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Ultradian Metabolic Rhythm in the Diazotrophic Cyanobacterium Cyanothece sp. ATCC 51142.

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Title: **Ultradian metabolic rhythm in the diazotrophic cyanobacterium *Cyanothece* sp. ATCC 51142**

Short title:

Ultradian metabolic rhythm in *Cyanothece*

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Abstract

The unicellular cyanobacterium *Cyanothece* sp. ATCC 51142 is capable of performing oxygenic photosynthesis during the day and microoxic nitrogen fixation at night. These mutually exclusive processes are possible only by temporal separation by circadian clock or another cellular program. We report an identification of a temperature-dependent ultradian metabolic rhythm that controls the alternating oxygenic and microoxic processes of *Cyanothece* sp. ATCC 51142 under continuous high irradiance and in high CO_2 concentration. During the oxygenic photosynthesis phase, nitrate deficiency limited protein synthesis and CO_2 assimilation was directed towards glycogen synthesis. The carbohydrate accumulation reduced over-excitation of the photosynthetic reactions until a respiration burst initiated a transition to microoxic N_2-fixation. In contrast to the circadian clock, this ultradian period is strongly temperature-dependent: 17h at 27°C, which continuously decreased to 10h at 39°C. The cycle was expressed by an oscillatory modulation of net O_2 evolution, CO_2 uptake, pH, fluorescence emission, glycogen content, cell division, and culture optical density. The corresponding ultradian modulation was also observed in the transcription of nitrogenase-related *nifB* and *nifH* genes and in nitrogenase activities. We propose that the control by the newly identified metabolic cycle adds another rhythmic component to the circadian clock that reflects the true metabolic state depending on the actual temperature, irradiance, and CO_2 availability.

\body

Introduction

Cyanobacteria helped to form the Earth's biosphere since they first evolved some 3 billion years ago. These microorganisms made our atmosphere oxygenic and their fixation of CO_2 and N_2 has made important contributions to the elemental cycles. In the past decades, cyanobacteria of the genus *Cyanothece* have been attracting a strong attention by their significant contributions to the nitrogen cycle (1), as unique models to study relationship between N_2 fixation, photosynthesis and respiration (2), as well as promising candidates for bioenergy production (3-5). Distinct potential has been found particularly in the strain *Cyanothece* ATCC 51142 (2, 4). *Cyanothece* sp. ATCC 51142 is a unicellular cyanobacterium where spatial compartmentalization of the mutually exclusive oxygenic photosynthesis and microoxic nitrogen fixation is impossible (6). The strategy used by this organism is to temporally separate the molecular oxygen released by photosynthesis from the nitrogenase that would otherwise be irreversibly O_2-inactivated. The capacity to separate the antagonistic metabolic processes in time is usually attributed to circadian control. The circadian clock in cyanobacteria relies on cyclic (de-) phosphorylation involving complexes of the KaiA, KaiB, KaiC proteins (7-9). The clock mechanism has been studied to a great detail in *Synechococcus elongatus* PCC 7942 and also *in vitro* (10). The clock period in this organism has been shown to be temperature compensated – a feature essential for controlling the daily rhythm particularly in organisms that do not sustain a stable temperature for their metabolism (11).

In *Cyanothece* sp. ATCC 51142, the *kai* genes exist in multiple copies (12) although the Kai proteins have not been studied in the detail achieved for *S. elongatus*. The daily modulation of the metabolic activity and gene transcription, namely alternation of photosynthetic and N_2-

fixation phases, has been attributed to the control by the circadian clock largely because the observed period was approximately 24 h, even in continuous light following a 12 h light / 12 h dark entrainment (13). Certain genes, especially those associated with nitrogenase assembly and function, demonstrated a circadian-type of regulation when the light-dark pattern was modified for growth under continuous light or short day-night periods (14).

However, there are no published data on *Cyanothece* sp. ATCC 51142 to show that the period of the oscillatory modulation in continuous light is temperature compensated as expected for circadian control. One can expect an involvement of metabolic processes (see also (2)) that may result in distinct ultradian rhythms such as known in yeast, which occur with temperature-dependent periods that are significantly shorter than 24 h. The hypothesis suggesting an involvement of an ultradian metabolic cycle in *Cyanothece* sp. ATCC 51142 is supported by oscillations with ca. 12 h periods that occur in continuous light following an initial 12 h light/ 12 h dark entrainment (15) as well as in continuous light in a batch culture (16). Components of about 12 h period have also been detected by a Fourier transform analysis in transcript data in continuous light following a 12 h light / 12 h dark entrainment (17).

In a search for a potential ultradian metabolic rhythm in *Cyanothece* sp. ATCC 51142, we have performed experiments similar to those in yeast in which ultradian oscillations were induced by a starvation period (18). *Cyanothece* sp. ATCC 51142 was grown to late exponential or linear phase in regular medium containing nitrate and supplied with saturating CO_2 and light. Strong ultradian oscillations occurred after the cells were moved to a minus-nitrate medium. The newly found ultradian metabolic rhythm is strongly temperature-dependent. We also show that the circadian cycle is well temperature-compensated. The contrasting temperature dependence documents that the ultradian and circadian cycles are independent. The ultradian rhythm

dominates in saturating CO_2 and light while the circadian rhythm prevails when irradiance and/or CO_2 concentration are lowered.

Results

Ultradian and circadian rhythms in a diurnally entrained culture. *Cyanothece* sp. ATCC 51142 (hereafter *Cyanothece*) was grown in flat-panel photobioreactors with highly time-resolved, automated sampling to follow cyclic processes over days and weeks (19). Before the experiment shown in Fig.1, the culture was entrained in 12 h light / 12 h dark cycles in a turbidostat mode in which the culture optical density was kept in a narrow range by a photobioreactor-controlled feedback dilution. The experiment started after the last period of the diurnal entrainment (interval 0-24h in Fig.1) by switching off the dilution allowing the culture batch growth and by keeping the culture in continuous light for the subsequent 10 days (interval 24 - 264h in Fig.1). In response to the pre-treatment, the culture exhibited a complex oscillatory pattern in which both, the shorter ultradian and the longer circadian rhythms combined. The periods in which the ultradian rhythm dominated (see the 72-96 h interval in the bottom left panel) were followed by periods dominated by the circadian rhythm (see the 216-264 h interval in the bottom right panel).

The concentration of dissolved oxygen oscillated with a minimum in the first half of subjective night in the circadian phase (216-264 h, see open circles in the lower right panel in Fig. 1). The minimum in the concentration of the dissolved oxygen during the ultradian phase (approx. 48–144 h, see the open circles in the lower left panel in Fig. 1) was not firmly anchored to the subjective day/night cycle, because its period was neither equal to 12 h nor to 24 h. The minima of the dissolved oxygen preceded by 1 to 2 hours the maxima of the dissolved CO_2 concentration (thin full line in Fig. 1). The oscillations of the medium pH (short-dashed line in the lower panels of Fig. 1) were nearly anti-parallel to the dissolved CO_2 concentration, as

anticipated from the water chemistry of inorganic carbon forms. During the rapid growth (lower left panel in Fig. 1) the optical density at 680 nm of the suspension (OD_{680}) was weakly modulated with a period of the ultradian rhythm so that the maxima of the dissolved oxygen concentration matched the steepest increase of the optical density. This suggests that high photosynthetic activity indicated by the high concentration of dissolved O_2 correlated with an increase in chlorophyll concentration represented by the surrogate OD_{680}. The maxima of the stationary pigment fluorescence emission (the long-dashed line in the lower panels in Fig. 1) approximately coincided with the O_2 minima and with the CO_2 maxima, a pattern that was not always reproduced in parallel experiments. This dynamic pattern can be tentatively interpreted as high fluorescence due to a reduction of the plastoquinone pool by high rates of respiration and low rates of photosynthesis and *vice versa*.

Metabolic rhythms in a diurnally non-entrained culture. A complex oscillatory pattern similar to the diurnally entrained culture (Fig.1) was also observed in continuous light, i.e. without the diurnal entrainment, when cyanobacteria rapidly growing in a nitrate-rich medium were transferred to a medium without nitrate (time 0 in Fig. 2). The dynamic pattern during the first 3 days following the media replacement exhibited a dominant ultradian character (period ~10.5 h). As the culture grew (OD_{680}~2.2), the oscillations became dominated by the circadian rhythm (lower right panel in Fig. 2). Note that the residual involvement of the ultradian dynamics remained also in the dense culture, as demonstrated by the two high amplitudes at 160.5^{th} and 234^{th} hours of the experiment. The time period separating the two high amplitudes corresponded to 7 ultradian periods (7 x 10.5 h = 73.5 h) and to, roughly, 3 circadian periods (3 x 24h = 72 h). Thus, at every 7^{th} ultradian and every 3^{rd} circadian period, the respective amplitudes combined rather than cancelled each other.

We argue that the transition from the dominant ultradian to circadian rhythm in Fig. 2 was induced by decreasing the mean effective irradiance in the dense culture. This hypothesis is supported by the results in Fig. 3 in which the culture density was stabilized by the turbidostat that was diluting the suspension to the same optical density and, thus, the mean irradiance was fully controlled by the photobioreactor to high irradiance in the initial days and to low irradiance towards the end of the experiment. The data demonstrate that the ultradian rhythm can be converted to circadian cycle by lowering of the incident irradiance in the photobioreactor. This result implies a question what is the decisive factor controlling the transition of the ultradian phenomena to the circadian rhythm: Is it light as the result in Fig. 3 suggests, CO_2 availability, or is it fast growth that requires both, high light and high CO_2? In Supplementary information (*SI Appendix*, Fig. S1 and Fig. S2), we suggest that high light and high CO_2, rather than fast growth, lead to the dominating ultradian rhythm. Another condition for the ultradian rhythm to occur is

lack of nitrate in the medium. An addition of nitrate quickly damped the ultradian oscillations to zero (*SI Appendix*, Fig. S3). Also, the ultradian oscillations in Fig. 2 were induced by transferring the cells to a nitrate-free medium.

Temperature is another important factor affecting the ultradian metabolic rhythm. The oscillations shown in Figs. 1-3 occurred at 36 °C with an ultradian period of 10.55 ± 0.05 hour (mean and standard deviation of 5 biological replicates). We found that the period of ultradian oscillations was strongly temperature dependent. It increased to 17 hours at 27 °C and decreased to 10 hours at 39 °C (Fig. 4). The temperature coefficient Q_{10} was 1.55, documenting the ultradian temperature dependence. We also measured the periods of the 24h rhythms in different temperatures and found Q_{10} close to 1, typical for the temperature-compensated circadian rhythm. The different response to temperature change shows that the ultradian rhythm is truly independent phenomenon that is not derived from the circadian clock.

Data in Fig. 5*A* demonstrate the ultradian oscillations of dissolved O_2 concentration, pH and partial synchronization of cell division (bars). The increasing dissolved oxygen and rising pH (i.e., decreasing dissolved CO_2) reflected active photosynthesis leading to an accumulation of biomass carbon between ca. 7^{th} and 13^{th} hour (closed squares in Fig. 5*B*). A large part of the organic carbon was stored in form of semi-amylopectin granules (20), frequently referred to as glycogen (open squares in the Fig. 5*B*). The proportion of glycogen carbon to total biomass carbon increased during the photosynthesis phase, likely due to the lack of nitrogen that limited protein synthesis. The imbalance between the surplus of fixed carbon and the lack of nitrate in the medium grew to a point where photosynthesis was down-regulated, the dissolved oxygen and, ca. 2 h later, also the pH started decreasing and the accumulation of organic carbon was suspended. The glycogen content started decreasing and oxygen dropped to a level allowing nitrogen fixation. Nitrogen fixation dominated between ca. 3^{rd} and 7^{th} hour and between 13.5^{th}

and 18th hour of the experiment – periods indicated by the dashed line rectangle surrounding the respective segments in Fig. 5. The rising nitrogen content of the biomass is indicated by the dashed line and full circles in Fig. 5*C*. The genes *nifH* and *nifB* necessary for nitrogen fixation are expressed to higher mRNA levels in the early phase of the nitrogen fixation periods.

Discussion

Linking the ultradian metabolic rhythm to other cyclic processes. This paper has demonstrated that *Cyanothece* sp. ATCC 51142 displays ultradian rhythms of alternating photosynthesis and N_2-fixation when grown without nitrate in continuous culture conditions**.** Whether the cultures were entrained (Fig. 1) or not (Fig. 2), the exponential growth phase showed ultradian rhythms that eventually degenerated into more circadian-like rhythms. This finding ties together many disparate results for *Cyanothece* sp. ATCC 51142 over the years and may provide a basis for the interrelationship of the circadian and ultradian types of temporal regulation.

The earlier experiments typically used low light / low CO_2 and showed circadian patterns without an obvious ultradian modulation (3, 21). Here, we demonstrated that the ultradian rhythm occurs when photosynthesis is saturated by high light and high CO_2 concentration. In respect to high carbon availability, it is important to note that an ultradian component was also observed over a circadian pattern when *Cyanothece* was grown heterotrophically with glycerol (22).

Both, the ultradian and circadian cycles exhibit many similar features. During the photosynthesis phase, cells accumulate large amounts of glycogen**.** The transition from photosynthetic to nitrogen fixation phase correlate with degradation of glycogen and a

respiration burst. When enough ATP and NADH/NADPH are accumulated and intracellular oxygen is lowered by respiration, the cells start fixing nitrogen.

Earlier studies of *Cyanothece* sp. ATCC 51142 have been performed mostly on 12 h light/ 12 h dark grown cells and also on cells grown under continuous light and short light/dark periods (21). Invariably, the nitrogenase genes in the 35-gene cluster demonstrated the most consistent circadian behavior (13, 14, 21). Interestingly, the nitrogenase genes were among the large majority of genes displaying a circadian rhythm even in experiments in which some other genes oscillated with a 12h period. The observation of gene oscillation with a 12h period led to an earlier proposal of an ultradian rhythm (17). Here, we probed expression of the *nifB* and *nifH* genes and both were clearly modulated by the ultradian rhythm, a feature consistent also with the ultradian modulation of the nitrogenase activity (Fig. 5). The ultradian modulation of the nitrogenase genes supports the notion that the ultradian rhythm is, under favorable conditions, dominant in all aspects attributed earlier exclusively to circadian control. In this sense, it is highly relevant to ask if the ultradian and circadian processes are not reflecting a single control mechanism (23).

To clarify the interrelationship between the ultradian and circadian rhythms, we measured temperature dependence of the respective periods (Fig. 4). The circadian period was shown to be well temperature-compensated ($Q_{10}\approx1$) while the ultradian period was found to be strongly temperature-dependent ($Q_{10}=1.55$). We conclude that the ultradian metabolic oscillation represents a process that is truly independent from the circadian cycle.

The ultradian rhythms were replaced or dominated by circadian rhythms when the cell irradiance was lowed either by self-shading in high cell density (Figs. 1,2) or by dimming the irradiance incident at the photobioreactor cuvette (Fig. 3). In Supplementary information, we

show an experiment which supports the conclusion that the ultradian rhythm is largely suppressed when photosynthesis and growth are not saturated by light (*SI Appendix, Fig. S1A*).

The ultradian rhythm may also depend on fast growth which requires both, high light and high CO_2. However, we show in (*SI Appendix, Fig. S1B*) that the growth rate is maximal at ca. 33ºC and declines towards 36ºC and 39ºC. This is a qualitatively contrasting temperature dependence compared to that of the ultradian period that declines monotonously between 27ºC and 39ºC. We conclude that the growth rate is not a driver of the ultradian phenomena. This conclusion is further supported by showing (*SI Appendix, Fig. S2*) that the change of the specific growth rate by increasing the sparging gas CO_2 concentration in the range from 700 to 1000 ppm is small compared to the much steeper decrease of the ultradian period.

It appears that two independent mechanisms of temporal separation of photosynthesis and nitrogen fixation are possible in *Cyanothece*: the ultradian rhythm that prevails when photosynthesis is saturated by light and CO_2 and the circadian rhythm that dominates when the rate of photosynthesis is limited.

Interestingly, the experiments reported here also shed light at an earlier observation that the circadian patterns of photosynthesis and nitrogen fixation often appeared even during the first entrainment period, a feature that was hard to understand without context shown in Fig. 1. The experimental cultures used to be inoculated with batch cultures that were 10-14 days old and, thus, closely resembled ca. >200 h cells in Fig. 1 that were probably already poised for the circadian regime prior to inoculation. It was the new photobioreactor that permitted us to work under a variety of environmental conditions and we can now assimilate the information from these different experiments.

On the homology between the metabolic rhythms in Cyanothece and in Saccharomyces cerevisiae.

Aerobically-growing, dense continuous cultures of *Saccharomyces cerevisiae* exhibit autonomous energy-metabolite ultradian oscillations of ca. 2-5 hours period that are observed under low nitrogen and low carbon conditions (18, 24-26). Though yeast are eukaryotic organisms that separate incompatible processes in different compartments, they also use temporal separation to carry out incompatible metabolic reaction and further alleviate futile cycles (27). A difference between the ultradian oscillations in yeast and in *Cyanothece* relates to the culture density. In contrast to yeast, the *Cyanothece* ultradian oscillations do not occur in high cell density that leads to self-shading of the penetrating light and to a reduction of the ultradian oscillatory amplitudes. Thus, this difference is only due to the role of light penetration through the culture that is critical for cyanobacteria and irrelevant to yeast.

In other aspects, several principal homologous features of metabolic oscillations of *Cyanothece* sp. ATCC 51142 and *Saccharomyces cerevisiae* can be found in spite of their largely different prokaryotic and eukaryotic character. The yeast oscillations, similarly to the oscillations reported here for *Cyanothece*, consisted of two distinct phases: the anabolic, carbohydrate-storing phase (reductive in the yeast and photosynthetic in the cyanobacterium) and catabolic phase (oxidative in the yeast and N_2-fixing in the cyanobacterium). The ultradian oscillations of dissolved O_2 and pH (CO_2) were observed in both organisms, accompanied by accumulation of carbohydrates in the anabolic/photosynthetic phase that was concluded by a burst of respiration, see (24) and (18) for yeast. Also, cell division occurred only in the anabolic/photosynthetic phase, see (18) for yeast. In yeast, many genes participating in the regulation of metabolic processes were oscillating with the same ultradian period as the energetic-metabolic oscillations. In *Cyanothece,* the nitrogenase-related genes *nifB* and *nifH* were also clearly modulated by the

ultradian rhythm, a feature consistent with the ultradian modulation of the nitrogenase activity. The homology between the metabolic ultradian oscillations in yeast and in cyanobacteria suggests that both organisms may have the same strategy of benefiting from the ultradian cycling in separating the mutually exclusive metabolic processes, anabolic or photosynthetic and catabolic or nitrogen-fixing reactions, and avoiding futile cycles (18, 27).

The finding of the ultradian metabolic cycle *Cyanothece* sp. ATCC 51142 also resonates with the recent report (2) about significant differences between six *Cyanothece* strains subjected to identical external cues. The differences were interpreted as indicating importance of metabolic signals that are specific for each strain rather than pure regulation by the circadian clock. An important question to ask in future research is on the role of such metabolic signals and of the ultradian cycle in natural conditions. *Cyanothece* sp. ATCC 51142 is a benthic organism that lives in mats or clusters on rocks in intertidal areas (28). Ultradian synchronization of alternating oxygenic and microoxic conditions in the *Cyanothece*-containing biofilms can be considered as an potentially important supplement to the circadian control in adjusting to actual temperature, light and, carbon availability in the environment.

Materials and Methods

Growth conditions, real time measurements and sampling. The *Cyanothece* sp. strain ATCC 51142 (28) was obtained from Jana Stöckel, Washington University, USA and maintained in the complete ASP2 medium (29, 30) buffered with TAPS and TAPSO as described earlier (16).

For growing and real time monitoring of cultures, we used the flat panel photobioreactors (FMT-150, Photon Systems Instruments, Brno, Czech Republic), which were described in detail

in (19). Unless specified otherwise, the culture temperature was stabilized at 36.0±0.3 °C, irradiance was 130-160 μmol(photons) m^{-2} s^{-1} of red light (≈627 nm) supplemented with 25 μmol(photons) m^{-2} s^{-1} of cool white or blue light (≈455 nm). High concentration of dissolved CO_2 was generated by sparging with 0.5 % CO_2.

For diurnal entrainment, the culture grown previously on a shaker in an Erlenmeyer flask was inoculated in a nitrate-free ASP2 medium in the photobioreactor with alternating 12 h light and 12 h dark periods. After the entrainment lasting at least 5 days, the lights were switched on continuously.

For ultradian metabolic cycles induced by removal of nitrate from the medium, the culture was first grown in photobioreactors in ASP2 medium with nitrate until late exponential or early linear phase. The cells were harvested by centrifugation and re-suspended in nitrate-free ASP2 medium. Cells continued to grow in continuous light without nitrate in batch or in turbidostat mode (OD680 = 0.7). Aliquots for cell counting, for quantification of chlorophyll, carbohydrate storage, total carbon and total nitrogen content, and for the measurement of nitrogenase activity were collected every 2-2.5 hours during 24-54 hours. Concentrations of dissolved oxygen and carbon dioxide were measured by the photobioreactor *in situ* every minute as described in (31).

Analytical measurements. Estimation of chlorophyll, carbohydrate, carbon and nitrogen content and cell counts were done as described previously (16).

The nitrogenase activity was determined using the standard acetylene reduction method: Culture aliquots of 15 ml were incubated in gas-tight glass vials of total 40 ml volume with 8 ml of acetylene for 2 hours in light and temperature that were used for growing. Then, 4 ml of

headspace gas were assayed by gas-chromatography. Potential ethylene contamination of acetylene was checked in 4 ml of gas phase taken immediately after the addition of the acetylene.

Quantitative Real Time PCRs were performed in a Mini-Opticon System (Bio-Rad). Normalized Relative Quantities (NRQ) and Standard errors of RQ were determined according to (32). Expression levels of 16S and *rpnA* genes were used as endogenous controls for normalizing the abundance of *nifB* and *nifH* transcripts. Detailed information about RNA extraction, RT, and PCR conditions is available in *SI Appendix*.

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Figure legends

Fig. 1. Transition from ultradian to circadian rhythms in diurnally entrained batch culture. *Cyanothece* culture was diurnally entrained in a turbidostat during 28 days with 12 h light /12 h dark, 36 °C, in the ASP2 medium without nitrate. The dark/light entrainment was followed by constant light 136 μmol(photons).m^{-2}.s^{-1} of 627 nm and 10 μmol(photons).m^{-2}.s^{-1} of cool white light with turbidostat switched off. The upper panel shows the experiment period starting with the last 24 h entrainment period and ending after 10 days of continuous light. The black rectangle between 12 and 24 h shows the last night dark period and the subsequent striped rectangles show subjective (anticipated) night periods. The lower two panels show typical ultradian (72-96 h) and circadian (216-264 h) rhythms. The solid line shows the culture growth dynamics as measured by optical density at 680 nm, OD_{680}. The thick line with open circles shows relative changes in the dissolved O_2. The other measurements are only shown in the lower two panels: The thin full line represents relative change in the dissolved CO_2, the dashed line represents relative change of pH and the long-dashed line shows relative change of the steady state pigment fluorescence emission.

Fig. 2. Batch culture transition from ultradian to circadian rhythms after an exchange to nitrate-less medium. The culture growing rapidly in nitrate supplemented ASP2 medium was harvested and re-suspended in nitrate-less medium (time 0). The transition introduced oscillations in dissolved O_2, in OD_{680} optical density, steady-state fluorescence emission F_t, and in pH. The dissolved CO_2 is represented here by the anti-parallel pH dynamics. The symbols are the same as in Fig. 1.

Fig. 3. Transition from ultradian to circadian rhythms after decrease of light in a turbidostat. The oscillations of dissolved O_2, CO_2, stationary fluorescence Ft, and pH were dominated by the ultradian rhythm during the first 64 hours when exposed to the high irradiance (160 μmol(photons).m^{-2}.s^{-1}). Switching to low irradiance (50 μmol(photons).m^{-2}.s^{-1}) lead to an decrease in the ultradian rhythm amplitude and to prevalence of the circadian oscillation. Noteworthy, the saw-like OD_{680} curve shows that the culture was automatically diluted whenever its density grew over 0.7. The dilutions were less frequent around minima in the concentration of dissolved O_2 indicating slow growth when photosynthesis is suppressed.

Fig. 4. Temperature dependence of the ultradian (open circles) and circadian (closed circles) cycle periods**.** The data were obtained as averages of at least two independent experiments. In 33 and 36°C, more independent experiments were performed to determine the standard deviation of ±0.05 h. The temperature coefficients (Q_{10}) were calculated to quantify the temperature dependence of a rate proxy 1/period.

Fig. 5. The processes occurring during the ultradian oscillations relative to the photosynthesis and nitrogen fixation phases. (*A*) Oscillation of the dissolved O_2 (open circles), pH (closed circles) and percentage of dividing cells as counted in a microscope (bars). (*B*) Total carbon in cells (closed rectangles) increased during the photosynthesis phase whereas it remained stagnant during the nitrogen fixation phase. Glycogen content (open rectangles) decreased during the nitrogen fixing phase. (*C*) Dynamics of the total nitrogen content of photobioreactor biomass together with expression levels of nitrogenase genes. The dashed line shows the increase of N content during the nitrogen fixation phase and stagnant level during the oxygenic photosynthesis

phase. The solid lines show expression levels (NRQ) of the *nifB* (x marks) and *nifH* (+ marks) genes together with measured nitrogenase activity (open triangles).