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Photoadaptation in the green alga *Spongiochloris* sp. A three-fluorometer study

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Abstract

The dark-adapted cells of the green alga *Spongiochloris* sp. were exposed to "white light" of 1000 μmol(photon) m^{-2} s^{-1} for 2 h and then dark adapted for 1.5 h. Changes of photochemical activities during photoadaptation were followed by measurement of chlorophyll (Chl) fluorescence kinetics, 77 K emission spectra, photosynthetic oxygen evolution, and pigment composition. We observed a build-up of slowly-relaxing non-photochemical quenching which led to a decrease of the F_v/F_m parameter and the connectivity. In contrast to the depression of F_v/F_m (35 %) and the rise of non-photochemical quenching (~ 1.6), we observed an increase in effective absorption cross-section (20 %), Hill reaction (30 %), photosynthetic oxygen

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Abbreviations: A_{max} = area above the Chl fluorescence induction curve; Chl = chlorophyll; DCMU = 3-(3,4-dichlorophenyl)-1,1-dimethylurea; ETR = electron transport rate; F_0, F_v, F_m = minimum, variable, and maximum Chl fluorescence in dark-adapted cultures; F_m' = maximum fluorescence of light-adapted cultures; LED = light emitting diode; LHC2 = major light-harvesting antennae of photosystem 2; NPQ = non-photochemical quenching, $(F_m/F_m') - 1$; PFD = photon flux density; PS = photosystem; Q_A, Q_B = primary and secondary quinone acceptor of PS2; S_m = the normalised area above the induction curve, A_{max}/F_v; t_{Fm} = time when F_m is reached; $V(t) = [F(t) - F_0]/F_v$, the normalised variable fluorescence; Φ_p^{max}, Φ_p' = maximum and effective photochemical yield of PS2; σ_{PS2} = effective absorption cross-section of PS2.
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evolution (80 %), and electron transport rate estimated from the Chl fluorescence analysis (80 %). We showed an inconsistency in the presently used interpretation schemes, and ascribe the discrepancy between the increase of effective absorption cross-section and the photosynthetic activities on one side and the effective non-photochemical quenching on the other side to the build-up of a quenching mechanism which dissipates energy in closed reaction centres. Such a type of quenching changes the ratio between thermal dissipation and fluorescence without any effect on photochemical yield. In this case the F_v/F_m ratio cannot be used as a measure of the maximum photochemical yield of PS2.

Additional key words: chlorophyll fluorescence; photochemical yield; non-photochemical quenching; connectivity; electron transport rate.

Introduction

Chlorophyll (Chl) fluorescence measurement is one of the most widely used techniques in photosynthesis and plant physiology research (Dau 1994a, Govindjee 1995). As pointed out by Holzwarth (1993), it lacks a general theoretical support since it is mostly based on semi-empirical relations. At present, several groups have their own interpretation schemes frequently related to protocols specific to a particular Chl fluorescence equipment.

The simplest but still widely used experimental approach is measurement of the Chl fluorescence induction transient (Kautsky effect) supported by a well-elaborated theoretical basis (Malkin and Kok 1966, Butler 1978, Strasser *et al.* 1995, 1999, reviewed in Lazár 1999). A typical example of an instrument is the Plant Efficiency Analyser (*PEA* fluorometer, *Hansatech*, UK) which offers measurement of fluorescence transients in the time scale from 40 μs to minutes. A progress in instrumentation and experimental techniques came with using of modulated measuring radiation. This set-up allows measurement of Chl fluorescence yield independently of various actinic radiation sources. At present, the most widely used instrument of this type is the *PAM* fluorometer (*Walz*, Germany). Its use is mostly based on a simple model of Chl fluorescence quenching analysis and measurement of F_v/F_m or $\Delta F/F_m'$ ratios (Bilger and Schreiber 1986, Schreiber *et al.* 1986, 1995). Another method represents the pump-and-probe technique of Chl fluorescence measurement (Mauzerall 1972, Ley and Mauzerall 1982, Mauzerall and Greenbaum 1989). The technique has been frequently used for algae and phytoplankton (Falkowski *et al.* 1986, Kolber and Falkowski 1993). Recent technological progress and the use of ultra-bright LEDs has allowed the construction of new programmable microprocessor-controlled instruments such as the double-modulated fluorometer (*P.S.I.*) designed by Nedbal and co-workers (Trtílek *et al.* 1997, Nedbal *et al.* 1999) and the Fast Repetition Rate (FRR) fluorometer constructed by Kolber *et al.* (1998). These new instruments can employ experimental protocols designed for older instrumentation in combination with single-turnover pulse induction technique.

The lack of solid theoretical base for Chl fluorescence phenomena and a gap between time-resolved and steady-state methods have been challenged by Trissl *et al.* (1993). Using computer simulations based on the exciton-radical pair equilibrium

model (Schatz *et al.* 1988), they questioned a number of assumptions presently used for interpretation of Chl fluorescence values. They disregarded: (*1*) estimation of the number of electrons flowing to the acceptor side according to the complementary area above the induction curve (Malkin and Kok 1966); (*2*) estimation of connectivity between PS2 units from the degree of sigmoidicity of the Chl fluorescence induction curve in the presence of DCMU (Joliot and Joliot 1964, Lavorel and Joliot 1972), and (*3*) calculation of photochemical yield from the F_v/F_m parameter (Malkin and Kok 1966, Kitajima and Butler 1975). In the subsequent discussion (Holzwarth 1993, Falkowski *et al.* 1994, Trissl 1994) Falkowski and his co-workers questioned some of these conclusions. Later, Trissl and Lavergne (1995) and Lavergne and Trissl (1995) derived analytical formulae for the Chl fluorescence induction curve in the presence of DCMU. Surprisingly, up-to-date these conclusions have not become a matter of appropriate discussion and have not been properly verified.

In this paper we studied photoadaptation of the green alga *Spongiochloris* sp. The photoadaptation of the culture was followed by measurement of Chl fluorescence with three commercially available instruments. *PAM* and *PEA* fluorometers were used in their standard applications and the values were elaborated according to their standard protocols. The *P.S.I.* fluorometer was used to record Chl fluorescence inductions in the presence of DCMU to determine effective absorption cross-section and connectivity. The values obtained are used to check the validity of individual semi-empirical parameters used in Chl fluorescence analysis as well as self-consistency of the current interpretation schemes (*PAM*, *PEA*). The results are interpreted using the model of Lavergne and Trissl.

Materials and methods

Culture of the chlorococcal alga *Spongiochloris* sp. (Culture Collection of the Institute of Botany, Academy of Sciences, Třeboň, Czech Republic) was grown at 30 °C in a mineral medium bubbled with air + 2 % CO_2 in a glass cylinder which was irradiated with tungsten filament bulbs to give about 20 μmol(photon) m^{-2} s^{-1}. Before the experiment, the cultures were kept in the dark for 10 h. Then, the cultures were diluted with fresh medium to about 6 g(Chl) m^{-3} and exposed to 1000 μmol(photon) m^{-2} s^{-1} in an 18-mm thick plate-parallel cuvette for 120 min under bubbling with air + 2 % CO_2. After the exposure the culture was kept for 90 min in the dark for relaxation. Irradiation was provided by tungsten filament bulbs and measured with the *Li-185B* quantum sensor (*Li-Cor*, USA).

Chl fluorescence measurements were done using three commercially available fluorometers: continuous irradiation (*PEA*, *Hansatech Instruments*, King's Lynn, Norfolk, UK), modulated (*PAM 101*, *Walz*, Effeltrich, Germany), and double-modulated (*P.S.I.*, Brno, Czech Republic). The *PEA* fluorometer was used for recording Chl fluorescence induction transient (Kautsky effect). Samples were prepared by collecting 2.5 cm^3 of algal suspension on filter paper disks (*Millipore*, 0.8 μm). Two disks per sample were prepared and each disk was cut in 4 quadrants,

giving a total of 8 replicates. Each sample was placed into a *Hansatech* leaf-clip. After 5 min of dark adaptation, the Chl fluorescence induction was measured during 1 s of red irradiation from LEDs (peak 650 nm) of about 2700 μmol(photon) m^{-2} s^{-1} (Strasser *et al.* 1995). In our experiments, the F_0 value given by the instrument was used. The parameters F_m, F_v, A_{max}, and t_{Fm} were calculated according to Strasser *et al.* (1995, 1999): the normalised fluorescence $V(t) = [F(t) - F_0]/F_v$, the trapping rate $TR_0/RC = (dV/dt)_0/V_J$ (determined as $[V(300\ \mu s) - V(50\ \mu s)]/V_J$), and the relative ETR as S_m/t_{Fm} where $S_m = A_{max}/F_v$ is the normalised area above the induction curve.

The fluorometer *PAM 101-103* coupled with the emitter-detector unit *ED-101US* (*Walz*, Germany) was used for measurements of F_v/F_m, NPQ, and ETR. The F_v/F_m ratio was determined in culture samples dark-adapted for 5 min. Constant fluorescence F_0 (minimum fluorescence in the dark-adapted state) was measured by modulated radiation [< 0.3 μmol(photon) m^{-2} s^{-1}, 1600 Hz] from a LED (peak wavelength at 655 nm). F_m was reached using a saturating radiation pulse [4000 μmol(photon) m^{-2} s^{-1}, 0.5 s duration]. Electron transport rate ($ETR = \Delta F/F_m' \times PFD$) was measured using actinic radiation of 1000 μmol(photon) m^{-2} s^{-1} provided by a halogen lamp (*FL-103, Schott*, Germany). After reaching the steady-state F level, a saturating pulse was applied to determine the maximum fluorescence yield in the light, F_m'.

The *P.S.I.* fluorometer (standard version 100 kHz, 16 bit) was used for recording of the Chl fluorescence induction curve in the presence of 10^{-5} M DCMU. After 5 min of dark adaptation the transient was induced by 100 sub-saturating flashes of 15 μs duration, 500 μs apart which were supplied by red (*Hewlett-Packard HLMP 8104*, 650 nm) LEDs. The induction curve was fitted by the simplified expressions of Lavergne and Leci (1993): $V(t) = (1 - q)/(1 + Jq)$ and $-dq/dt = \sigma_{PS2}(1 + J)\, q/(1 + Jq)$ where q is the fraction of open reaction centres, V(t) represents normalised variable Chl fluorescence, σ_{PS2} is the effective absorption cross-section (relative value), and J is a connectivity parameter. Only the PS2α reaction centres were taken into consideration.

Oxygen evolution: The radiant energy-saturated [3500 μmol(photon) m^{-2} s^{-1}], steady-state rate of photosynthetic oxygen evolution was measured at 30 °C in cell suspensions using a Clark-type electrode (*YSI*, USA) in a temperature-controlled chamber (Bartoš *et al.* 1975). For the measurement of oxygen evolution in the presence of an artificial electron acceptor (Hill reaction activity, HRA) *p*-benzoquinone (7 mM final concentration) was added just before the measurement.

Pigment analysis (Chl *a*+*b*, total carotenoids) was determined spectrophotometrically in 80 % acetone (Lichtenthaler 1987). The amount of individual carotenoids was assessed by HPLC using the procedure of Gilmore and Yamamoto (1991).

77 K chlorophyll fluorescence emission spectra: For these measurements the samples were adapted in the dark for 5 min. *Rhodamin 6G* was added to final concentration of 5 μM as an internal fluorescence standard. Then, 90 mm^3 of the cell suspension was injected into a 0.5-mm deep groove on metal holder, and the sample was rapidly frozen in liquid nitrogen. Spectra were recorded on the spectrofluorometer *Fluorolog* (*SPEX*, USA). The excitation wavelength was 440 nm. The spectrum was scanned

from 540 to 780 nm. The excitation and emission slits were set to 4 and 2 nm, respectively. The spectra were normalised according to a fluorescence peak of *Rhodamine 6G* at 588 nm.

Results

F_v/F_m ratio: It is generally assumed that the F_v/F_m ratio reflects maximum photochemical yield of PS2. We determined this parameter using each of the three instruments. The parameter was determined after 5 min of dark adaptation either in the absence (*PAM, PEA*) or in the presence of DCMU (*P.S.I.*). The time-course of the F_v/F_m changes was the same in all the instruments, only the values measured by the *PAM* fluorometer were by 10 to 15 % higher than those obtained using the *PEA* and *P.S.I.* fluorometers (Fig. 1). The F_v/F_m ratio was greatly depressed (by about 35 %) during high-irradiance exposure, reaching its minimum after 90 min. When the light was switched off after 2 h, F_v/F_m slowly relaxed with a half-time longer than 2 h.

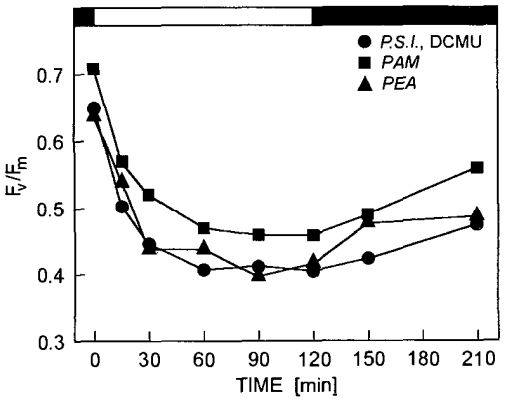

Fig. 1. Determination of F_v/F_m by the *PAM, PEA,* and *P.S.I.* fluorometers during high irradiance treatment of *Spongiochloris* cells. F_v/F_m in algal cells was determined after 5 min of dark adaptation. *PAM* - 0.5 s pulse of "white" saturating radiation, *PEA* - 1 s pulse of red measuring radiation from red LEDs, *P.S.I.* - 50 ms induction elicited by 100 sub-saturating flashes (650 nm) in the presence of 10^{-5} M DCMU. The *upper bar* indicates light [1000 μmol(photon) m^{-2} s^{-1}, *white section*] and dark (*black sections*) periods of the experiment.

Non-photochemical quenching was determined by the *PAM* fluorometer. The total quenching referred to the starting value of F_m^{start} (0 min) was calculated according the Stern-Volmer formalism: $NPQ_{total} = (F_m^{start} - F_m')/F_m'$. The rapidly relaxing component was determined from the F_m value obtained for each sample after 5 min of dark adaptation using the formula: $NPQ_{fast} = (F_m - F_m')/F_m'$. The time-course of both quenching parameters is shown in Fig. 2. The total non-photochemical quenching (NPQ_{total}) raised rapidly during the irradiation and reached its maximum at 90 min; then it started to decline. On the contrary, the rapidly relaxing, 'fast' non-photochemical quenching (NPQ_{fast}) declined throughout the experiment.

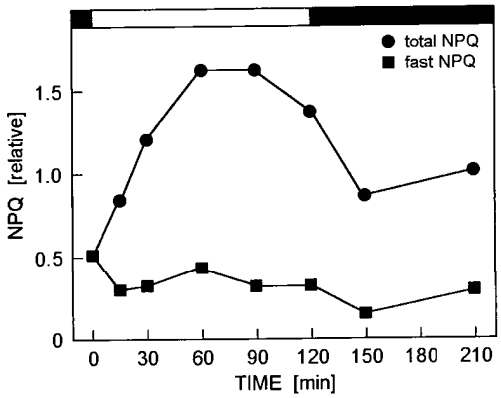

Fig. 2. The time-course of NPQ_{total} and NPQ_{fast} during high irradiance treatment of the *Spongiochloris* culture. F_m' was determined at the irradiance of 1000 μmol(photon) m^{-2} s^{-1} of "white light" by 0.5 s saturating pulse. The total quenching was calculated according to the Stern-Volmer formalism using the starting value of F_m^{start} in the dark (0 min): $NPQ_{total} = (F_m^{start} - F_m')/F_m'$. The rapidly relaxing component was determined from the F_m value obtained for each sample after 5 min of dark adaptation according to the formula: $NPQ_{fast} = (F_m - F_m')/F_m'$. The *upper bar* indicates light [1000 μmol(photon) m^{-2} s^{-1}, *white section*] and dark (*black sections*) periods of the experiment.

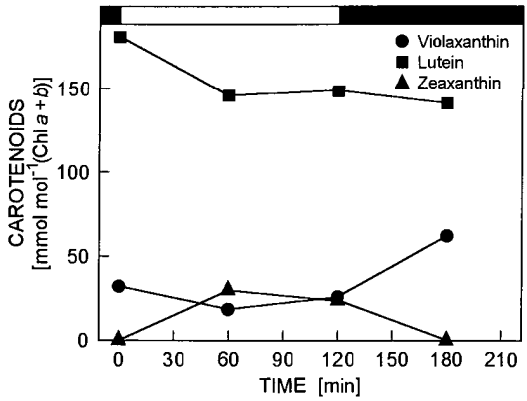

Fig. 3. Changes in the violaxanthin, zeaxanthin, and lutein contents during high irradiance treatment of the *Spongiochloris* culture. β-carotene and neoxanthin are not shown since their contents did not change [35 and 50 mmol mol^{-1}(Chl)]. Antheraxanthin was present only in trace amounts (not quantified). The *upper bar* indicates light [1000 μmol(photon) m^{-2} s^{-1}, *white section*] and dark (*black sections*) periods of the experiment.

Pigments: During the irradiation the content of Chl and the Chl *a/b* ratio remained stable (2.0 between 0 and 180 min of irradiation). We observed an increase in amount of total carotenoids reflected in a decrease of the $(a+b)/(x+c)$ ratio from 6.4 at the start of the experiment to 6.0 at 60 min and 5.8 at 120 and 180 min. The changes in the amount of individual carotenoids during the experiment were related to Chl $(a+b)$ content (Fig. 3). We found nearly constant amounts of β-carotene

[35 mmol mol^{-1}(Chl)] and neoxanthin [50 mmol mol^{-1}(Chl)] (not shown). The amount of lutein decreased by about 20 % during the first 60 min of irradiation and then remained stable (Fig. 3). The amount of violaxanthin was reduced during the initial 60 min of the experiment in an antiparallel way to the increase of zeaxanthin. The zeaxanthin content reached its maximum after 60 min of irradiation [30 mmol mol^{-1}(Chl)], then slightly declined after another 60 min [25 mmol mol^{-1}(Chl)]. During the following 60 min of dark relaxation zeaxanthin completely disappeared. Antheraxanthin was present only in trace amounts (not quantified).

The effective absorption cross-section σ_{PS2} (antenna size, trapping rate) was determined from the rate of Q_A reduction during the 50-ms induction of Chl fluorescence in the presence of 10^{-5} M DCMU using the *P.S.I.* fluorometer. We found about 20 % increase of effective absorption cross-section (Fig. 4) linearly related with the increase of the corresponding values of F_0 (Fig. 5).

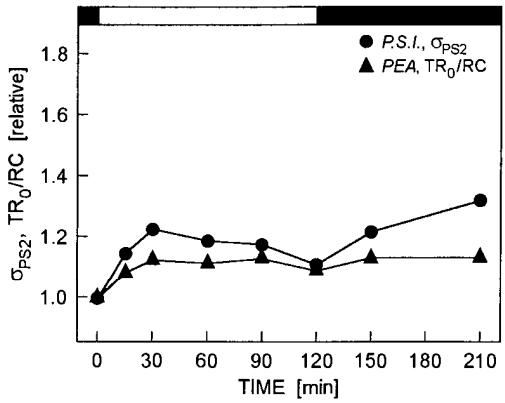

Fig. 4. Time-course of the effective absorption cross-section σ_{PS2} (antenna size) and trapping rate (TR_0/RC, *PEA*) during high irradiance treatment of the *Spongiochloris* culture. The relative value of effective absorption cross-section was calculated from a rate of PS2 closure during the Chl fluorescence induction in the presence of 10^{-5} M DCMU. After 5 min of dark adaptation the transient was induced by 100 sub-saturating flashes of 15 μs duration, 500 μs apart provided by the *P.S.I.* fluorometer. The effective absorption cross section was also estimated indirectly from the initial slope of the O-J region of the induction curve (*PEA* measurement) calculating the TR_0/RC parameter ([V(300 μs) – V(50 μs)]/V_J). The *upper bar* indicates light [1000 μmol(photon) m^{-2} s^{-1}, *white section*] and dark (*black sections*) periods of the experiment.

The effective absorption cross section was also estimated from Chl fluorescence induction measurement (*PEA*), using the parameter $TR_0/RC = (dV/dt)_0/V_J$ (Fig. 4). This parameter reflects a measure of the initial rate of Q_A reduction, when all the reaction centres are open; thus, it can be considered as an alternative of effective absorption cross-section. The irradiation caused an increase by about 15 % of the TR_0/RC value which was in good agreement with the increase of the effective absorption cross section determined by the *P.S.I.* fluorometer.

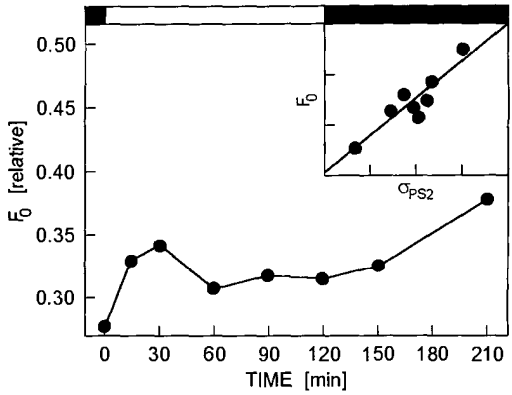

Fig. 5. Time course of F_0 measured by *P.S.I.* using the red measuring radiation during the high irradiance treatment of the *Spongiochloris* culture. The *upper bar* indicates light [1000 μmol(photon) m^{-2} s^{-1}, *white section*] and dark (*black sections*) periods of the experiment. The *insert* shows the relationship between F_0 and the effective absorption cross-section (σ_{PS2}).

Connectivity between PS2 units: The second parameter determined from the Chl fluorescence induction curve in the presence of DCMU was the connectivity (grouping) parameter J. The parameter depends on connectivity (grouping) probability p_{2G} and reaction centre parameters ($J = p_{2G} F_v/F_0$) (Strasser and Greppin 1981, Trissl and Lavergne 1995). Plotting the values of J against corresponding values of F_v/F_0 we found a linear relationship between these two parameters (Fig. 6). Using linear regression we calculated the connectivity probability $p_{2G} = 0.35$.

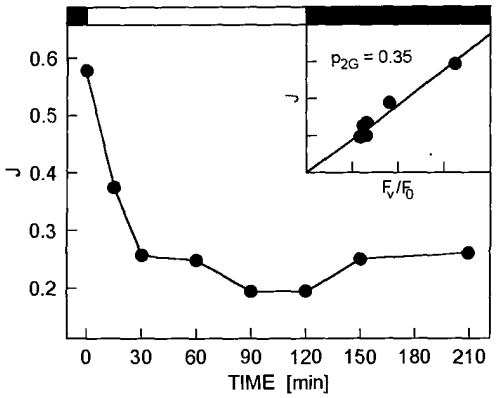

Fig. 6. Time-course of the connectivity parameter J during high irradiance treatment of the *Spongiochloris* culture. The parameter J was determined from the sigmoidicity of the Chl fluorescence induction curve recorded in the presence of 10^{-5} M DCMU after 5 min of dark adaptation. The *upper bar* indicates light [1000 μmol(photon) m^{-2} s^{-1}, *white section*] and dark (*black sections*) periods of the experiment. The *insert* shows the relationship between the connectivity J and the F_v/F_0 ratio.

77 **K Chl fluorescence emission spectra** were recorded at the beginning and at the end of irradiation (after 120 min). Surprisingly, we did not observe any significant difference between these two spectra (Fig. 7). Moreover, we noticed an unusual shape of the spectrum with only two major bands at 688 and 698 nm where the peak at 720-725 nm, usually ascribed to PS1, was nearly absent forming only a negligible shoulder on the major peak at 698 nm.

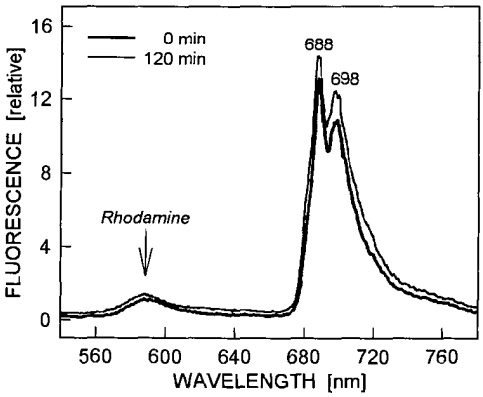

Fig. 7. 77 K chlorophyll fluorescence emission spectra of the *Spongiochloris* cells before irradiation (0 min, *thick line*), and afterwards [1000 μmol(photon) m^{-2} s^{-1}, 120 min + 5 min of dark relaxation, *thin line*). The excitation wavelength was 440 nm. The spectra were normalised to the emission peak of *Rhodamin 6G* used as an internal fluorescence standard. The spectra are slightly vertically shifted to show the difference.

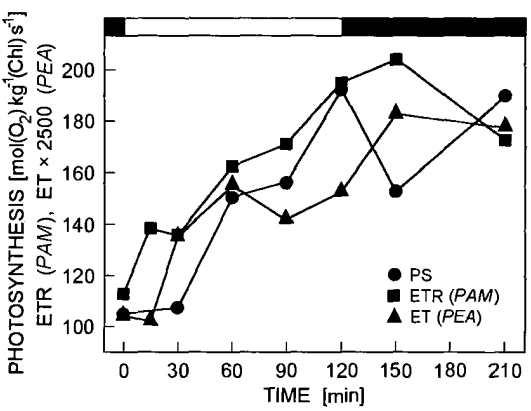

Fig. 8. The time course of ETR (*PAM* and *PEA* measurements) and photosynthetic oxygen evolution during high irradiance treatment of the *Spongiochloris* culture. The steady-state rate of oxygen evolution was measured at 30 °C under saturating irradiance [3500 μmol(photon) m^{-2} s^{-1}]. The ETR value (*PAM* measurement) was calculated from the $\Delta F/F_m'$ ratio determined under 1000 μmol(photon) m^{-2} s^{-1} ($ETR = \Delta F/F_m' \times PFD$). The ETR value (*PEA*) was calculated from normalised area above the induction curve. The upper bar indicates light [1000 μmol(photon) m^{-2} s^{-1}, *white section*] and dark (*black sections*) periods of the experiment.

Photosynthesis and ETR: To characterise photosynthetic activity during the experiment we measured the maximum rate of photosynthetic oxygen evolution. The rate of photosynthesis grew during the irradiation starting from 105 to 190 mol(O_2) kg^{-1}(Chl) s^{-1} (about an 80 % increase) at the end of the irradiation (120 min) (Fig. 8). During the dark relaxation the activity of photosynthesis remained stable.

For comparison we also determined rates of electron transport from Chl fluorescence values using two approximation methods: (*1*) as the normalised area above the induction curve S_m/t_{Fm} (*PEA*), and (*2*) as the $\Delta F/F_m'$ parameter (*PAM*) (Fig. 8). Both parameters showed a similar course (about 80 % increase) as photosynthetic activity. We also determined Hill reaction activity (PS2) in the presence of 7 mM benzoquinone. Similarly to the activity of photosynthesis we observed an about 30 % increase in Hill reaction activity during the irradiation (not shown).

Chl fluorescence induction curves were measured at 0, 120, and 210 min during the experiment and normalised on the variable Chl fluorescence (Fig. 9). At the end of the irradiation (t = 120 min), we observed an increase in V_J = V (2 ms) and a decrease in V_I = V (30 ms). After dark relaxation (t = 210 min) the V_J increase partly recovered while the V_I value fell to even lower value.

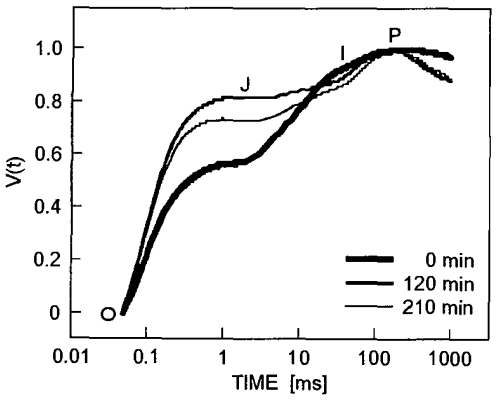

Fig. 9. Chlorophyll (Chl) fluorescence transients (*PEA*) of the *Spongiochloris* cells. The Chl fluorescence induction curves were recorded at the start (0 min) and end of irradiation [1000 μmol(photon) m^{-2} s^{-1}, 120 min] and after the end of the following 90 min dark relaxation (210 min). The curves are normalised to the variable Chl fluorescence and plotted in logarithmic time-scale from 10 μs to 1 s. The points O, J, I, P are indicated.

Discussion

We analysed and compared various Chl fluorescence parameters obtained by measurement with three types of Chl fluorometer during adaptation of *Spongiochloris* sp. to irradiation. One of the most interesting observations was a non-correspondence between the F_v/F_m parameter and the effective absorption cross-section. Similar non-

correspondence also exists between the rise of photosynthesis and the increase of NPQ. The F_v/F_m ratio has usually been considered to reflect the maximum photochemical yield of PS2 (Φ_p^{max}). The effective absorption cross-section (σ_{PS2}) depends upon the optical absorption cross-section of PS2 (a_{PS2}) and its photochemical yield: $\sigma_{PS2} = a_{PS2}\Phi_p^{max}$ (Kolber *et al.* 1998). The F_v/F_m parameter was greatly depressed during the irradiation by about 35 % whereas the σ_{PS2} parameter increased by about 20 %. A similar phenomenon was already observed during photoadaptation in other green algae (*e.g.*, *Chlorella* and *Scenedesmus* - Masojídek *et al.* 1999). Considering the quasi-linear relationship between σ_{PS2} and F_0, we can assume that observed changes of σ_{PS2} originated mostly from changes of a_{PS2}; Φ_p^{max} remained constant during the experiment. The depression of F_v/F_m clearly corresponded to the build-up of slow non-photochemical quenching. The existence of NPQ has usually been ascribed to an adaptation mechanism(s) protecting the photosynthetic apparatus against excess radiant energy (Dau 1994b). The pH-gradient dependent quenching, the zeaxanthin-dependent quenching (Young and Frank 1996, Gilmore 1997), state transitions connected with rearrangement of LHC2 (Allen 1992), or photoinhibition (Prášil *et al.* 1992) have been considered as main non-photochemical quenching mechanisms. The first two mechanisms can be excluded as they should contribute only to the fast (= rapidly relaxing) non-photochemical quenching. The pH-dependent quenching usually relaxes within tens of second (Krause *et al.* 1982). The appearance of the zeaxanthin-dependent quenching is associated with presence of pH gradient (Gilmore 1997). Moreover, the present zeaxanthin disappeared within 60 min of dark relaxation whereas the slow NPQ relaxed with a half-time greater than 2 h. The maximum amount of zeaxanthin observed in this study [30 mmol mol^{-1}(Chl)] was significantly lower than values [up to 200 mmol mol^{-1}(Chl)] reported for higher plants (Demmig-Adams 1990, Schindler and Lichtenthaler 1996). We conclude that the zeaxanthin-dependent quenching plays a little role in *Spongiochloris*. A minor importance of the zeaxanthin-dependent quenching in green algae was already found in *Scenedesmus* and *Chlorella* (Masojídek *et al.* 1999) and in *Dunaliella* (Casper-Lindley and Björkman 1998). Taking into account the results of Hill reaction (30 % increase) and photosynthesis (80 % increase) measurements, we can also exclude photoinhibition as a plausible quenching mechanism. State transitions represent the mechanism of redistribution of excitation energy between PS1 and PS2. Considering the increase in effective absorption cross-section and minimal change in 77 K emission spectrum, we can also rule out this mechanism. As the presence of slow NPQ did not decrease the level of F_0 (Fig. 5), it seems that the observed type of quenching is caused by an unknown mechanism operating probably directly in the reaction centre. This quenching mechanism is likely to be temperature-dependent as it does not affect the intensity of 77 K fluorescence emission.

The F_v/F_m parameter is mostly accepted as a measure of the photochemical yield of PS2 (Kolber and Falkowski 1993, Schreiber *et al.* 1995, Strasser *et al.* 1999). It is often assumed that the closure of PS2 reaction centre does not change other rate constants. In this case F_v/F_m equals $k_p/(k_p + k_d + k_f) = \Phi_p^{max}$. In contrast to this assumption, a determination of rate constants from picosecond Chl fluorescence kinetics has shown that thermal dissipation rate constants (k_d) differ in closed and open reaction

centres (Roelofs *et al.* 1992). Later, Lavergne and Trissl (1995) derived their equations assuming different thermal dissipation rates in open (k_d^{ox}) and closed (k_d^{red}) reaction centres and demonstrated that k_d^{red} strongly affects the F_m value (and NPQ), F_v/F_m, and J. Similarly, in Appendix of this paper we have elaborated the simplified expression for F_v/F_m supposing $k_d^{ox} \neq k_d^{red}$. In this case F_v/F_m is not equal to photochemical yield since it is decreased due to the difference in thermal dissipation rates (See Appendix, Eqs. 4 and 5). We assume that the observed slow non-photochemical quenching is caused only by a change of k_d^{red}. From the phenomenological point of view the proposed quenching strongly resembles (decrease of F_m, constant F_0, no correspondence of photochemical yield and F_v/F_m) the original Butler's concept of reaction centre quenching despite of the different model used (Butler and Kitajima 1975, Butler 1978).

Also the decrease of connectivity parameter J (Fig. 6) is clearly connected with the build-up of non-photochemical quenching. J characterises hyperbolic dependence of Chl fluorescence on fraction of closed reaction centres (Lazár 1999, here denoted as C). It is usually interpreted in terms of connectivity concept introduced by Joliot and Joliot (1964) and Lavorel and Joliot (1972). J depends on connectivity (grouping) probability p_{2G} and reaction centre parameters [$J = p_{2G}(F_v/F_o)$ - Strasser 1978, Strasser and Geppin 1981, Lavergne and Trissl 1995, Lazár 1999]. These conclusions fully agree with our result showing the linear relationship between F_v/F_0 and J. It suggests that the connectivity probability stays constant during the experiment and the decline of J is caused by the build-up of slow non-photochemical quenching. The lower values of connectivity (J_{start}~0.6), when compared to usually reported values 1.0-1.5 for dark adapted material (Strasser and Greppin 1981, Trissl and Lavergne 1995), could be ascribed to irradiance-gradients. *Spongiochloris* cells have a large diameter of about 100 μm (*Chlorella* ~5 μm), hence a relatively deep irradiance-gradient can be formed across them. As a general rule, a Chl fluorescence induction curve formed by averaging fluorescence induction curves with the same J but different rise kinetics due to absorption of radiation in the sample artificially displays smaller values of J (H.-W. Trissl, personal communication).

There exist two basic methods to determine the electron transport activity from Chl fluorescence values. The one described by Malkin and Kok (1966) (used in *PEA* measurement) estimates the electron transport from relative area above the induction curve. In the other approach (used in *PAM* measurements), the ETR is calculated from the photochemical yield of PS2 in the light, $\Delta F/F_m'$ (a parameter originally proposed by Weber 1960). It has become frequently used since 1989 when Genty *et al.* (1989) proved its correlation with the rate of CO_2 fixation in higher plants. Since both ways were questioned by Trissl *et al.* (1993), we determined the maximum rate of photosynthesis by measurement of oxygen evolution and compared with the estimation provided by fluorescence methods. Both methods adequately describe the increase in ETR during the irradiation. The fact that $\Delta F/F_m'$ well follows the photosynthesis signalises a significant portion of Δk_d quenching is present already in dark-adapted material at the beginning of the experiment. The relation between $\Delta F/F_m'$ and the effective photochemical yield can be expressed: $(\Delta F/F_m')/\Phi_p' = (k_p' - k_d')/k_p' = 1 - k_d'/k_p'$ (see Appendix, Eq. 6). This ratio stays constant if the $\Delta k_d'/k_p'$ ratio is also

constant, so, considering 80 % increase of k_p' during the irradiation, $\Delta k_d'$ should increase in the same extent. Measurements of maximum photochemical yield give its value in range 0.9-1.0 (Kramer and Mathis 1980, Roelofs *et al.* 1992, Groot *et al.* 1997). Using $\Phi_p^{max} = 0.95$ and measured values of F_v/F_m (start 0.67, end 0.43) we can calculate that $(\Delta k_d^{end} - \Delta k_d^{start})/\Delta k_d^{start} = 0.83$ which corresponds to the approximate 80 % increase of k_p'. This also means that the rapidly relaxing NPQ_{fast} consists predominantly from the normal type of quenching affecting in the same way open and closed reaction centres.

As the observed increase of F_0 probably reflects only the changes of optical absorption cross-section, it seems that a number of PS2 units stays constant. Then, keeping this in mind, the observed increase in photosynthetic oxygen evolution may be caused by an activation of Calvin cycle and/or electron transport carriers. The increase in Hill reaction activity should rather be ascribed to the activation of some units than to the assembly of new ones. This is in agreement with changes in the shape of Chl fluorescence induction curves (*PEA*, Fig. 10) as we observed a decrease of the V_I level. According to Strasser *et al.* (1995), V_I reflects heterogeneity of the PQ-reducing centres; therefore, its decrease during irradiation seems to correspond with the proposed activation of PS2 units. The O-J phase of the induction kinetics (t = 0-2 ms) allegedly represents the photochemical phase, leading to the reduction of Q_A to Q_A^- (Strasser *et al.* 1995). The increase of V_J has usually been ascribed to an inhibition of forward electron transfer ($Q_A \rightarrow Q_B$) by adverse physiological conditions. As we found the increase of the ETR, we explain this effect as a combination of the increase of the PS2 trapping rate (*e.g.*, effective absorption cross-section) and the decrease of connectivity (which causes relative up-shift of the J step).

In this study we examined the photoadaptation of *Spongiochloris* sp. We observed a great discrepancy of the individual Chl-fluorescence parameters (decrease of F_v/F_m *vs.* increase of σ_{PS2}, increase of NPQ together with increase $\Delta F/F_m'$). We suppose that this problem arises from the existence of an unknown slowly-relaxing non-photochemical quenching mechanism which can dissipate energy in closed reaction centres. Such a type of non-photochemical quenching does not affect photochemical yield, despite it greatly decreases the F_v/F_m ratio, highly increases an apparent NPQ, and decreases the effective connectivity among PS2 units. We also show that the maximum photochemical yield remained constant during the irradiation as well as the connectivity probability and the number of units. The increase of effective absorption cross-section was probably caused by the increase of optical absorption cross-section. We also show that the xanthophyll cycle is operative in *Spongiochloris* sp., but its contribution to total non-photochemical quenching is small.

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Appendix

The Chl fluorescence yield in open reaction centres (Φ_{f0}) is given by the ratio of the fluorescence rate constant (k_f) and the sum of rate constants of all de-excitation processes (k_p, rate constant of photochemistry; k_d^{ox} rate constant of thermal dissipation in open reaction centres). Similarly, the Chl fluorescence yield in close reaction centres (Φ_{fm}) is given by the ratio of the fluorescence rate constant and the sum of rate constants of Chl fluorescence and thermal dissipation in closed reaction centres (k_d^{red}).

$$\Phi_{f0} = \frac{k_f}{k_p + k_d^{ox} + k_f}; \qquad \Phi_{fm} = \frac{k_f}{k_d^{red} + k_f} \tag{1}$$

The F_v/F_m ratio is calculated from measured Chl fluorescence yields according to the formula:

$$F_v/F_m = \frac{\Phi_{fm} - \Phi_{f0}}{\Phi_{fm}} \tag{2}$$

After the substitution of fluorescence yields using Eq. 1 and subtracting we obtain:

$$F_v/F_m = \frac{k_p + k_d^{ox} - k_d^{red}}{k_p + k_d^{ox} + k_f} \tag{3}$$

Assuming that the rate constants of thermal dissipation in closed and open reaction centres can differ, we can substitute their difference with the parameter $\Delta k_d = k_d^{red} - k_d^{ox}$.

$$F_v/F_m = \frac{k_p}{k_p + k_d^{ox} + k_f} \times \frac{k_p - \Delta k_d}{k_p} \tag{4}$$

We can also derive expression for the maximum photochemical yield

$$\Phi_p^{max} = F_v/F_m \times \frac{k_p}{k_p - \Delta k_d} \quad (5)$$

In the ideal case when $k_d^{red} = k_d^{ox}$, the parameter Δk_d becomes zero. Then, we can obtain the well-known relationship for the maximum photochemical yield $\Phi_p^{max} = (F_v/F_m)$.

Eq. (5) can be generalised to obtain expression for the effective photochemical yield. (k_p' - effective rate constant of photochemistry; $\Delta k_d'$ effective Δk_d)

$$\Phi_p' = \Delta F/F_m' \times \frac{k_p'}{k_p' - \Delta k_d'} \quad (6)$$