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An axenic cyclostat of *Prochlorococcus* PCC 9511 with a simulator of natural light regimes

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Abstract

A cyclostat was designed for growing the oceanic oxyphotobacterium *Prochlorococcus* PCC 9511. Culture of this organism, known to be difficult to grow, was mastered for a large volume. *Prochlorococcus* grew well and axenic conditions were maintained for up to 15 days. We designed an illumination system allowing a smooth bell-shaped irradiance curve reaching almost 1000 μmol quanta m^{-2} s^{-1} to be obtained. Cell division was strongly synchronised under these illumination conditions, which were close to those found at low latitude in the upper layer of ocean. The described device is particularly well suited to make experiments requiring up to 6 L per day of well synchronised, exponentially-growing *Prochlorococcus* culture.

Introduction

Automatic systems for the culture of unicellular algae are commonly used in experimental phycology for replacing batch cultures when stable and/or controlled growth conditions are required. In such systems, renewal rate of the culture medium is fixed, or continuously adjusted according to prescribed values of growth (chemostat) or biomass (turbidostat) criteria. Chemostat is well designed to study the static or dynamic effect of nutrient deficiency on microalgal physiology (e.g. Herzig & Falkowski, 1989; Sciandra et al., 1997). Turbidostat, which allows to maintain cultures at maximal growth rate, has often been used for the study of physiological processes governed by light (e.g. Falkowski & Owens, 1980).

A major limitation in the use of automatic culture systems is the difficulty to maintain axenic conditions. This is because these systems always involve a fresh medium input and several outputs for waste, punctual sampling and continuous analyses. Moreover, the special dishes used are often cumbersome, complicated to handle (to set up and remove) and not proper for sterilisation treatments. Although Groeneweg and Soeder (1978) have successfully run axenic turbidostats and chemostats for up to 2 months using a special, small-volume (ca. 350 mL) glassware, continuous culture in axenic conditions remains little used, especially for phytoplankton like *Prochlorococcus*, partly because the deployment of such a system is expensive and laborious. Non-axenicity has many drawbacks. When

phototrophs have a size close to that of contaminant heterotrophic bacteria, as is the case for some small cyanobacteria, discrimination is not possible using most particle counters. Analysis of the particulate carbon concentration is also useless in this case as a significant part of the biomass in fact belongs to bacteria. Measured respiration results both from phototrophs and bacteria catabolism. Finally, for molecular studies dealing with expression of non-photosynthetic genes, presence of bacteria makes interpretation difficult because of the risk of cross-hybridisation of gene probes with contaminant DNA or RNA.

Another difficulty when running continuously cultures for the study of environmental forcing is encountered with light. The smooth temporal variations and the spectrum of solar radiation are hard to simulate. Therefore, turbidostats are often run under binary or stepwise light:dark cycles. Kroon et al. (1992) introduced a 'cyclostat' with computer-controlled venetian blinds that allows the natural sun cycle to be accurately simulated (see also Kroon & Dijkman, 1996). New technologies now allow such a system to be deployed much more easily and at a low cost.

In this paper, we present a large-volume axenic cyclostat with a very simple design, that uses common non-toxic consumables now commercially available, and is equipped with an easy to handle computer-controlled illumination system that relies upon the use of dimmable neon tubes. This cyclostat was developed for the culture of *Prochlorococcus* PCC 9511, the first *Prochlorococcus* strain having been made axenic (Rippka et al., 2000). Until recently, *Prochlorococcus* was known to be difficult to grow in the laboratory as it is an algae very sensitive to any small change in its culturing conditions (R. Rippka, pers. comm.). With an average cell size of ca. 0.6 μm, *Prochlorococcus* is the smallest photosynthetic organism known to date (Partensky et al., 1999a). It is particularly abundant in the oligotrophic tropical ocean from the well-illuminated surface waters down to the 'deep chlorophyll maximum', where irradiance is often below 1% of the incident solar light in surface (Partensky et al., 1999b). Furthermore, at all depths, *Prochlorococcus* cell division has been found to be highly synchronised by the natural alternation of day and night (Vaulot et al., 1995).

The problem to be overcome was to grow *Prochlorococcus* PCC 9511 in continuous culture at a maximum irradiance approaching 1000 μmol quanta m^{-2} s^{-1}, i.e. close to that observed in the upper layer of open ocean. Furthermore, this cyclostat had to remain axenic during the entire duration of the experiment (PROchlorococcus MOLecular ECology, lab-workshop held in Roscoff France in May 1999), and to produce a sufficient amount of culture to concomitantly perform a large variety of measurements (including flow cytometric cell counting, optical and photosynthetic parameters determination, pigments, carbon and nitrogen content evaluation as well as expression of photosynthetic and cell cycle genes).

Methods

Characteristics of the axenic *Prochlorococcus* PCC 9511 have been recently described (Rippka et al., 2000). Two experiments using this strain are presented below. In the first one, we determined some parameters of the turbidostat on a moderate culture volume and under continuous illumination. In the second experiment, we tested the system for large volume and exposed it to a simulated natural light cycle (cyclostat mode).

First experiment

The culture medium was directly prepared into a 20-L polycarbonate tank using sea water from the English Channel collected at 5 m in the Bay of Morlaix (France). Sea water was stored during more than one month at room temperature, then filtered through a Millipore 0.2 μm filtering cartridge and autoclaved at 120 °C during one hour. The PCR-S11 stock solutions (Partensky et al., 1999a; Rippka et al., 2000) were added after filtration through 0.2 μm sterile filters (vitamins and metals solutions) or autoclaving [HEPES, Na_2.EDTA/$FeCl_3$, $(NH_4)_2$–SO_4 and Phosphate buffer]. The only modifications with regard to the original PCR-S11 recipe were that Na_2EDTA/$FeCl_3$ and vitamin B12 were supplied at 2 μM and 0.7 nM, respectively.

Prochlorococcus PCC 9511 was grown at 21 °C in a 2.8-L cylindrical polycarbonate bottle filled with 1.7 L culture medium. The entire culture set up, including the vessel, the different input and output tubes and filters, was autoclaved at 110 °C for 20 min. All parts were made of either Teflon, silicone, polycarbonate or polyethylene. The renewal medium was introduced continuously into the culture using a peristaltic pump (Gilson Minipulse 2) through a 0.2 μm sterile filtration cartridge (Pal Gelman SpiralCap®). Air could exchange with the culture vessel through a sterile 0.3μm

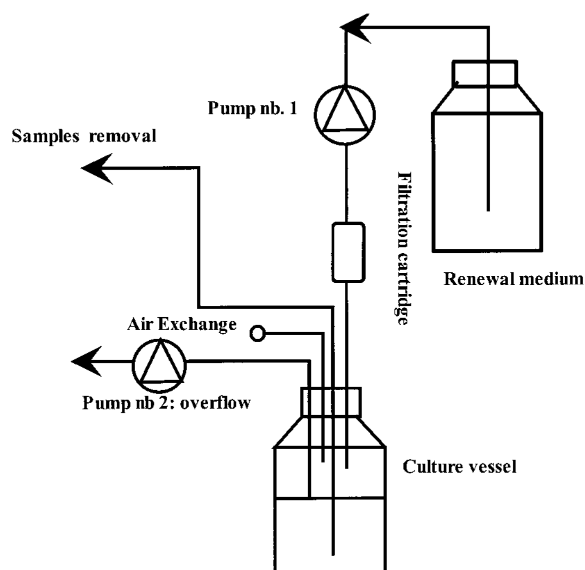

Figure 1. Schematic diagram of the turbidostat.

Whatman Hepa-Vent™. Continuous illumination was provided by three mercury lamps (OSRAM HQI – TS) placed above the culture system, through a Plexiglas cover and sheets of blue supple plastic filter (Lee Filters, n° 183). Photosynthetically available radiation (PAR, irradiance integrated between 400 and 700 nm), as measured using a quantum scalar irradiance meter (Biospherical Instruments, QSL 100), was set at 45 ± 5 μmol quanta $m^{-2}s^{-1}$, a value close to the optimal continuous growth irradiance under blue light for the closely related strain MED4 (Moore et al., 1995). The volume of the culture was stabilised at 1.7 L by pumping excess out (Figure 1).

Samples were collected twice a day, after a gentle stirring of the vessel, although culture seemed homogeneous by eye. To reduce the risk of contamination, the end of the collector tubing was pinched with two clamps. For sampling, the part of the tube located between the two clamps was cut with flamed scissors. After sampling near a Bunsen, the removed clamp was put back upstream the remaining clamp till the next sampling. The spectral absorbance, $A(\lambda)$, due to algal cells was measured in a 1-cm quartz cuvette using a dual beam spectrophotometer equipped with an integrating sphere (Varian, DMS-100), with 1-nm increments between 380 and 750 nm. Filtered medium from the culture (0.2 μm syringe filters; Pal Gelman Acrodisc®) was used as reference. $A(750)$ was subtracted from $A(\lambda)$ to correct for residual scattering (see e.g. Stramski & Reynolds, 1993). $A(\lambda)$ measurements, used here as biomass index, were duplicated (as well as the reference) and averaged. During the batch phase, the growth rate, μ, was calculated from:

$$\mu(t) = 1n\left[\frac{A(442)_t}{A(442)_{t-\Delta t}}\right]\Delta t^{-1}$$

where $A(442)_t$ and $A(442)_{t-\Delta t}$ are the absorbances at time t and t-Δt, respectively, Δt being the time elapsed between current and previous measurements.

During the turbidostat phase, the dilution flow rate was controlled twice a day by weighing, and adjusted to a new value if necessary, to compensate for $A(442)$ variations.

Axenicity of the culture was checked daily. Petri plates were filled with PCR-S11 medium with 8 gL^{-1} agar and enriched with 1 g D+ glucose and 0.2 g of casein acid hydrolysate per litre of medium. Two different inoculum volumes were tested, 50 and 200 μL. Absence of bacterial colonies on plates 6 days after inoculation, as compared to a positive control consisting in a similar volume of non-bacteria-free *Prochlorococcus* culture, indicated that the initial sample was axenic.

Second experiment

The turbidostat culture was then modified in order to yield large daily volumes of *Prochlorococcus* culture. Twelve polycarbonate Nalgene carboys filled with 10 L of sterile PCR-S11 medium were connected by an equal length of silicone tubing to a peristaltic pump (Masterflex model 7523-37, Cole-Parmer Instrument Co), set at a mean rate of ca. 8 mL min^{-1} and running on continuously. Note that the total quantity of fresh medium needed for the entire experiment must be planned before the start in order to avoid changes in medium composition. The medium was then split into two lines, each having two autoclaved 0.2 μm SpiralCap™ capsules mounted serially and being coupled to a 20 L Nalgene polycarbonate jerry-can. Both jerry-cans (cyclostats 1 and 2) were immersed into a glass aquarium filled with running water thermostated at 21 ± 1 °C using a circulating bath (Huber, Minichiller). The entire culture set up (tubing, connections and containers) was rinsed before use (HCl and Milli-Q water) and autoclaved to decrease the risks of contamination. Assemblages of the different tubing should be done under the laminar hood or near a Bunsen. The excess volume of culture was continuously pumped out with a peristaltic pump in a closed carboy which was sterilely emptied by pumping when needed. In our experiment, the culture vessels were

not stirred at any time since healthy *Prochlorococcus* cells generally do not settle down. Use of this culture system for studies of nutrient limited cultures may require the cultures to be gently stirred to allow a better homogenisation of nutrients in the culture.

The most original part of the set-up was the illumination system, which transformed the turbidostat into a ‘cyclostat’ (sensu Kroon et al., 1992). It consisted of two white plates placed on opposite faces of the culture containers, each supporting a set of six 50-cm U-shaped, dimmable neon tubes (OSRAM, DULUX® L, 2G11, 55 W / 12 – 950, LUMILUX DE LUXE, daylight). The tubes were disposed head to tail with a ca. 20 m^{-1} density. Each tube was connected to an electronic ballast (OSRAM, Quicktronic®, QT 1 × 55 / 230 – 240 DIM), power supplied with 220 VAC. Their 0–10 VDC analogue input allows to modulate the neon tube output between 1 to 100%. The ballast’s were controlled using a data acquisition board (ADAC, 5525MF-V, 26330) equipped with two 12-bit digital-to-analogue channels and sixteen 12-bit analogue-to-digital channels. The ADAC card was driven by a personal computer using the Labtech Notebook software (Laboratory Technologies Corp.) version 10.11. To generate a simulated natural light-dark cycle, two functions were sequentially applied: a PAR vs. time function and an input-VDC vs. PAR one. The PAR vs. solar time relationship was calculated with 300-s time steps using a radiative transfer code (Sequoia Scientific Inc., Hydrolight, Version 3) for June 1, at equator, under standard clear sky atmospheric conditions (as defined in Morel, 1991), and just below ocean surface. The resulting lookup table was read every second using linear interpolation. The input-VDC vs. PAR relationship of the neon tube – ballast set was determined using a laboratory power supply coupled to an oscilloscope (LeCroy, 9310 C) and a quantum scalar irradiance meter. A least squares spline function was fitted to the experimental relationship and a lookup table was produced with 1×10^{-5} increments on a PAR 0–1 relative scale. At the beginning of the light period, the shift from 0 to 1% was done in two time steps by turning on one neon tube over two. All tubes were then controlled together using the functions described above.

The light provided by this system was diffused through a plastic foil (Lee Filters, ‘white frost’, n° 220) wrapped around the aquarium where *Prochlorococcus* cultures were immersed. Just after the end of the experiment, PAR was measured inside the culture at full light power (equivalent to solar noon) using a quantum scalar irradiance meter (LI-COR LI-1000) equipped with a 4-π spherical probe. Average irradiance was 970 and 912 μmol quanta m^{-2} s^{-1} in cyclostats 1 and 2, respectively. PAR temporal variations were monitored in relative units using another quantum scalar irradiance meter (Biospherical Instruments, PNF-300 reference collector) connected to the analogue-to-digital channels of the data acquisition board. The irradiance spectrum of the neon tube was measured between 350 and 750 nm with 1-nm increments using a spectroradiometer (LI-COR, LI-1800UW).

Prochlorococcus cells were acclimated for 15 days to the light:dark cycle before a four-day period of extensive sampling. Culture was first grown in batch for 4 days then run for 7 days in cyclostat mode with a maximal level in the Jerry-cans set at 2 L, then the volume was progressively increased up to 10 L within one day and remained at this level for three days prior to the beginning of extensive sampling. With this volume and when accounting for wastes, approximately 6 L of culture growing at an estimated growth rate of one division per day (i.e. μ = 0.69 d^{-1}) could be sampled daily without affecting population growth (but see results). During the dark period, sampling was made under low green illumination, which is not absorbed by *Prochlorococcus* cells (Morel et al., 1993; Shimada et al., 1996) and therefore did not disturb cell growth. The two cyclostats were not sampled at the same frequency. Cyclostat 1 was sampled every second hour (except at 04:00) for a variety of analyses (including optics, flow cytometry, photosynthetic parameters, pigments, C/N, TEM, etc.) and cyclostat 2 every fourth hour for flow cytometry and RNA. In the present paper, we will only report absorbance at 442 nm, cell number, individual cell near-forward light scattering (hereafter denoted NFS) and DNA cellular content. Absorbance was measured as described above but using a Perkin Elmer Lambda 19 spectrophotometer. Cell number, NFS and DNA cellular content were determined using a FacScan flow cytometer (Becton Dickinson). Immediately after sampling, cells were fixed with a mixture of 1% paraformaldehyde and 0.1% glutaraldehyde for 15 min (both chemicals from Sigma), then frozen in liquid nitrogen and stored at –80 °C. Once thawed, cells were stained using a 1/10 000th dilution of the commercial solution of the DNA dye SYBR-Green I (Molecular Probes Inc.), then analysed, as detailed elsewhere (Marie et al., 1999). The same measurement

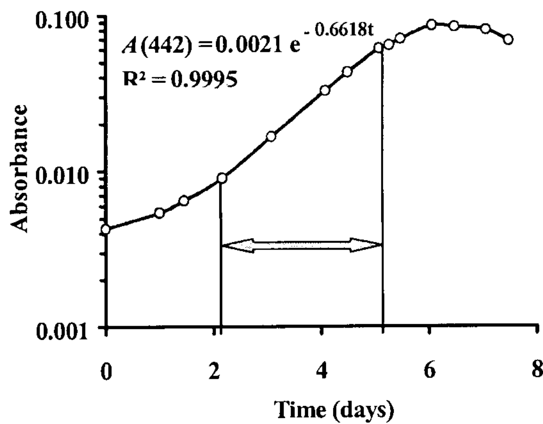

Figure 2. Changes in absorbance at 442 nm with time for the batch culture grown during the first experiment (determination of the optimal conditions). An exponential function was fitted to experimental data within the window illustrated on the plot to determine the growth rate (μ).

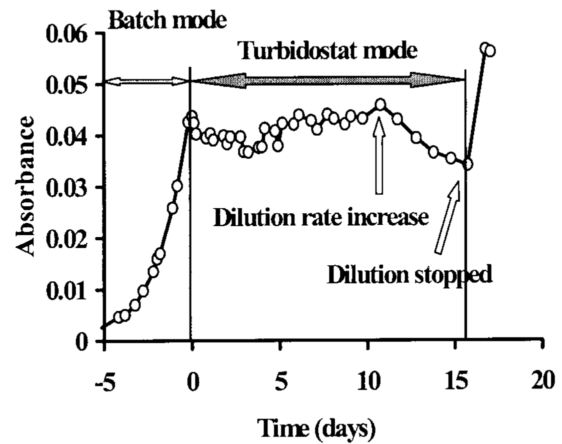

Figure 3. Changes in absorbance at 442 nm with time for the turbidostat grown during the first experiment. Absorbance first increases during the batch mode culture. Absorbance is stable during the turbidostat mode culture. At day 10,75 the dilution rate was increased. At day 15,75, the dilution was stopped.

allowed to determine *Prochlorococcus* cell number using divinyl-chlorophyll a red fluorescence, their NFS, and their DNA cellular content using green fluorescence, and to determine whether contaminant bacteria were present in the culture.

Results and discussion

First experiment

Prior to starting the turbidostat, a batch culture was first done to determine the exponential-growth range and the maximum carrying capacity (MCC) of the medium in terms of $A(442)$, as well as the maximal growth rate (μ_{max}) of the *Prochlorococcus* PCC 9511 under the experimental conditions. Figure 2 shows $A(442)$ as a function of time for the batch culture. The growth was exponential up to a $A(442)$ value of 0.06 and the MCC was 0.09. μ_{max} was on average 0.66 d^{-1} (one doubling per day) during the exponential phase, as estimated by fitting an exponential function to $A(442)$ vs. time data within the time window shown in Figure 2. Although the exponential-growth range extended up to 0.06 in terms of $A(442)$, we adopted 0.04 as upper limit for turbidostat operation 1) to anticipate variations in the culture medium composition that may induce a slower growth, 2) to account for the fact that, at $A(442) = 0.06$, cells are probably growing on internal reserves, and 3) to keep the culture optically thin.

To start the turbidostat, a second batch culture was initiated under conditions similar to the first one. μ_{max} was estimated to 0.55 d^{-1} during the exponential phase. This discrepancy of μ_{max} values between the 2 runs is not significant and might be due to error in the determination of the growth rate (up to 10%, Sciandra, pers. comm.), and to the use of absorbance which is a better indicator of the biovolume than of the cell number. Once the density reached $A(442) = 0.04$, the culture was then diluted with new medium at a rate of ca. 0.5 d^{-1} with daily adjustments. Note that in theory, the renewal rate (r), when defined as:

$$r = \frac{\Delta v}{V \Delta t}$$

where v is the renewed volume and V is total volume of the culture, must equal μ_{max} to maintain the biomass constant but must not exceed μ_{max} to prevent flushing the culture.

Figure 3 shows that a nearly constant $A(442)$ was maintained within ca. 10%. After 10.75 days, the dilution rate was increased to 0.6 d^{-1} to see if the growth could be enhanced. μ did not change significantly (data not shown), which indicates that the culture was not nutrient limited, and consistently $A(442)$ decreased. After 15.75 days, dilution was stopped to evaluate the capacity of the remaining medium in the turbidostat to support higher biomass. After the dilution was stopped μ was 0.51 d^{-1}, and the biomass increased up to 0.06 only, which emphasises the occurrence of natural and experimental variations. The axenicity was maintained until day 13.

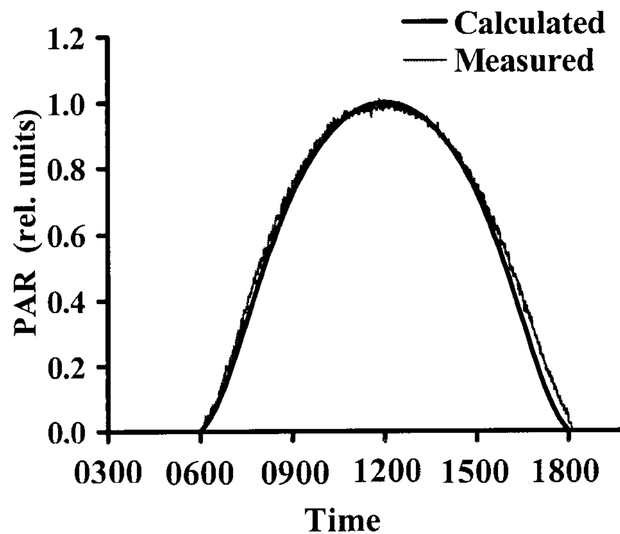

Figure 4. PAR as a function of time during the cyclostat experiment.

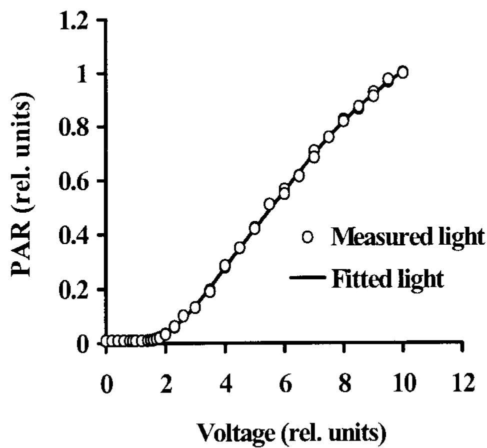

Figure 5. PAR vs. Input-VDC response of the ballast – neon tube set. The data from two independent measurement series have been pooled to calculate the least-square spline function.

Second experiment

Figure 4 shows PAR as a function of time, as determined from radiative transfer calculations and as measured during the second experiment. Figure 5 illustrates the input-VDC vs. PAR response of the ballast – neon tube set and the fitted spline function. The measured PAR vs. time function shows some departure from the calculated one (< 7%), as well as a slight asymmetry. This discrepancy between the input and output PAR vs. time relationships is partly due to the fact that the PAR vs. input-VDC response was characterized on a single ballast-neon-tube unit, i.e. in a configuration different from the final one (with

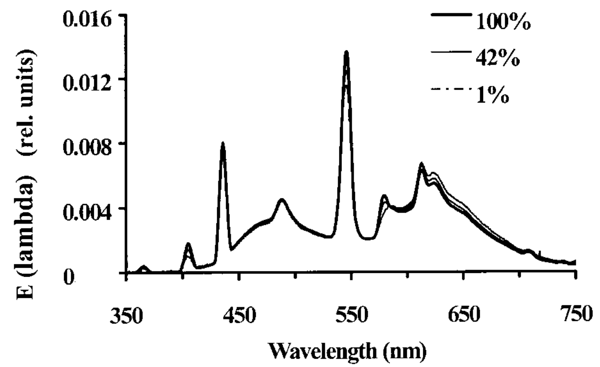

Figure 6. Spectral values of irradiance for the neon tube used in the second experiment, measured at 3 different input-VDC (1, 5 and 10 VDC) corresponding to 1, 42 and 100% of maximal neon tube irradiance.

12 units). The asymmetry in the measured PAR vs. time relationship is likely due to some hysteresis in the PAR vs. input-VDC response of the ballast-neon tube set. It could be compensated for by deriving separate PAR vs. input-VDC responses for the increasing and decreasing components of the PAR vs. time relationship.

Figure 6 illustrates normalised irradiance [E(λ)] spectra of the neon tube, measured at 3 different input-VDC (1, 5 and 10 VDC) corresponding to 1, 42 and 100% of maximal neon tube irradiance. It shows that the shape of the E(λ) spectrum does not change significantly with input-VDC. Although these spectra are much featured compared with the sun irradiance spectrum, they cover well the PAR range. To better simulate the underwater irradiance spectrum, one can complement the system for instance with blue filters such as the 'Moonlight Blue' (Lee Filters, n° 183) which allows to simulate realistic underwater spectral irradiance. Note that the illumination system described here can reach PAR values of up to about 3 000 μmol quanta m^{-2} s^{-1} closer to the source, which is plenty to obtain high PAR even with a blue filter.

Figure 7 shows $A(442)$ variations in cyclostat 1 during the four-day period of intensive sampling. It varied first around 0.02, then dropped down around 0.013 at the end of the second light period, and progressively decreased to 0.01. Cell number in both cyclostat showed a similar pattern with a clear oscillation during the first two days. The night increase was due to cell division, which was highly synchronised, whereas the day decrease was due to dilution with fresh medium. This oscillation was obscured during the last 48 h (and especially last day) of the

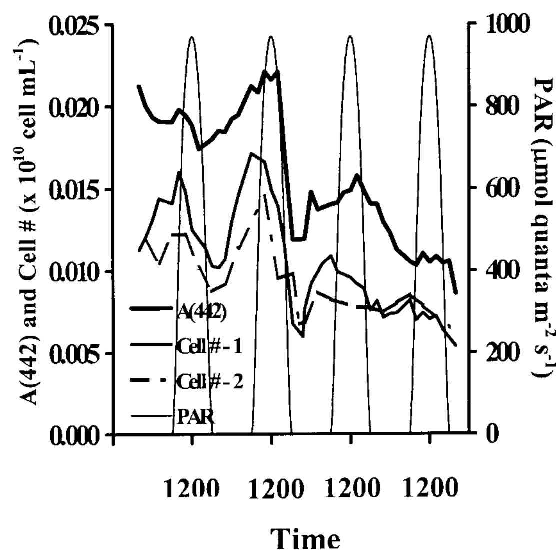

Figure 7. Temporal variations over 4 complete days of sampling in cyclostat 1 and 2 of *Prochlorococcus* cell number, and cyclostat 1 *A*(442) and PAR.

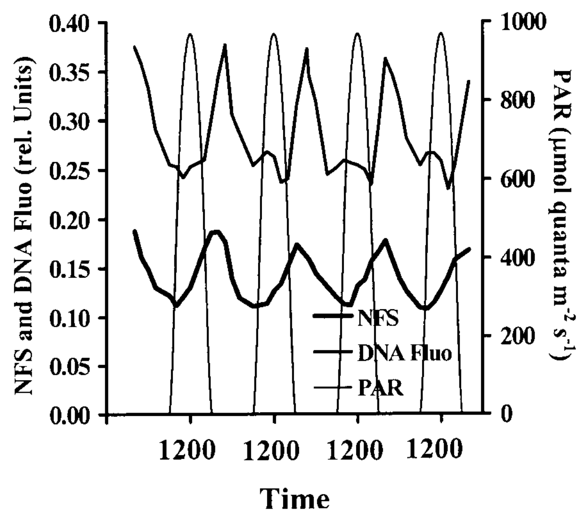

Figure 8. Temporal variations over 4 complete days of sampling in cyclostat 1 of *Prochlorococcus* NFS, DNA cellular content, and PAR.

experiment, likely because of oversampling combined with excessive dilution to maintain the volume of the culture. Nevertheless, as will be shown below and detailed elsewhere, cells still divided at high rates and most cellular parameters (cell size, pigments, photosynthetic parameters) continued to show strong and reproducible diel patterns (see below). It is worth noting here the reproducibility between the two cyclostats in terms of cell number (Figure 7).

The fact that biomass indicators oscillates when phototrophs are submitted to a light-dark cycle was previously noted by Kroon et al. (1992). He suggested that continuous monitoring as well as continuous adjustment of biomass could not be conducted without ambiguity when a dynamic light regime is applied. In fact, the cyclic light regime induces cyclic variations in all biomass descriptors. Using such a biomass descriptor to continuously adjust dilution would obviously lead to cyclic variations in the dilution rate that may in turn eventually affect the biomass index. To avoid any such loop in the culture physiology and biomass control, dilution rate must be adjusted only once a day, and always at the same time of the cell cycle, based on some optical measurement or cell counts. Here, we adopted a continuous dilution strategy where dilution was adjusted (according to cell counts) at the dark to light transition. This strategy proved to be relatively efficient in terms of biomass and physiological stability at least during the pre-experiment phase as well as for the first 48 h of the extensive sampling.

The cyclostat remained perfectly axenic from inoculation until the beginning of the sampling period (15 days in all). During the 4-day experiment, presence of bacteria was extemporarily followed by flow cytometry. No bacteria were detected till 36 h after sampling start. After this time, a bacterial population, probably introduced during the intensive sampling, was systematically observed in cyclostat 1 (not determined for cyclostat 2) and its density slowly increased from less than 1.6% of the *Prochlorococcus* cell population at t = 38 h to ca. 5.6% at the end of the experiment (96 h).

Figure 8 shows diel variations in NFS and DNA cellular content observed during 4 days in order to illustrate the stability of the cyclostat presented here in physiological terms. In the *Prochlorococcus* size range, NFS depends on cell size (Ackleson & Spinrad, 1988), and on cellular carbon content as it depends on the refractive index (Stramski & Reynolds, 1993). Therefore the diel cycle in NFS was likely produced by 1) the accumulation of cellular carbon during the light period which resulted from photosynthetic activity, and 2) a decrease during the dark period due to cell division followed by the consumption of carbon by catabolism. The diel pattern of the average DNA content per *Prochlorococcus* cell was even more reproducible. It was characterized by a sharp increase starting just before the end of the light period and that

lasted 4 h. This increase corresponded to the DNA synthesis phase. It was followed by a strong decrease of DNA content due to cell division, which lasted approximately the same time as DNA synthesis.

Conclusion

This study has demonstrated the feasibility of maintaining the difficult-to-grow *Prochlorococcus* in large volume (10 L) axenic containers. Special care must be taken to avoid contamination during intensive sampling, as it apparently happened after two days in at least one of the cyclostats. We also recommend that the volume of sample taken each day should rather be slightly *below* than *at* the expected growth rate of the species, as oversampling may lead to dilution of the population. However, the latter may not be a major problem, since even at lower concentration, the population remained healthy, very well synchronised (Figure 8), and therefore usable to study a number of processes such as e.g. cell cycle, photosynthesis, gene expression, etc. The illumination system we designed is suitable for simulating any kind of natural irradiance variations in the aquatic environment. This system can be used to precisely simulate a natural light-dark cycle in order to study in detail the synchronization of the cell division as observed in the field. It can also be very useful to study the effects of clouds and vertical mixing as well as photoinhibition. The overall system is cheap and easy to deploy. Its main drawbacks are 1) the highly featured irradiance spectrum of the neon tube which does not match the solar spectrum, and 2) the quasi-absence of any ultraviolet radiation. Metal halide discharge lamps can be found with a spectrum that mimics much better the solar one. However, these lamps are expensive, never dimmable and produce much heat. Their use to generate smooth light variations implies the use of more complicated optical set up such as, for instance, venetian blinds. Other dimmable neon tubes with better spectral properties may exist or appear on the market.

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