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VARIABILITY OF PHOTOSYSTEM II QUANTUM YIELD AND RELATED PROCESSES IN *CHLORELLA PYRENOIDOSA* (CHLOROPHYTA) ACCLIMATED TO AN OSCILLATING LIGHT REGIME SIMULATING A MIXED PHOTIC ZONE¹

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ABSTRACT

The regulation of photosystem II (PSII)-related parameters was characterized for *Chlorella pyrenoidosa* Chick grown in continuous nutrient-replete culture and acclimated to a light field simulating fluctuations of a natural environment. The maximum quantum yield for charge separation at PSII showed a light-driven decrease during the first half of the photoperiod when incident irradiance (integrated hourly) was increasing to a midday maximum. This change was accompanied by a decrease in the effective cross-section for all PSII and the connectivity between PSII, while the cross-section for inactive centers increased. Furthermore, a decrease in the number of active PSII centers and the amount of Q_B bound to D1 became evident. During the second half of the day, as irradiance declined toward the end of the photoperiod, all the observed changes in PSII photochemistry were reversed. The low quantum yield values at the middle of the photoperiod returned to 85% of the value at the end of the photoperiod by the addition of far-red light, suggesting that a State I–II–I transition took place during the day. Measured rates of oxygen production correlated well with fluorescence-derived estimates of photosynthesis. The overall results suggest that PSII photochemistry is optimized to minimize the susceptibility to photoinhibition and to allow balanced growth under fluctuating irradiation and that the impact of these alterations on primary production can be adequately monitored through bio-optical measurements alone.

Key index words: *Chlorella pyrenoidosa*; dynamic light regimes; fluorescence; photosystem II regulation; primary production; simulated mixed photic zone; variable quantum yield

Irradiance fluctuations in many highly productive regions of oceans and lakes, as well as biotechnologically valuable algal mass cultures, take place on a time scale of less than 1 h (Kirk 1983). In these environments, where phytoplankton can be exposed for short periods of time to high and low irradiances due to mixing, overall growth often is light-limited. Photosynthetic adaptation to changes of irradiance over time scales of hours to days are fairly well described: it might proceed through structural changes in cellular characteristics, like the size and number of photosynthetic units or the amount of photosynthetic electron flow components (Falkowski 1981, Prézélin 1981). It is unlikely that biosynthetic mech-

anisms would provide the basis for optimal photosynthetic performance under short-term changes in irradiance. Fast-responding and energetically inexpensive functional adjustments can stabilize and optimize photosynthesis under these conditions. The fact that linear electron transport starts with the formation of stable charge separations at photosystem II (PSII; Table 1 summarizes significant symbols) makes its function a prime target for regulation. For example, the effective cross-section of PSII (the product of quantum yield for charge separation and optical cross-section) should be large during low light exposure to allow optimal rates of photosynthesis. But the D1 protein in PSII, without which linear electron transport cannot proceed, is known for its susceptibility to be damaged by high photon fluxes. Hence, a fast responding and tight regulation of PSII activity is needed to allow efficient growth of phytoplankton in an environment where irradiance is fluctuating between limiting and potentially inhibiting intensities. The regulation may alter the light-harvesting function or alter the rate of electrons that are donated by PSII to the plastoquinone pool. Light harvesting depends on pigments that are bound to a number of proteins within a PSII. Transfer of energy may proceed to the reaction center or between pigment molecules that structurally belong to different PSII (connectivity). Changes in connectivity will influence light harvesting without structural adaptation by functionally modifying the size of the antennae that transfer energy to a reaction center. If high light exposure causes disproportionate excitation of PSII relative to PSI, then redistribution of absorbed energy from PSII to PSI (State I–II transition) may restore the balance and increase ATP phosphorylation by cyclic PSI activity. Regulation of the rate of electron transfer from PSII to the plastoquinone pool may occur as a result of structural damage and repair to PSII or as a result of changes in the redox state of the plastoquinone pool under high irradiance exposure. A low redox potential of the plastoquinone pool will slow down the rate of electrons accepted from PSII and may be related to a decreased sensitivity for photoinhibition (Adir et al. 1990). Both mechanisms, regulation at the level of light harvesting or related to electron transfer from PSII to the plastoquinone pool, simply are the combined result of exposure to light and the structural composition of the photosynthetic machinery.

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TABLE 1. *Symbol definitions.*

PAM	Pulse amplitude modulated.
PSII, PSI	Photosystem II, photosystem I.
Q_A	Quinone A, first stable electron acceptor of PSII.
Q_B	Quinone B, second stable electron acceptor of PSII.
F	Fluorescence level. Subscripts o, i, d, and p represent the fluorescence level during light induction at the origin, intermediate peak, dip, and plateau levels, respectively. Subscripts v, s, and m indicate the variable, steady-state, and maximum levels, respectively. If the symbol is followed by a prime, the measurement was made in the presence of actinic illumination.
k	Kinetic rate constant. Subscripts o-i, p, d, f, and t represent the rate constant for the rise from F_o to F_i , photochemistry, radiationless energy dissipation, fluorescence, and energy transfer from PSII to PSI, respectively.
k_{o-i}	Kinetic rate constant for the rise from F_o to F_i .
q_p	Photochemical quenching.
$\phi_{II,c}$	Quantum yield for electron transport generated at PSII. If denoted with a superscript o, the symbol represents the quantum yield with all reaction centers in an open state.
Ant	Normalized complementary area above the induction curve in the presence of DCMU at time t.
Am	Fraction of PSII with α -type characteristics (core antennae plus peripheral light-harvesting complex) and rate constant $k\alpha$.
Bm	Fraction of PSII with β -type characteristics (core antennae only) and rate constant $k\beta$.
P	Connectivity between PSII units.
ω	Quantum yield for electron transfer from closed PSII to open PSII.
A	Fraction of PSII characterized by a fast fluorescence decay after a single turnover saturation flash at a rate of K_a .
B	Fraction of PSII characterized by a slow fluorescence decay after a single turnover saturation flash at a rate of K_b .
P-I	Photosynthesis versus irradiance.
α	Light-limited slope of P-I curve.
lk	Light level at which photosynthesis saturates.
τ	Minimum turnover time for linear electron transport.
P_{max}	Maximum rate of photosynthesis.
σ_{PSII}	Effective cross-section of PSII.
FR	Far-red light.
Γ	Electron yield, mole oxygen per mole electron.
f_{II}	Fraction of absorbed light funneled to PSII.
Di	Constant needed to attain uniform dimensions.
k_c	Spectral averaged absorption coefficient.

The natural variability of quantum yields in aquatic ecosystems plays an incisive role in the precision at which predictions can be made of the magnitude of primary production. This variability has been documented (e.g. see Schofield et al. 1993) and is related to chromatic adaptation (Kroon et al. 1993), nutrient starvation (Geider et al. 1993), and fluctuations in solar irradiance (Henley et al. 1991, Hanelt 1993). The introduction of fluorescence-based techniques opens the possibility to better document the *in situ* values of the quantum yield (Kolber and Falkowski 1993), yet at the same time it demon-

strates the current lack of knowledge in answering why, how, and to what extent environmental variables such as light or nutrients induce changes in the quantum yield. The present study is based on the premise that algal cells adapted to a light:dark cycle with continuous fluctuations in irradiance during the light period will display the photosynthetic regulatory processes by which balanced energy conversion and optimal photochemistry are achieved in natural populations under nutrient-replete conditions. Unlike information derived from natural populations, irradiance was the only experimental variable, and the data exemplify the possible impact of light-driven changes in PSII-related parameters. In previous reports using a similar experimental approach (Kroon et al. 1992a, b) we investigated the effect of dynamic light regimes on optical properties, the quantum requirement for oxygen, and photosynthesis-irradiance (P-I) relationships. We showed that they vary drastically during a dynamic light regime even though overall growth rates seem to be unaffected by the light regime. While these experiments revealed the effects of dynamic light regimes on photosynthetic characteristics (and their limited interpretive value; see also Henley 1993), they did not reveal the mechanisms responsible for the observed changes. The present study aims to reveal the regulatory processes related to PSII activity and structure in *Chlorella pyrenoidosa* grown in a continuous culture and adapted to a dynamic light regime. We used pulsed amplitude-modulated (PAM; see Schreiber et al. 1986) fluorescence measurements to derive information on PSII antennae size and activity, amount of active PSII, quantum yield for charge separation, state transitions, and electron transfer rates between Q_A and Q_B . Oxygenic photosynthesis was characterized in terms of maximum production (P_{max}), light-limited efficiency (α), and the minimum time required for complete linear electron transport (τ). We also tested the robustness of fluorescence-derived estimates of photosynthetic rates to predict rates of oxygen production of *C. pyrenoidosa* observed over the light period. A hypothetical sequence of PSII-related events is proposed that allows protection against photoinhibition and that allows for efficient overall growth under fluctuating irradiance conditions.

MATERIALS AND METHODS

A continuous turbidostat culture (10:14 h LD; fixed dilution rate of 0.8 d⁻¹) of *Chlorella pyrenoidosa* Chick was grown under an oscillating photoperiod on a mineral medium (Van Liere and Mur 1978) containing excess nutrients. The pH of the culture remained constant at 7.8 $\pm$ 0.1 as a result of the bicarbonate buffer in the medium and the supply of carbon dioxide from pressurized air as small air bubbles at a rate of 150 L·h⁻¹. The air flow also provided intimate mixing. The temperature was kept constant at 20.0° $\pm$ 0.1° C by a water jacket (23.7 $\times$ 43.5 $\times$ 1.5 cm) arranged between the light source and the rectangular culture vessel (23.7 $\times$ 45.0 cm and a thickness of 3.0 cm). The oscillations in photon flux density (PPFD) resulted from three functions: a sine function with a period of 10 h (reflecting changes

in solar irradiation), a sine function with a period of 1 h (reflecting the position of a hypothetical layer in a water column that circulates through depth), and a linear function (which determines the PFD at a given depth). The total daily light dose incident on the culture was $10.5 \text{ mol} \cdot \text{m}^{-2} \cdot \text{d}^{-1}$. The optical density of the culture reduced the average irradiance inside the culture vessel to 87% of the incident value. Changes in growth irradiance (Osram Metalogen HMI 1200 W/GS) were computer-controlled by angular slit displacement of a venetian blind that was arranged between the light source and the culture unit. A detailed description of the culture unit is presented elsewhere (Kroon et al. 1992c). The photosynthetic PFD impinging on the surface of the culture vessel was continuously measured with a Licor Li-190SB quantum sensor, the output of which was recorded with a chart recorder. The interaction of the growth-light source with the venetian blind caused an unequal distribution of light at the surface of the culture vessel. The measured PFD was corrected to yield the surface-averaged value.

After a 30 min hydrolysis of freshly collected cells in 0.1 M NaOH (1 h, 100°C) protein concentration was determined according to the Folin method (Herbert et al. 1971) using bovine serum albumin as a standard. After a 30 min acid hydrolysis (1 M HCl, 100°C) glucose concentration (in the hydrolysate from starch) was determined using the GOD-perid-method of the Boehringer test combination (Werner et al. 1970). Pigments were analyzed by high-performance liquid chromatography (HPLC) on an ODS-hypersil column ($250 \times 4.6 \text{ mm}$) with $5\text{-}\mu\text{m}$ particles. After extraction for 30 min in 100% methanol saturated with magnesium carbonate and centrifugation of the extract, 300 mL of ionpairing reagents (tetrabutyl ammonium acetate [1.5% w/v] and ammonium acetate [7.7% w/v]) was added to 1 mL of supernatant 3 min prior to injecting into the HPLC. Gradient elution ($1.5 \text{ mL} \cdot \text{min}^{-1}$) was achieved by linearly mixing phase A (90% methanol, 10% water, and 1% [v/v] of ionpairing reagents) with phase B (80% methanol, 20% acetone) in 15 min and subsequent elution with phase B for 16 min. Peak areas were used to calculate pigment concentrations. Protein, glucose, and pigments were determined in duplicate. Whole-cell *in vivo* absorption spectra ($400\text{--}750 \text{ nm}$; 0.35 nm resolution) of *C. pyrenoidosa* were measured with an Aminco DW2000 spectrophotometer with the cuvette placed directly in front of the photomultiplier. Stirring prevented settling of the cells. Spectral averaged absorption coefficients were calculated for the light source used as in Kroon et al. (1992a).

Room temperature chlorophyll *a* fluorescence measurements were made with a PAM 101 fluorometer (Heinz Walz, Effeltrich, Germany). Details of PAM fluorometry can be found in Schreiber et al. (1986). The applied fluorescence nomenclature is according to Van Kooten and Snel (1990). PSII fluorescence is detected by a low-intensity, pulsed measuring beam emanating from a light-emitting diode (peak wavelength 650 nm , half-bandwidth 20 nm ; frequency of 1.6 or 100 kHz). The measuring beam alone ($<0.3 \mu\text{mol} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$) did not induce variable fluorescence (F_v). A four-arm fiber optic transduced the measuring beam and additional actinic illumination to the sample and collected the fluorescence signals from the sample. During *in situ* pulse saturation measurements, the fiber optic was placed directly against one side of the culture vessel at a 90° angle to the growth-light source and saturation pulses of 500 ms duration (Schott KL-1500; $12,000 \mu\text{mol} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$ white light) were administered every 150 s . When previously dark-adapted cells are light-exposed, their fluorescence intensity will increase from a minimum level F_o (Q_A fully oxidized, i.e. all PSII reaction centers open) to a maximum level F_m (Q_A transiently fully reduced, i.e. all PSII reaction centers closed) upon illumination with a saturating light pulse. Any increase from F_o to F_m is termed variable fluorescence, or F_v . In the continued presence of light, a steady-state level F_s is reached after several minutes. Administering a saturating light pulse at F_s will increase F_s to F_m' , which is generally lower than F_m . The difference between F_m' and F_m results from energy dissipation processes other

than photochemistry (Bradbury and Baker 1981, Schreiber et al. 1986). From the relative changes in F_m' and F_s , the amount of photochemical quenching (q_P) can be calculated with Eq. 1 (Van Kooten and Snel, 1990):

$$q_P = \frac{F_m' - F_s}{F_m' - F_o} \quad (1)$$

The efficiency of photon trapping by open PSII is calculated according to

$$\phi_{exc}^0 = \frac{F_m - F_o}{F_m'} \quad (2)$$

The apparent quantum yield of PSII for charge separation ($\phi_{II,e}$) now follows by definition from Eqs. 1 and 2 (Genty et al. 1989, see also Havaux et al. 1991):

$$\phi_{II,e} = q_P \cdot \phi_{exc}^0 = \frac{F_m' - F_s}{F_m'} \quad (3)$$

and the maximum quantum yield for charge separation is

$$\phi_{II,e}^0 = \frac{F_m - F_o}{F_m} \quad (4)$$

Fluorescence induction measurements were made on samples ($4 \mu\text{g}$ total chlorophyll) that had been dark-adapted for 15 min in the presence or absence of DCMU ($20 \mu\text{M}$ final concentration) and then dark-filtered on Whatman GF/F filters immediately prior to the measurement. An example of the applied analysis (based on Owens 1986) is presented in Figure 1. Cells that have been dark-adapted in the presence of DCMU displayed a fast fluorescence rise upon illumination because electron transport between the primary stable electron acceptor Q_A and the secondary acceptor Q_B has been completely blocked (Fig. 1A). The normalized area (with respect to the total area) above the induction curve (Ant) is a direct measure for the fraction of reduced Q_A (Melis and Schreiber 1979). The rise in Ant usually is biphasic and is represented by Eq. 5 (Melis and Homann 1975, Owens 1986):

$$\text{Ant} = \text{Am}(1 - e^{-k\alpha t}) + \text{Bm}(1 - e^{-k\beta t}), \quad (5)$$

where Am is fraction of PSII_a (PSII with core and peripheral antennae chlorophylls) with rate constant $k\alpha$, and Bm is fraction PSII_b (PSII only with core antennae) with rate constant $k\beta$. The rate constant $k\alpha$ is severalfold larger than $k\beta$. As a result, the contribution of PSII_a centers to the rise in Ant will be negligible at some time after the start of the fluorescence induction (indicated by the inflection of the curve in Fig. 1B). The exponent containing $k\alpha$ in Eq. 5 will reduce to 0 if the PSII_a contribution to the Ant increase is negligible. Subsequently, Bm and $k\beta$ can be determined from Eq. 6 (after substituting $\text{Am} = 1 - \text{Bm}$ in Eq. 5):

$$\text{Ant} = 1 - \text{Bm} \cdot e^{-k\beta t} \quad (6)$$

Connectivity (P) between PSII units is derived from $F_{v,t}$ and Ant using Eq. 7 (Hipkins 1978):

$$F_{v,t} = \frac{(1 - P) \cdot [\text{Ant}]}{1 - P \cdot [\text{Ant}]} \quad (7)$$

$$P = \phi_{II,e}^0 \cdot \omega \quad (7a)$$

where ω is the energy transfer yield from a closed to an open reaction center (Fig. 1G, H). DCMU induction curves were made in triplicate, and the reproducibility of derived parameters was within 5%.

Fluorescence induction after dark adaptation without DCMU proceeds from the constant fluorescence F_o , to an intermediate peak (F_i), to a lower level, or dip (F_d), and finally to the maximum fluorescence, or peak level (F_p). Inactive PSII centers are impaired in their ability to reduce plastoquinone on a fast time scale (Graan and Ort 1986). The initial very fast increase from F_o to F_i probably

FIG. 1. The complete analyses of a DCMU induction curve measured at 0800. After normalizing the variable fluorescence (A), the natural logarithm of the complementary area (which is a measure of the fraction of closed reaction centers) is determined during the induction period (B). The two dashed lines in B indicate the limits that were set to perform linear regression analysis from which the amplitude and rate constant of the β -phase were derived. The first derivative of B is shown C, which was used to ascertain the linearity of the β -phase (a constant value of the derivative). In the next step, the contribution of the β -phase is subtracted from A and the remaining fluorescence is normalized (D). After a second natural logarithmic transformation, the kinetics of the α -phase can be determined (E). If curve E was non-linear, the initial and final rate constants of the α -kinetics were determined (indicated by the tangent of the dashed lines). Connectivity (P) was investigated by plotting the complementary area versus the normalized fluorescence before (F) and after (G) subtraction of the β -phase. The 1:1 lines in (F) and (G) indicate the relation between Ant and $F_{v,t}$ without connectivity ($P = 0$). In that case, $F_{v,t}$ would be a direct measure for the fraction of closed reaction centers. Values for connectivity were derived by application of Eq. 7 to the data of curve (G) and P was taken as the average of the data in H that correspond to data in G of normalized variable fluorescence ranging from 0.2 to 0.8. A biphasic curve such as that shown in B was only found at 0800, 0900, and 1800. The sequence of the analyses applied to all other induction curves proceeded in the following order: A–E–G–H.

FIG. 2. An example of the decay of fluorescence in relative units after a single-turnover flash in the absence of DCMU for samples taken at 1200 and 1600. The traces are the average of 16 individual decay profiles. Deconvolution was performed by a commercially available nonlinear fitting program. The derived values for the amplitudes and rate constants of the fast (A, Ka) and slow (B, Kb) components are presented in the figure.

originates from those inactive centers (Cao and Govindjee 1990). Very shortly after the addition of light during the F_0 to F_i rise, the rate of closure exceeds the capacity of inactive centers to transfer electrons from Q_A^- to the plastoquinone pool. Following F_i , the rate of plastoquinone reduction by inactive centers is not negligible, and fluorescence decreases slightly to F_d . At the same time and for the rest of the induction period, the plastoquinone pool progressively reduces, which results in a temporary closure of all reaction centers and an increase to F_p . Both F_i and F_p were transient fluorescence maxima, induced by closed reaction centers of inactive and all PSII, respectively (Melis 1985, Cao and Govindjee 1990). During these maxima, the nonlinearity between variable fluorescence and the fraction of reduced Q_A due to connectivity disappears (see Eq. 7). Thus, the ratio of $(F_i - F_0)$ and $(F_p - F_0)$ represents the fraction-inactive PSII (Guenther et al. 1988). Even though DCMU was absent, the fluorescence rise from F_0 to F_i reflects closure of Q_A from inactive PSII and may be subjected to the analysis that was used for DCMU-treated samples. Kinetic analysis of the F_0 to F_i rise with Eq. 5 determines the rate constant k_{a-i} , which is an approximate measure for the effective cross-section for inactive PSII centers. Fluorescence inductions without DCMU were made in duplicate, and the reproducibility of derived parameters was within 5%.

Fluorescence decay was measured in the absence of DCMU at 17 μ s resolution during 10 ms, starting 120 μ s after the firing of a single-turnover flash (General Electric FT-230 flash tube, 5 μ s half-width, discharge voltage 1.4 kV). The duration of the flash is too short to excite reaction centers more than once, and its high intensity provides photons for all reaction centers. To reduce the signal-to-noise ratio, we averaged 16 replicate decay curve measurements (Fig. 2). The interval between flashes was 20 s. A pulse generator triggered the actinic flashes and started data acquisition by the PAM 101. During the dark period following a saturating single-turnover flash, fluorescence will decay to F_0 . The decay kinetics reflect the re-oxidation of Q_A^- by Q_B or Q_B^- . Complete Q_A^- re-oxidation can only be described by a triphasic exponential decay function. However, during the first 10 ms, the decay could be accurately described by omitting the slowest decay component and applying a biphasic function:

$$F_v = A \cdot e^{-K_a \cdot t} + B \cdot e^{-K_b \cdot t} + F_0 \quad (8)$$

where A and B are the amplitudes and Ka and Kb the decay rate constants for the fast and slow phase, respectively.

P-I curves were performed as described by Dubinsky et al. (1987), with one slight modification. The outputs from the oxygen electrode and the light sensor were connected to a 12-bits A/D converter. Each point of the P-I curve was determined

FIG. 3. The protein (▲), glucose (●), and chlorophyll ($a + b$) (○) concentration [$\text{mg} \cdot \text{L}^{-1}$] during an oscillating photoperiod. Data are the average of 2 consecutive days ($n = 4$). The range of deviation from replicates relative to the average values over the photoperiod was 3.3% for chlorophyll, 5.6% for glucose, and 2.4% for protein.

during 2.5 min at increasing irradiances. Dark oxygen uptake was determined after 15 min adaptation in darkness prior to the P-I measurements. After completion of the P-I curve, a computer program determined the P-I parameters α , $P_{\max}$, and I_k (Kroon et al. 1992a). After 2.5 min incubation at each irradiance, three saturating pulses were administered in order to perform the fluorescence pulse saturation technique as described previously. Far-red illumination ($40 \mu\text{mol} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$) was provided by light from an Osram HLX 64640 slide projector, passing through a Schott PAL double-band interference filter (714 nm, 20 nm half-bandwidth). Oxygen flash yields were determined with single-turnover flashes (as used for fluorescence decay measurements) at frequencies between 2 and 14 s^{-1} with increments of 2 s^{-1} . The minimum turnover time for linear electron transport (τ) was calculated as the ratio of the oxygen flash yield and $P_{\max}$ obtained under continuous illumination.

RESULTS

Biomass and P-I parameters. The green alga *Chlorella pyrenoidosa* was grown under a light regime characterized by hourly reoccurring peak irradiances. The total daily-integrated light dose limited the growth rate to 0.8 d^{-1} . With the growth rate being less than 1 d^{-1} , synchronization of cell division and dominance of cell cycle events over the light: dark cycle did not determine the physiological response. Photosynthesis was light-limited during the start and toward the end of the photoperiod. The PFD between 1100 and 1600, if supplied during a P-I determination, was sufficiently high ($>250 \mu\text{mol} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$) to result in rates of oxygen evolution that were less than light-saturated rates of photosynthesis.

The volumetric protein and chlorophyll ($a + b$)

FIG. 4. The photosynthetic parameters α , $P_{\max}$, I_k , and τ determined on samples taken at hourly intervals during the photoperiod. Note the use of different y-axes for each parameter. The data are single point measurements, and the standard deviation inherent to the measurement is 10%.

concentration of the culture was not influenced by the large oscillations in PFD during the photoperiod (Fig. 3). This directly indicates balanced growth of the cells during the entire day. The amount of glucose that was detected in the hydrolysate of starch began to increase 2 h after the beginning of the light period, increased linearly over most of the photoperiod, and appeared to decline during the last hour of the photoperiod. The efficiency of light-limited photosynthesis (α), $P_{\max}$, I_k , and the minimum turnover time for linear electron transport were constant during the photoperiod (Fig. 4). Apparently, no extensive photodamage accumulated and photochemical activity was regulated during the photoperiod.

PSII quantum yield and electron transport. During the photoperiod, *in situ* F_m' values were always higher than F_s values, and both parameters showed symmetry over the day and within hourly PFD oscillations (Fig. 5A). F_s values were instantaneously and positively coupled to PFD. Exceptions to this generalization were observed between 1200 and 1400, when F_s values were more quenched than during the rest of the photoperiod. In contrast to F_s values, F_m' values tended to be inversely related to PFD and the relative dynamics in F_m' did not reflect the magnitude of the variations in PFD.

The daily pattern in $\phi_{II,e}$ as derived from F_s and F_m' (Eq. 3) showed that at any time during the photoperiod, $\phi_{II,e}$ is promptly and inversely coupled to PFD (Fig. 5B). At high PFD, $\phi_{II,e}$ is low, and vice versa. Similar to the changes in F_s and F_m' , the dynamics of $\phi_{II,e}$ were symmetrical within each oscillation and over the entire photoperiod. The noisiness of the data (Fig. 5) is the result of the interference of air bubbles that were used to achieve mixing of the culture.

PSII effective antennae size and connectivity. Fluorescence induction curves in the presence of DCMU (see example in Fig. 1) were made on samples taken every 30 min throughout the photoperiod, coincid-

FIG. 5. Fluorescence pulse saturation analysis made with the fiber optic placed directly against one side of the culture vessel at a 90° angle to the growth-light source. A) *In situ* changes in the fluorescence parameters in relative units as a result of the growth irradiance alone (F_m' , lower trace) or in combination with a saturating light pulse (F_m' , upper trace) over the photoperiod. The light pulses were repeated with an interval of 150 s. B) PSII quantum yield for charge separation, $\phi_{II,c}$.

FIG. 6. Changes in the A) initial, B) final, and C) ratio between final and rate constants of the fluorescence induction curves measured with DCMU. The final rate constant was determined in that part of the induction, where $\ln(1 - \text{Ant})$ equaled -4 (see Eqs. 5 and 6 and Fig. 1E). Values represent the mean of triplicate measurements, with standard deviations of 5–10%. D) The energy transfer yield from a closed to an open reaction center (ω , ●) and connectivity (P , ○). Both time series for P and ω are averaged values from 2 consecutive days.

ing with the beginning, middle, and end of each hourly oscillation in PFD. Kinetic analysis of the complementary area (Ant) rise during an induction (see Eq. 5) revealed the presence of PSII $_{\beta}$ only at the beginning (0800–0900) and end (1800) of the photoperiod. At these times, PSII $_{\beta}$ comprised only 5–15% of total PSII, and the values for k_{β} were about 5 s $^{-1}$. For the remaining part of the day, the Ant rise was analyzed with Eq. 5, without subtracting a β -phase, assuming the existence of PSII with α -type kinetics only. The rate constant (k_{α}) for the Ant rise, which is a relative measure for the effective cross-section of PSII (σ_{PSII}), appeared to continuously increase from the start to the end of each induction curve. The initial and final k_{α} values (Fig. 6A, B) varied over the photoperiod. In addition, the ratio of the (fast) final PSII closure relative to the (slow) rate at the start of the fluorescence induction varied during the photoperiod (Fig. 6C). The changes in the initial and final values for k_{α} can result from changes in quantum yield for photochemistry or from changes in the number of pigment molecules that transferred energy to the reaction center of PSII. A change in rate constants for processes that compete with photochemistry will induce a change in the quantum yield. The time needed to reach F_m in the presence of DCMU was around 75 ms under our experimental conditions. It is unlikely that rate constants for processes that compete with photochemistry would alter within this

time frame. We therefore interpreted the relative change in k_{α} during the DCMU induction as being a measure for the number of PSII units capable of transferring excitation energy between each other, assuming the existence of a common pigment bed shared by several PSII reaction centers (Melis and Anderson 1983). The data (Fig. 6A, B) indicate that σ_{PSII} varied as an inverse function of PFD over the photoperiod, being lowest at times of highest irradiances. Furthermore, our measurements suggest that the number of PSII units that functionally shared their antennae decreased at least 30% during the middle of the photoperiod (Fig. 6C).

The fact that a number of PSII units can functionally share their antennae point to the existence of a probability to transfer excitation energy between neighboring antennae domains. We may then group PSII units into clusters that are statistically able to share excitation energy between their antennae complexes. Changes in the statistical number of clustered PSII units should be closely related to changes in connectivity (P and ω ; see Eq. 7) or interunit excitation energy transfer. During the photoperiod, lowest P values were observed at highest growth irradiances (Fig. 6D). Parallel changes were found for ω . The absolute value for ω depends on the value of the rate constant for excitation energy transfer from a closed to an open reaction center, relative to the sum of rate constants for all other

FIG. 7. Fluorescence induction parameters without DCMU after addition of a saturating light flash to samples that were withdrawn from the culture vessel at 30-min intervals. The samples were allowed 15 min dark adaptation prior to the measurement. A) Changes in the fluorescence levels F_p , F_i , F_a , and F_o (from top to bottom). See Table 1 for explanation of symbols. B) Photoperiod-dependent changes in the maximum quantum yield for open reaction centers.

de-excitation processes, excluding the rate constant for exciton capture by open centers (Hipkins 1978). Thus, ω is influenced by spillover and radiationless excitation decay processes. The fact that ω was less than unity proves that energy transfer was restricted to a limited number of photosynthetic units.

Maximum PSII quantum yield and inactive PSII. F_o was relatively stable over the day, while F_i , F_a , and F_p varied as an inverse function of PFD (Fig. 7A). F_p was always equal or even higher than the F_m level obtained in the presence of DCMU (data not shown), implying that the Q_A pool was maximally reduced at F_p . The maximum quantum yield for charge separation, $\phi_{II,e}$, was therefore calculated using Eq. 4, with F_p substituting F_m . $\phi_{II,e}$ was not constant but also varied inversely to the light regime (Fig. 7B).

The fraction-inactive PSII was calculated from data in Figure 7A and was high during midday and slightly lower at the end of the photoperiod than at the beginning (Fig. 8A). The effective cross-section of inactive PSII units was twice as large at midday compared to the start of the day (Fig. 8B) and had an opposite dependency to PFD than the cross-section for total PSII.

Electron transfer between Q_A and Q_B . Fluorescence decay measurements after a single-turnover flash (see example in Fig. 2) were repeated every 15 min during the photoperiod. Equation 8 was used to deconvolute the observed fluorescence decay kinetics within the 10-ms range during which the decay was

FIG. 8. The fluorescence induction curves in the absence of DCMU were used to derive A) the fractions of inactive PSII over the photoperiod and B) the rate constants of the F_i increase during the photoperiod. The standard deviations were within 5–10% of mean values. Fluorescence decay profiles were made at 15-min intervals over the photoperiod. C) Changes in the ratio of the amplitude of the fast and slow fluorescence decay component. The vertical dashed lines separate the hourly oscillations of the growth irradiance. D) Changes in the fast fluorescence decay rate constant (see Eq. 8) over the photoperiod. The error in the rate constants and amplitudes was generally within 5% of the mean of duplicate measurements.

monitored. The ratio of the fast and slow component was inversely related to the hourly light dose (Fig. 8C). The component with slow Q_A reoxidation kinetics dominated between 1030 and 1500, whereas the fast component dominated at the start and end of the photoperiod. Within the hourly irradiance oscillations between 0900 and 1600, the ratio was maximal at 15 min after the start of an oscillation and minimal 15 min before the start of the next oscillation. The changes in the relative abundance of both decay components, as reflected by their amplitudes in the decay curves, occurred independently of each other over the day (data not shown). From the onset of the light period, K_a decreased drastically from 2.7 to 1.2 ms^{-1} at 1100, then remained constant and increased during the last 3 h of the day to 1.8 ms^{-1} (Fig. 8D). The values for K_b oscillated around 60 s^{-1} from 1000 up to the end of the photoperiod after reaching a temporal highest value of 90 s^{-1} at 0900 (data not shown). The fast component reflects the reoxidation kinetics of Q_A when Q_B was bound in the reaction center prior to the flash. The slow kinetics reflect the time needed for the return of Q_B to the binding site after the flash (Cao and Govindjee 1990) or may reflect the existence of isolated quinone domains with a low ratio of plastoquinone per PSII (Joliot et al. 1992, Lavergne et al. 1992). In either case, the fluorescence decay measurements indicate that the fraction of reaction centers with an occupied Q_B site is vari-

TABLE 2. Effect of FR treatment. Fluorescence induction parameters were obtained after 15 min dark adaptation (–FR) or in the presence of 714 nm illumination (+FR), which is preferentially absorbed by PSI. Duplicate measurements were performed during the photoperiod at 1300, at 1400, and at the end of it at 1800. The data were reproducible within 5%. The columns headed by the % symbol contain the percentile difference of the +FR relative to the –FR treatment. Symbols and calculations are explained in Table 1 and the text.

	1300			1400			1800		
	–FR	+FR	%	–FR	+FR	%	–FR	+FR	%
F_o	0.218	0.313	11.4	0.271	0.303	11.8	0.237	0.232	–2.1
F_i	0.420	0.557	32.6	0.398	0.529	32.9	0.474	0.430	–9.3
F_p	0.538	0.802	49.1	0.514	0.763	32.6	0.841	0.801	–4.8
$\phi_{II,e}^o$	0.480	0.610	27.1	0.470	0.600	27.7	0.720	0.710	–1.4
PSII _{inactive}	0.540	0.500	–7.4	0.520	0.490	–5.8	0.390	0.350	–10.3
k_{o-i}	341	421	23.5	326	389	19.3	199	175	–12.1

able during the photoperiod such that more centers have an empty site during the highest irradiance exposure.

Effect of far-red light exposure. If the observed changes in the fluorescence parameters (Figs. 5–8) are influenced by State I–II–I transition during the photoperiod, then it should be possible to reverse the observed changes with PSI light within several minutes. Therefore, we investigated the effect of far-red (FR) illumination on the fluorescence parameters in the absence of DCMU (Table 2). If the FR treatment induces a State II–I transition, then we expect an increase in the maximum variable fluorescence and an increase in the PSII cross-section. The effect of FR illumination compared to a dark incubation was similar for samples withdrawn at midday. After FR illumination, they showed a relatively higher increase in F_p compared to F_o , leading to a higher $\phi_{II,e}^o$ compared to the dark incubation. FR light restored $\phi_{II,e}^o$ values at midday to 85% of its value at the end of the photoperiod. The induction of a State I with FR illumination led to a small decrease in the fraction of inactive PSII centers, which acquired a higher cross-section as indicated by an increase in k_{o-i} . FR illumination did not significantly alter F_o , F_i , F_p , and $\phi_{II,e}^o$ at 1800. In con-

trast to the midday samples, the effective cross-section for inactive PSII units now decreased due to the FR treatment.

Fluorescence yield and oxygen evolution. At this stage of the experiments, it is clear that variable fluorescence yield is drastically quenched over the photoperiod. Results indicate a dynamic dissipation of absorbed energy by processes other than photochemistry. Photochemical processes are responsible for decreasing quantum yields for light-limited oxygen production. The progressive closing of PSII reaction centers as PFD increases will cause the quantum yield for oxygen production to decrease. Equation 9, however, should hold regardless of the redox state of PSII:

$$\frac{d}{dt} O_2 = \Gamma \cdot \frac{d}{dt} e^-, \quad (9)$$

where Γ represents the electron yield [oxygen per electron]. The rate of electrons generated by PSII can be written more extensively and when substituted in Eq. 9 gives (Kroon et al. 1993)

$$\frac{d}{dt} O_2 = \Gamma \cdot \phi_{II,e} \cdot PFD \cdot k_c \cdot f_{II} \cdot D_i, \quad (10)$$

where k_c is the spectral averaged absorption coefficient [cm^{-1}], f_{II} [–] is the fraction of absorbed light directly funneled to PSII, and D_i is a constant needed to attain uniform dimensions (equal to 11.52).

Simultaneous measurements of the oxygen production rate over a range of PFD and $\phi_{II,e}$ values allowed us to determine the correlation between these parameters (Table 3). The ratio between the rate of oxygen production and $PFD \cdot \phi_{II,e} \cdot k_c$ is constant during the day ($\Gamma \cdot f_{II}$, last column of Table 2). The total average of $\Gamma \cdot f_{II}$ over the entire photoperiod is 0.170 with a standard deviation of 0.011.

DISCUSSION AND CONCLUSIONS

To investigate the presence and capability of photochemical mechanisms that regulate photosynthesis, measurable parameters must be acquired on time scales shorter than observable changes in photosynthesis (10–30 min). Any change in the composition of PSII, in the relative activity of PSI and PSII, or in metabolic activity of the organism exerting an effect on the redox state or adenylate energy charge,

TABLE 3. Correlation between fluorescence and oxygen production. Linear regression analysis was performed for oxygen production rates as a function of $PFD \cdot \phi_{II,e}$ during the photoperiod. The second column shows the inverse value of the gradient (grad^{-1}); the third column, the standard deviation (SD); the fourth column, the correlation coefficient R^2 . The spectrally averaged absorption coefficient k_c (cm^{-1}) is shown in the fifth column. Oxygen production rates were measured as $\text{mg O}_2 \cdot \text{L}^{-1} \cdot \text{h}^{-1}$. The resulting D_i was 11.52, which was used (see Eq. 9) to calculate $\Gamma \cdot f_{II}$ (last column).

Local time	Grad ⁻¹	SD	R ²	k_c	$\Gamma \cdot f_{II}$
0800	5.28	0.10	0.995	0.0992	0.166
0900	5.83	0.16	0.990	0.0991	0.150
1000	4.75	0.14	0.989	0.0967	0.189
1100	4.83	0.19	0.985	0.0991	0.181
1200	5.25	0.18	0.981	0.1045	0.158
1300	5.27	0.16	0.984	0.0987	0.167
1400	5.34	0.15	0.988	0.0984	0.165
1500	5.15	0.13	0.991	0.0966	0.174
1600	4.98	0.13	0.991	0.1001	0.174
1700	4.82	0.15	0.986	0.0985	0.183
1800	5.26	0.09	0.996	0.0978	0.169

is likely to influence the chlorophyll *a* fluorescence properties immediately (Krause and Weis 1991). One main determinant for the magnitude of phytoplankton fluorescence signals under field conditions is irradiance, which is influenced by the solar incident irradiance, optical characteristics, and mixing of the water column. Due to its nonintrusive character and ability to provide information on the activity and composition of the photochemical system on a short time scale, fluorescence measurements are indispensable in studies aiming to elucidate the effect of rapidly changing irradiance on algal physiology. This study reports effects on PSII-related processes that are exclusively driven by changes in irradiance for a *Chlorella* culture that was adapted to a dynamic light regime.

It appears that the growth irradiance never accomplished a complete closure of PSII. This is evidenced by the observation that F_s was always less than F_m' (Fig. 5A). The quantum yield for electron transport of PSII declined at increasing irradiances (Fig. 5B). The values for F_s , F_m' , and $\phi_{II,e}$ responded instantaneously to changes in the growth irradiance and were symmetrical within each hourly oscillation and over the photoperiod. We suggest that the immediate response and the symmetry indicate that repair processes exceeded the rate of photodamage and that photoinhibition (characterized by the condition when the rate of damage exceeds the rate of repair) had no major effect on the rate of photochemistry. Photoinhibition would decrease the number of components that perform photochemistry. As it would take time to restore damaged photochemical components, the changes in $\phi_{II,e}$ would lag behind changes in growth irradiance. Further support for a minor impact of photoinhibition is given by the finding that none of the photosynthetic parameters α , P_{max} , I_k , or τ or carbohydrate/protein levels were negatively influenced by the dynamic light regime (Figs. 3, 4).

During the main part of the photoperiod, our analysis of the complementary area growth in the presence of DCMU did not reveal the existence of PSII _{β} . PSII _{β} centers are characterized by the absence of peripheral light-harvesting complexes (while having the core antenna) and incapability of intrasystem energy transfer and are postulated to be located mainly in the nonappressed regions of the stroma thylakoids (Melis and Homann 1975, Thielen and van Gorkom 1981, Melis and Anderson 1983). Small contributions of PSII _{β} kinetics in *Chlorella* were reported earlier (Saito et al. 1983), and the kinetics were suggested not to be properties of structural components but, rather, reflections of differences in intrasystem energy transfer (Kyle et al. 1982). We did observe major changes in the relative abundance of inactive PSII (Fig. 8A). Our results do not support the view that inactive PSII would be equivalent to PSII _{β} (Melis 1985) or that inactive PSII would be a fraction of PSII _{β} (Guenther et al. 1988).

To discuss which PFD-driven mechanisms led to

the observed changes in the quantum yield, it is instructive to consider why quantum yields for PSII would change. As a matter of fact, the PSII quantum yield is a ratio of probabilities, or rate constants, for the process of photochemistry relative to all possible energy-dissipating processes: photochemistry (k_p), radiationless energy dissipation (k_d), fluorescence (k_f), and transfer of energy from PSII to PSI (k_t). Any increase in the rate constant of photochemistry-competing processes will lower the PSII quantum yield. The relationship between k_d and zeaxanthin content has been reviewed in depth by Demmig-Adams (1990), and zeaxanthin itself, which increases under high light conditions (Schubert et al. 1994), is suggested to dissipate excessive energy. The light-driven changes of PSII quantum yields shown here indicate that k_p (via xanthophyll-cycle pigments), k_t (via state transitions), or both were controlled by the light regime. However, FR light is known to induce a State I, and, thus far, no effect of FR light on the xanthophyll cycle has been described. The value for $\phi_{II,e}$ was an inverse function of PFD (Fig. 7B), and its dynamics were largely driven by changes in F_p (Fig. 7A). Induction of State I in midday samples with FR light increased $\phi_{II,e}$ to 85% of the value at the end of the day (Table 2), and the incomplete reversal may indicate the impact of energy dissipation through components of the xanthophyll cycle. The fact that F_o values were constant during the photoperiod does not exclude the occurrence of state transitions. Even when state transitions are the only cause for changes in fluorescence, the change in F_o is less than a third of the change in F_m for *Chlorella* (Saito et al. 1983), and we cannot exclude other mechanisms that would lead to the observed constant values for F_o . Changes in connectivity between PSII complexes (Fig. 6D) coincided with the F_p dynamics, thus indicating lower PSII connectivity in State II than in State I (Kyle et al. 1982). The decrease in F_p was accompanied with a decrease of ω , which is in agreement that a State II increased the probability of direct energy spillover from PSII to PSI. The State II–I transition required both ATP and oxidized intersystem electron carriers (Bulte et al. 1990). This is evidenced by the fact that State I was present at the beginning and end of the photoperiod and that *Chlorella* cells remained in State II after 15 min dark incubation (Table 2) but transformed into State I after FR light incubation. Therefore, I suggest that a State I–II transition occurred from the start of the day to midday and a State II–I from midday to the end of it.

According to a model proposed by Adir et al. (1990), light exposure of PSII will lead to conformational changes of the D1 protein, a major PSII component that contains the binding site of Q_B and turns over continuously in the presence of light (Mattoo and Edelman 1987). The model predicts that, depending on the intensity of light exposure, the D1 modification is reversible (a) or irreversible (b). In the latter case, the result is a translocation of

FIG. 9. Summary of observed changes in *C. pyrenoidosa* that was adapted to an oscillating light regime. The middle three columns are located underneath the PFD graph at positions relative to the local time, for which the given values were determined (symbols explained in Table 1). a, data from Table 2. b, data from Figure 7B. c, values calculated from Figure 6A. The effective cross-section for PSII is equivalent to the initial rate constant for the fluorescence increase in the presence of DCMU. The value at the start of the photoperiod is set to 100%. d, values calculated from Figure 8B. The effective cross-section for inactive PSII is equivalent to the rate constant for the fluorescence increase from F_0 to F_t . The value at the start of the photoperiod is set to 100%. e, values calculated from Figure 8A. f, values calculated from Figure 8C. The amount of Q_B bound to the D1 protein at the start of the photoperiod is set to 100%. g, data from Figure 6D.

the damaged reaction center to the stroma thylakoids, replacement of D1 by a precursor, and a translocation back to the grana thylakoids. In this scenario, stages (a) and (b) would be under control of electron flow via Q_B . During the highest growth irradiances in our experimental setup, it is likely that *Chlorella* cells would be susceptible to photodamage. An empty Q_B site is proposed to provide protection against the initial photoinhibitory stages (Adir et al. 1990). In this study, the reversible increase in inactive PSII centers (Fig. 8A) and the relative increase in the slow fluorescence decay component (Fig. 8C) indicate that a larger fraction of PSII reaction centers had no Q_B bound during the middle part of the photoperiod. Despite a larger effective cross-section (Fig. 8B), the inactive PSIIs were not irreversibly damaged during midday. The decrease in connectivity might have offset the increase in the rate of excitons reaching inactive PSII due to its larger effective cross-section. Our data suggest that the Q_B -D1 pocket is less occupied during bright light exposure and that this process is related to an *in vivo* mechanism to prevent irreversible photodamage to PSII. These results support the hypothesis that inactivation of PSII units plays a role in minimizing the deteriorating effects of photoinhibition on lon-

ger time scales (Oquist et al. 1992). Furthermore, our data indicate that the photoregulation of PSII activity that minimized D1 photodamage is sensitive to instantaneous changes in irradiance.

In higher plants, the efficiency of excitation capture by open PSII reaction centers (F_v/F_m) multiplied with the amount of open reaction centers is a good indicator for the quantum yield of nonlinear electron transport (Genty et al. 1989). Based on this observation, Keiller and Walker (1990) determined a linear relationship between the pulse saturation parameters $(F_m' - F_s)/F_m'$ and carbon dioxide assimilation under a variety of physiological conditions in leaves. The gradient in their equation in fact partly represents $1/(\Gamma \cdot k_e \cdot f_{II} \cdot D_i)$ (see Eq. 10). This formulation of the relationship between oxygen production rate and $(F_m' - F_s)/F_m'$ contains two parameters, which I did not measure directly: the stoichiometric value for the ratio of oxygen and electrons needed to generate oxygen (Γ , which by definition has a value of 0.25), and f_{II} , the fraction of absorbed light directed to PSII. I found high values for $\Gamma \cdot f_{II}$, which did not show consistent variations during the photoperiod (Table 3). My results suggested the occurrence of State I–II–I transitions. Thus, f_{II} is likely to be variable and Γ counteracted these variations. Assuming that Eq. 10 is correct, I suggest that even in State II, f_{II} was at least 0.5. Nevertheless, the constancy of $\Gamma \cdot f_{II}$ opens the possibility to accurately predict *in situ* primary productivity based on fluorescence and absorption measurements. Using a different set of equations, fluorescence-derived predictions of primary production can be made with a “pump and probe” fluorometer (Kolber and Falkowski 1993).

Combining the different processes that I discussed previously, I suggest that the following sequence of events occurred in *Chlorella pyrenoidosa* during the oscillating photoperiod based on the available data (Fig. 9). During the first 2 h, the plastoquinone pool progressively became reduced. The lowered redox state of the plastoquinone pool led to a State I–II transition. The effective cross-section of total PSII was smaller in State II than in State I. As the hourly peak irradiances increased, the combination of a high *trans* thylakoid electrochemical potential and a low plastoquinone redox potential decreased the amount of Q_B bound to the D1 protein. The relative abundance of active PSII was quantitatively equal to the number of PSII that had Q_B bound to the D1 protein. The effective cross-section and fraction of inactive PSII increased toward the middle of the photoperiod. Meanwhile, around the middle of the photoperiod, the connectivity between PSII lowered, which results in a decreased probability of exciton capture by the reaction centers. All these processes, possibly combined with a fast-responding high-energy state-quenching and/or increased heat dissipation via the xanthophyll cycle at peak irradiances, prevented severe photodamage at high ir-

radiances. Around midday, light irradiance no longer limits carbon dioxide fixation, and both the effect of State II and slower electron transfer through Q_B might have induced cyclic PSI electron transport. The reducing equivalents generated under this condition and the excess produced ATP could be used for increased nitrate reduction and amino acid incorporation into protein, respectively. During the second half of the photoperiod, all processes reversed, leading to a State I at the end of the photoperiod. The ultimate effect of such a regulation of photosynthesis would be a balanced growth despite large fluctuations in light.

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