith and Williams, 2006). Heterotrophic bacteria have also been shown to have decreased DNA fluorescence under various forms of stress, and this decrease is suspected to be due to changes in DNA topology (Lebaron and Joux, 1994; Abboudi *et al.*, 2008). Changes in DNA staining fluorescence are known to be affected by DNA supercoiling (Sandhu *et al.*, 1985). We observed similar changes in DNA-binding dye fluorescence in *C. watsonii* WH 8501 and *Cyanothece* spp. using FCM, which are consistent with these previous reports of topological changes in the DNA supercoiling status.

The cyclic patterns of DNA fluorescence in *S. elongatus* PCC 7942 were linked to the phosphorylation cycle of the KaiABC protein complex (Smith and Williams, 2006). The KaiABC phosphorylation cycle has been demonstrated to be an important feature of the endogenous circadian timing mechanism in cyanobacteria (Ishiura *et al.*, 1998; Xu *et al.*, 2003; Nishiwaki *et al.*, 2004). The 'oscilloid model' suggests that circadian rhythms in gene expression in many cyanobacteria are maintained through changes in chromosomal and plasmid DNA topology (Mori and Johnson, 2001). The results reported here and those of Woelfle and colleagues (2007) provide support for the generality of the oscilloid model of circadian rhythms generation in cyanobacteria. The cyclic diel changes in DNA fluorescence we found in *C. watsonii* WH 8501 and *Cyanothece* sp. ATCC 51142 may reflect regulatory oscillations in DNA topology that are controlled by a circadian clock.

Experimental procedures

Cyanobacterial cultures were grown under different light-dark conditions. Batch cultures were grown in sterile polystyrene cell culture flasks (Corning, Corning, NY) in a temperature- and light-controlled incubator and sampled aseptically for DNA staining and FCM, RNA and PAM fluorometry. *Crocospaera watsonii* WH 8501 and *Cyanothece* spp. ATCC 51142 cultures were grown in nitrogen-deplete YBC-II media (Chen *et al.*, 1998). All cultures were incubated at 27°C and maintained at 100 $\mu\text{mol photon m}^{-2} \text{s}^{-1}$ photosynthetic photon flux density using cool-white fluorescent lights. Light intensity was measured with a spherical PAR

sensor (Biospherical Instruments, Quantum Scalar Laboratory Sensor, San Diego, CA) inside culture flasks filled with sterile media. Culture stocks were maintained under a 12:12 light-dark cycle (30 ml in 60 ml flasks) and then transferred and subcultured (grown in 300–600 ml batches in 500–1000 ml flasks) for individual experiments.

Cultures for experiments were grown under LD or LL for 3–4 days (100 $\mu\text{mol photon m}^{-2} \text{s}^{-1}$) before sampling to determine cell characteristics during the diel cycle under different light levels. Light levels for experiments were varied for different treatments using a combination of distance from light source and neutral density screening. The irradiances were 6, 20, 90 and 120 $\mu\text{mol photon m}^{-2} \text{s}^{-1}$ for the light level growth experiment. For the light-phase experiment, cultures were grown at 100 $\mu\text{mol photon m}^{-2} \text{s}^{-1}$ except for the low-level LD culture which was grown at 60 $\mu\text{mol photon m}^{-2} \text{s}^{-1}$. The early dark treatment (eD) was a LD culture grown at 100 $\mu\text{mol photon m}^{-2} \text{s}^{-1}$ which was shifted to the dark phase 7 h prior to the normal period by placing the culture in a black opaque plastic bag inside the growth chamber. Each assayed culture was gently shaken once daily or immediately prior to sampling. Dark-phase samples were carefully taken using dim illumination provided by a Bunsen burner.

Flow cytometry was used to determine culture cell density and to determine the fluorescence properties of cells. For FCM, 1.8 ml of cell culture was pipetted into 2 ml cryovials containing 0.2 ml of 2.5% Ultra-pure TEM-grade glutaraldehyde (0.25% final concentration) and incubated at room temperature for 10 min prior to flash-freezing in liquid nitrogen. Frozen samples were stored at -80°C until analysed.

A Cytopeia Influx cell sorter (Cytopeia, Seattle, WA) equipped with 488 nm and 380 nm wavelength solid-state laser light sources was used for flow cytometric analysis of cyanobacterial cultures. The 488 nm laser was used to determine forward scatter, side scatter and the fluorescence emissions of SYBR I Green DNA-binding dye, phycoerythrin and chlorophyll fluorescence using 530/40, 572/20 and 692/50 nm bandpass filters respectively. Hoechst 33342 DNA-binding dye stained cells were detected with a 355 nm wavelength laser using a 450/60 nm bandpass filter. The flow cytometer was operated using Spigot software (Cytopeia, Seattle, WA) and list mode run files were analysed using Flow Jo software (Treestar, Ashland, OR). Cell populations in each sample were identified using scatter and autofluorescence properties by comparison to standard 3.0 μm diameter Sphero[®] Ultra Rainbow beads (Spherotech, Lake Forest, IL).

Flow cytometry samples were thawed on ice and sub-sampled for DNA analysis. Hoechst 33342 was added to a final concentration of 1 $\mu\text{g ml}^{-1}$ and incubated for 60 min at room temperature prior to flow cytometric analysis (Monger and Landry, 1993). One microlitre of undiluted SYBR I Green (Invitrogen) was added to 99 μl of 0.22 μm water to make a stock solution for DNA staining samples. The stock solution was diluted 1:100 with culture samples (Marie *et al.*, 1997). SYBR I Green-stained samples were incubated for 10 min at room temperature prior to cytometric analysis. The normalized DNA fluorescence was calculated by taking the difference between the mean of the stained and unstained cells and dividing by the mean of the reference beads (3 μm diam-

eter Sphero® UltraRainbow beads, Spherotech). At least 10 000 events were used for each normalized calculation.

Images of DAPI (4',6-diamidino-2-phenylindole)-stained *Cyanothece* sp. ATCC 51142 cells were obtained using deconvolution fluorescence microscopy as described by Smith and Williams (2006). *Cyanothece* sp. ATCC 51142 cultures were grown under constant light for 32 h. DAPI fluorescence was imaged every 4 h using the DAPI channel on the fluorescence microscope (excitation at 360 nm and emission at 457 nm).

The transcription of the *nifH* gene in *C. watsonii* WH 8501 cultures was assayed using qRT-PCR using Taqman® probes (Church *et al.*, 2005). Samples of cell cultures (15 ml) were filtered through 25-mm-diameter 0.2-µm-pore-size Supor (Pall, East Hills, NY) polyethersulfone filters. Filter samples were collected using acid-washed and autoclaved fritted glass filter towers and placed into prepared bead beater tubes. The 2 ml bead beater tubes were autoclaved with a 50 µl mix of 0.5-mm- and 0.1-mm-diameter glass beads, cooled overnight, then 350 µl of Qiagen buffer RLT with 1% β-mercaptoethanol was added to the samples tube before inserting the filter sample. RNA filter sample tubes were immediately flash-frozen in liquid nitrogen after collection and stored at -80°C until extraction.

Frozen RNA samples were thawed on ice, then bead-beaten twice using a Biospec Products (Bartlesville, OK) shaker for 30 s with cooling in an ice-bath between rounds of bead-beating. The filters were removed using sterile syringe needles and the supernatant was extracted using a Qiagen RNeasy mini-plant kit with on-column DNase I treatment. RNA samples were eluted into 50 µl of RNase-free water. The RNA extract was rerun over the elution column and subsampled for immediate quantification using a Nanodrop ND-1000 UV-Vis spectrophotometer (Thermo Scientific). Up to 20 µg of RNA extract was used as template for SuperScript III First-Strand cDNA Synthesis System for RT-PCR. The *nifH*-specific primers *nifH2* and *nifH3* were used for cDNA synthesis (Zehr and Turner, 2001). Control samples included reactions without template and without the reverse transcriptase enzyme and were run with each set of reactions to check for reagent and DNA contamination respectively.

Quantitative PCR of reverse-transcribed RNA to quantify *C. watsonii nifH* transcripts was performed similar to previously reported methods (Church *et al.*, 2005). qPCR reactions (25 µl) were run with a 7500 ABI qPCR instrument. Taqman® primer-probe sets specifically designed to amplify *C. watsonii* WH 8501 (and closely related 'Group B' unicellular N₂-fixing cyanobacteria) *nifH* were used for quantifying *nifH* transcript abundance (Church *et al.*, 2005). qPCR cycle threshold (Ct) values of analytical replicates were averaged and transcript copy number estimated using standard curves generated with *C. watsonii nifH* genes cloned in plasmids (Short and Zehr, 2005). Transcript abundances were normalized to amount of RNA used in the reverse transcription reaction.

Pulse-amplitude modulation (PAM) fluorometry (Schreiber *et al.*, 1995) was used to examine photosynthetic efficiency and oxygen evolution capacity of cyanobacterial cultures. PAM measurements were made using a WATER-PAM fluorometer and WinControl software (Heinz Walz, GmbH). At each time point, two culture samples (1 ml each) were col-

lected into two separate glass cuvettes and dark-adapted for at least 10 min prior to measuring fluorescence parameters. Fluorescence yields (i.e. Fv/Fm and ΦPSII) were determined from saturating pulses (intensity 3000 µmol photon m⁻² s⁻¹ for a duration of 0.8 s). An automated program was used to determine all chlorophyll fluorescence parameters. The PAM fluorometer was set to zero with filtered YBC media prior to assaying experimental samples. For the first sample, Fv/Fm was measured with a saturating pulse in the dark-adapted state. Cells were then exposed to 4 min of actinic light (approximately 100 µmol photon m⁻² s⁻¹) followed by a saturating pulse to measure ΦPSII-100 in the light-adapted state. The program was then repeated for the second sample, except that the actinic light intensity was increased to approximately 1000 µmol photon m⁻² s⁻¹ for 4 min for the ΦPSII-1000 measurement. The values of Fm, F_o, Fm' and F_s were extracted from each fluorescence trace, and fluorescence parameters were calculated using standard equations as follows: Fv/Fm = (Fm - F_o)/Fm and ΦPSII = (Fm' - F_s)/Fm' (Campbell *et al.*, 1998; Maxwell and Johnson, 2000). The reported values of Fv/Fm are averages of the two Fv/Fm values obtained from the two traces at each time point.

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