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REGULAR PAPER

Probing of photosynthetic reactions in four phytoplanktonic algae with a PEA fluorometer

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Abstract High-resolution light-induced kinetics of chlorophyll fluorescence (OJIP transients) were recorded and analyzed in cultures of diatoms (*Thalassiosira weissflogii*, *Chaetoceros mulleri*) and dinoflagellates (*Amphidinium carterae*, *Prorocentrum minimum*). Fluorescence transients showed the rapid exponential initial rise from the point O indicating low connectivity between PS II units and high absorption cross-section of PS II antenna. Dark-adapted dinoflagellates revealed capability to maintain the PS I-mediated re-oxidation of the PQ pool at the exposure to strong actinic light that may lead to the underestimation of F_M value. In OJIP transients recorded in phytoplanktonic algae the fluorescence yield at the point O exceeded F_O level because Q_A has been already partly reduced at 50 μs after the illumination onset. PEA was also employed to study the recovery of photosynthetic reactions in *T. weissflogii* during incubation of nitrogen starved cells in N-replete medium. N limitation caused the impairment of electron transport between Q_A and PQs, accumulation of closed PS II centers, and the reduced ability to generate transmembrane ΔpH upon illumination, almost fully restored during the recovery period. The recovered cells showed much higher values of NPQ than control ones suggesting maximization of photoprotection mechanisms in the population with a 'stress history.'

Keywords Chlorophyll *a* fluorescence · OJIP transients · Phytoplankton · Nitrogen starvation

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Abbreviations

Chl	Chlorophyll
PS	Photosystem
P_{680}	The primary electron donor in PS II
Q_A	The primary quinone electron acceptor in PS II
Q_B	The secondary quinone electron acceptor in PS II
PQ	Plastoquinone
OEC	Oxygen evolving complex
FNR	Ferredoxin-NADP reductase
DCMU	3-(3,4-Dichlorophenyl)-1,1-dimethylurea
N	Nitrogen
NPQ	Non-photochemical Chl fluorescence quenching
PPFD	Photosynthetic photon flux density
O, J, D, I, P	Particular steps in light-induced Chl fluorescence curve
PAM	Pulse amplitude modulated fluorometer
PEA	Plant efficiency analyzer

Introduction

Chlorophyll (Chl) *a* fluorescence is widely used for the assessment of photosynthetic capacity of phytoplankton in situ (Kolber et al. 1990; Falkowski et al. 1991). Such parameters as F_O, which reflects light absorbance by photosynthetic pigments (Matorin et al. 2004), and F_V/F_M, the maximum quantum yield of photosystem (PS) II photochemistry (Genty et al. 1989), are commonly involved in phytoplankton monitoring with different types of fluorometers. Moreover, light-induced thermal energy dissipation in PS II antenna can be examined by measuring non-photochemical Chl fluorescence quenching with a

Pulse Amplitude Modulated fluorometer (PAM) (Schreiber et al. 1995). Furthermore, additional information can be obtained by recording high-resolution light-induced kinetics of Chl fluorescence (OJIP transients) using Plant Efficiency Analyzer (PEA) (Strasser and Govindjee 1992). OJIP transient reflects changes in electron transport in PS II over a wide range of time from microseconds to seconds that allows to evaluate such important characteristics of PS II as energy trapping, electron transport, and ΔpH-dependent dissipation of excitation energy into heat in the antenna complex (Strasser and Strasser 1995; Strasser et al. 2005). However, employment of PEA to monitor photosynthetic capacity of phytoplankton is hindered by the fact that current interpretations of OJIP transients are based on data obtained with higher plants and green algae, which differ from phytoplankton species, such as diatoms and dinoflagellates, in organization of their photosynthetic apparatus. It is necessary to study OJIP transients in cultures of various marine algae.

Particular attention should be given to the assessment of photosynthetic capacity of marine algae as a function of nutrient supply that can be used to monitor phytoplankton in situ. Nitrogen (N) is known to be a limiting factor for the majority of marine ecosystems. The intermittent supply of N results in periods of depletion, followed by episodic resupply due to, for example, upwelling events. Experiments with cultures of various algae showed that N starvation causes inhibition of protein synthesis and, consequently, suppression of cell growth (Falkowski et al. 1989, 1991). In the photosynthetic apparatus, N deficiency reduces the number of reaction centers of PS II and induces changes in the light-harvesting antenna (Kolber et al. 1988), including the decrease in Chl *a*/*b* or *a*/*c* ratio, as well as increase in the relative content of carotenoids and phycobilins (Cleveland and Perry 1987).

In this work, we studied OJIP transients recorded in four cultures of marine microalgae including diatoms (*Thalassiosira weissflogii*, *Chaetoceros mulleri*) and dinoflagellates (*Amphidinium carterae*, *Prorocentrum minimum*). Moreover, the changes in OJIP kinetics were analyzed during regeneration of N-deprived *T. weissflogii* in the medium supplied with nitrate.

Materials and methods

Culture conditions and nitrogen depletion

C. reinhardtii strain Dang 137+ WT was grown photoheterotrophycally in tris–acetate–phosphate medium, pH 7.0, in Erlenmeyer flasks at 30°C under continuous illumination (photosynthetic photon flux density (PPFD) 30 μE m^{-2} s^{-1}) and constant shaking.

The diatoms *Thalassiosira weissflogii* (Grunow) Fryxell et Hasle, *Chaetoceros mulleri* Lemmermann, and the dinofagellates *Prorocentrum minimum* (Pavillard) Schiller, *Amphidinium carterae* Hulbert were maintained as batch cultures in f/2 medium (Guillard and Ryther 1962), artificial seawater (salinity 17‰) at 20°C and constant shaking. Illumination was 30 μE m^{-2} s^{-1} provided by white fluorescent lamps. Light and dark periods were 14 and 10 h, respectively.

N-deprived cells of *T. weissflogii* were obtained by growing in N-limited f/2 medium (Guillard and Ryther 1962), supplied with 0.176 mM of nitrate, that corresponds to its content in f/10 medium. Cells were cultivated without re-addition of N during 34 days until the rate of culture growth decreased to zero and cell concentration began to decline indicating N starvation. Starved cells were collected by centrifugation, transferred to f/10 medium, and incubated during 28 days.

Measurement of Chl fluorescence

OJIP transients were recorded using a Plant Efficiency Analyzer (Hansatech, King's Lynn, Norfolk, UK). Red light peaking at 650 nm served as an excitation source. The Chl fluorescence signal was detected using a PIN photocell after passing through a long-wavelength cut-off filter. The period of illumination was 6 s. To increase the signal to noise ratio, cells were concentrated on a glass fiber filters (Millipore).

In order to analyze OJIP transients JIP test was introduced by Strasser and Strasser (1995). Table 1 shows a list of selected JIP-test parameters (M_O, S_M, and ψ_0), formulae used to calculate each parameter and a brief explanatory notes.

When indicated, OJIP transients were measured with an experimental fluorometer MEGA-25 designed in Biophysical Dept of Faculty of Biology (Moscow State University, Russia). In this instrument a series of short pulses (normally 80–100) with duration of 5 μs was applied to dark-adapted sample with an interval of 100 ms in order to excite Chl fluorescence yield at open PS II reaction centers, F_O. Thereafter, a 750 μs pulse of light was fired to induce the initial phase of the fluorescence rise (OJ). At this stage the signal was detected with intervals of 0.75 μs. Thereafter, Chl fluorescence was excited with a long pulse with the duration of 1.5 s, which allows to detect JIP phase in the kinetic curve. Two blue light-emitting diodes (455 nm, Luxeon) served as an excitation source. PPFD of actinic light can be varied in the interval 600–12,000 μE m^{-2} s^{-1}. The Chl fluorescence signal was detected using a photomultiplier R7400U-20 (Hamamatsu) supplied with a high-voltage power source C4900-01 (Hamamatsu). The

Table 1 Listing of characteristic points of OJIP transient and formulas for calculation of selected JIP-test parameters with short explanations

$F_{50\mu s}$, $F_{300\mu s}$, F_J	Fluorescence yield at 50 μs (point O)*, 300 μs, and at point J
F_P	Maximum in OJIP transient
$S_M = (\text{Area})/(F_P - F_{50\mu s})$	Normalized area between OJIP curve and F_P value, reflecting multiple turnover Q_A reduction events
$\psi_0 = (F_P - F_J)/(F_P - F_{50\mu s})$	The probability of electron transfer from PS II to PQ pool
$M_O = 4 \times (F_{300\mu s}-F_{50\mu s})/(F_P - F_{50\mu s})$	An approximation of the slope at the origin of fluorescence rise attributed to the rate of Q_A reduction

* In standard PEA fluorescence yield measured at 50 μs after the light onset is assigned to the minimal fluorescence yield, F_O

sensitivity of the detector can be adjusted over a broad range of Chl *a* content between 0.1 and 1,000 μg l^{-1}.

Maximal quantum yield of PS II photochemistry, F_V/F_M (Genty et al. 1989), and coefficient of non-photochemical Chl fluorescence quenching, NPQ (Bilger and Björkman 1990), were estimated using a Pulse Amplitude Modulated fluorometers Water-PAM and PAM-2000, respectively (Walz, Effeltrich, Germany). The weak (0.1 μE m^{-2} s^{-1}) modulated probe flashes (3 μs pulses from a 655-nm light-emitting diode at frequencies of 600 Hz or 20 kHz) were applied to excite Chl fluorescence. The following parameters were recorded: F_O, the fluorescence yield of dark-adapted cells; F_M, the fluorescence yield of dark-adapted cells under a long 0.8 s pulse of saturating actinic light; F_M', the fluorescence yield under a 0.8 s pulse of saturating light applied to cells exposed to continuous actinic light illumination.

Light-induced kinetics of delayed light emission were measured with a computer-controlled rotating-disc phosphoroscope under red light excitation (PPFD = 300 μE m^{-2} s^{-1}). The excitation/dark period was 16/4 ms.

Prior measurements of Chl fluorescence samples were placed in the dark for 15 min.

Results and discussion

Characteristics of OJIP transients recorded in *C. reinhardtii*, *T. weissflogii*, *C. mulleri*, *A. carterae*, and *P. minimum*

In algae and higher plants grown under optimal conditions and adapted shortly to the dark conditions PS II centers are in the open state, i.e., Q_A is fully oxidized, and the Chl fluorescence yield is minimal, F_O. Under strong illumination sufficient to insure the closure of all PS II centers the maximum fluorescence yield, F_M, is achieved. The time-resolved light induced increase in Chl fluorescence measured in dark-adapted green algae and plants with PEA demonstrates complex kinetics with several inflection points, known as the OJIP transient, in which a fluorescence signal at the point of minimum O ($F_{50\mu s}$), and at the point of maximum P, F_P, corresponds to F_O and F_M, respectively (Strasser and Govindjee 1992) (see Table 1). The rapid OJ rise has been ascribed to the reduction of Q_A while the rate of its re-oxidation is maximal, whereas the following JIP phase reflects the further reduction of Q_A due to the decrease in the rate of its re-oxidation, which is modulated mainly by the redox state of plastoquinone (PQ) pool (Antal and Rubin 2008). The quenching of Chl fluorescence during several seconds after the P peak can be ascribed to the ΔpH-dependent energy dissipation in PS II antenna (qE) and/or to the re-oxidation of photosynthetic electron transport chain due to the activation of photosynthetic reactions in chloroplast stroma. Present interpretations of the OJIP transients are related to data obtained on *Chloroplastida* (reviewed in Laźar 2003, Strasser et al. 2005), whereas other groups of photosynthetic organisms are poorly studied in this respect. This makes practical application of the method difficult to gain information about photosynthetic capacity of phytoplankton species, in which diatoms and dinoflagellates prevail.

Figure 1 shows OJIP transients recorded in green alga *C. reinhardtii*, diatoms (*T. weissflogii*, *C. mulleri*), and dinoflagellates (*P. minimum*, *A. carterae*). Kinetics obtained

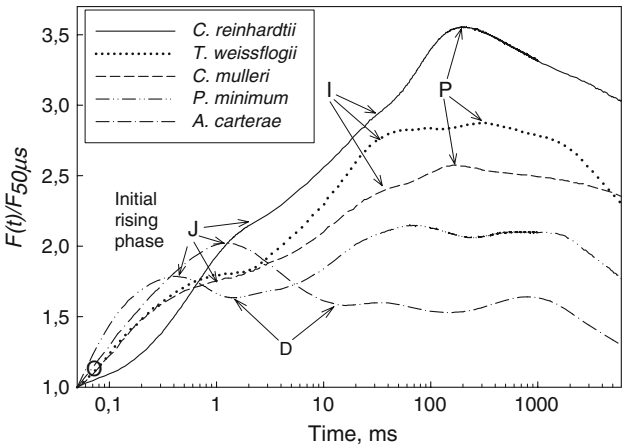

Fig. 1 OJIP transients recorded in *C. reinhardtii* (green algae), *T. weissflogii* (diatom), *C. mulleri* (diatom), *P. minimum* (dinoflagellate), and *A. carterae* (dinoflagellate) with a standard PEA. Fluorescence intensity is normalized to the value at the point O, $F_{50\mu s}$. OJIP transients were induced by PPFD 3000 μE m^{-2} s^{-1}. Before measurements cells were placed in the dark for 15 min

in *C. reinhardtii* is typical of green algae and higher plants showing three distinct phases of the fluorescence rise from $F_{50\mu s}$ at the O point ($\sim F_O$) to the F_P at the P point ($\sim F_M$), followed by the decrease in fluorescence yield. A difference between fluorescence yield $F(t)$ during application of actinic light and initial yield $F_{50\mu s}$ is a variable fluorescence: $F_V(t) = F(t) - F_{50\mu s}$. In Fig. 1 $F(t)$ is normalized to $F_{50\mu s}$, hence, $F_V(t)/F_{50\mu s}=F(t)/F_{50\mu} - 1$. Unlike *C. reinhardtii*, transients recorded in diatoms and dinoflagellates showed the reduced magnitude of variable fluorescence, which decreased successively in sequence *C. reinhardtii* (green algae)–*T. weissflogii* and *C. mulleri* (diatoms)–*P. minimum* and *A. carterae* (dinoflagellate). Moreover, diatoms *T. weissflogii* and *C. mulleri* showed the reduced value of the IP phase, while dinoflagellates *P. minimum* and *A. carterae* did not distinguished I and P peaks of usual shape. Furthermore, dinoflagellates revealed the pronounced dip after the J step (so-called dip D), which can be observed in green algae and plants under certain treatments damaging an OEC (Strasser 1997).

The slope of the initial Chl fluorescence rise in OJIP curve reflects photochemical reduction of Q_A, which is limited by the rate of exciton formation in the PS II antennae (Lazár and Pospìšil 1999). This characteristic depends first on the PPFD and on the PS II antenna absorption cross-section. All examined marine microalgae were characterized by the increased rate of the initial fluorescence rise which started from the point O without the lag phase and showed the highest values in dinoflagellates (Fig. 1). This data suggests the elevated PS II antennae size in phytoplanktonic algae as compared to *C. reinhardtii* grown under the same illumination, that indicates extensive capacity for the acclimation to moderate intensities of ambient light. In this respect, the appearance of the dip D in dinoflagellates can be explained by the high rate of the electron transport from P_{680} to Q_A that may cause the imbalance between oxidation and reduction of the primary electron donor P_{680} resulting in the transient accumulation of P_{680}^+, a fluorescence quencher (Butler 1972), and, hence, in the appearance of the dip.

As known, the shape of the initial fluorescence rise depends on the energetic connectivity between PS II units (Joliot and Joliot 1964). In plants and green algae cultivated under optimal conditions the shape of the initial fluorescence rise shows sigmoidicity (see, e.g., *C. reinhardtii* in Fig. 1) that was explained by the high probability of exciton exchange between PS II units in a supercomplex. Unlike *C. reinhardtii*, the initial phase of the Chl fluorescence kinetic curves measured in diatoms and dinoflagellates revealed the exponential rise (see example in Fig. 2), that may reflect distinct organization of the photosynthetic membranes, which lack the PS II enriched grana fraction of thylakoids. The absence of PSs segregation would rather reduce energetic connectivity between single PS II units.

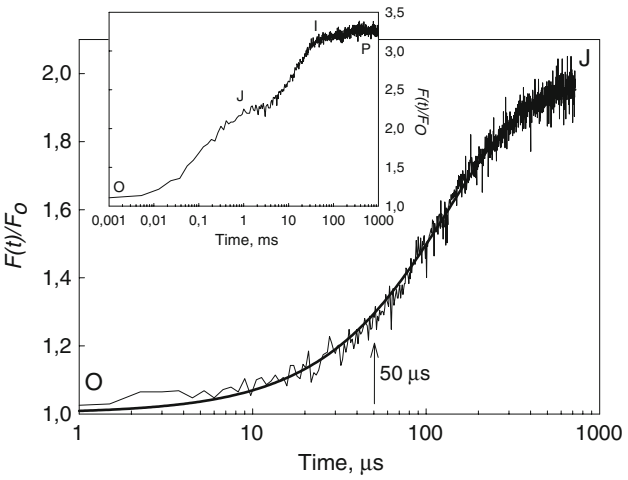

Fig. 2 Initial fluorescence rise (OJ) recorded in *T. weissflogii* with a MEGA-25 fluorometer and result of its one exponential fit. *Arrow* indicates fluorescence yield at 50 µs after the onset of illumination. *Inset* shows the complete fluorescence induction curve. PPFD of actinic light was 3000 µE m^{-2} s^{-1}. Fluorescence intensity is normalized to the F_O value; the latter was determined as described in 'Materials and methods.' Before measurements cells were placed in the dark for 15 min

The maximal magnitude of the variable fluorescence in OJIP transients was reciprocally related to the slope of the initial fluorescence rise (Fig. 1). Thus, the variable fluorescence was the lowest in dinoflagellates, which showed the most rapid initial rise of fluorescence. The low values of variable fluorescence observed in examined marine algae can be explained assuming, that the fluorescence yield at the point O ($F_{50\mu s}$) which is assigned to the F_O yield in PEA exceeds F_O level, because Q_A has been already partly reduced at 50 µs after the illumination onset. This may lead to the overestimation of true F_O value and, thus, to the reduced values of variable fluorescence. In order to test this assumption, we employed MEGA-25 fluorometer recently designed in Biophysical Dept. of Moscow State University to measure high-resolved OJIP transients. In this fluorometer F_O value is measured as a fluorescence signal excited by a short (5 µs) pulse of light (PAM principle). Thereafter, 750 µs and 1.5 s pulses are applied subsequently to induce OJ and JIP phases of fluorescence transient, respectively. A 0.75 µs resolution of signal is reached at the OJ phase and the first point is detected 1 µs after switching on the actinic light. These characteristics allow to estimate changes in fluorescence yield within 1–50 µs interval of illumination, which is beyond the time resolution of a standard PEA. Figure 2 shows the OJ phase of the fluorescence rise recorded on *T. weissflogii* with MEGA-25 at PPFD 3000 µE m^{-2} s^{-1}. The fluorescence yield was not noticeably altered during the first 10 µs of illumination. However, it began to rise after 10 µs of illumination attaining about 20% of OJ

amplitude and 10% of OP amplitude at 50 μs of illumination which is used to determine F_O value in a standard PEA. This result testifies for the overestimation of F_O value by a standard PEA when cells demonstrate high rate of the initial fluorescence rise.

In dark-adapted higher plants, complete reduction of the PQ pool under high light is promoted by the block imposed by ferredoxin-NADP reductase (FNR), which turns to inactive state during few minutes of incubation in the dark (Talts et al. 2007). This insures the complete reduction of the photosynthetic electron transport chain including Q_A upon short exposure of cells to strong light, and, therefore, allows to attain F_M. In the case, when FNR remains active the incomplete reduction of the plastoquinones and Q_A occurs under high irradiation resulting in the underestimation of F_M value (Schansker et al. 2006). In dinoflagellates the essentially reduced slow rising phase was observed in OJIP transients (Fig. 1) that probably reflects the capacity of these organisms to maintain PS I-mediated PQs re-oxidation under strong illumination. To test this assumption, we compared fluorescence transients recorded in *A. carterae* and *P. minimum* at different PPFD of actinic light in the presence of DBMIB, an inhibitor of PQs re-oxidation, and methyl viologen, which promotes PS I-mediated PQ re-oxidation. The red light used in PEA has a maximum at 650 nm, so that PS II receives the excess excitation as compared to PS I. Therefore, the rate of electron transport via PS I should be saturated at higher intensities of light than the electron transport in PS II. We measured fluorescence transients in *P. minimum* and *A. carterae* at two intensities of actinic light close to the upper and lower limits in PEA: 750 and 3000 μE m^{-2} s^{-1}. Fluorescence kinetic curves measured at 750 μE m^{-2} s^{-1} retained the main kinetic steps and were characterized by the essential increase in the magnitude of variable fluorescence as compared to the transients induced by 3000 μE m^{-2} s^{-1} (Fig. 3a, c). In Fig. 3b, d kinetic curves recorded at different intensities of actinic light are normalized to the OJ amplitude. As seen from this figure, the transients measured at lower PPFD showed the reduced slope of the initial fluorescence rise that results in a higher estimation of F_O and F_V values and in retardation of the dip D, as discussed above.

Fluorescence kinetic curves recorded at 3000 μE m^{-2} s^{-1} were characterized by the reduced magnitude of the slow rising phase; the latter is attributed to the reduction of the PQ pool. This data can be explained assuming that PS I efficiently re-oxidizes PQ pool at this light intensity. In *P. minimum* the short incubation (2 min) with 5 μM DBMIB did not affect the magnitude of the transients induced by 750 μE m^{-2} s^{-1}, whereas kinetics recorded at stronger light showed the essentially increased magnitude after the addition of the reagent (Fig. 3a). Furthermore, kinetic curves measured on *A. carterae* at 750 μE m^{-2} s^{-1} were weakly altered by DBMIB, while transients induced by 3000 μE m^{-2} s^{-1} revealed a noticeably increased slow rising phase in the presence of an inhibitor (Fig. 3c). Incubation of *P. minimum* with 1.0 mM methyl viologen did not cause visible changes in fluorescence transients (data not shown). However, induction curves recorded on *A. carterae* at 3000 μE m^{-2} s^{-1} revealed the reduced magnitude of the slow rising phase after treatment with methyl viologen, while kinetics induced by 750 μE m^{-2} s^{-1} were not altered by this reagent (data not shown). Therefore, fluorescence transients measured at 3000 μE m^{-2} s^{-1} were influenced by both DBMIB and methyl viologen to a greater extent than those induced by 750 μE m^{-2} s^{-1}. This result indicates that dark-adapted dinoflagellates rather maintains PS I-mediated electron transport at high intensity of actinic light. This process may potentially lead to the incomplete reduction of the PQ pool during measurements of OJIP transients and to the underestimation of the F_M value ($F_P < F_M$).

Incorrect evaluation of F_O and F_M by standard PEA in phytoplanktonic microalgae should result in underestimation of F_V/F_M, a widely used measure of PS II photochemical activity. Table 2 shows F_V/F_M values evaluated in *C. reinhardtii* and phytoplanktonic microalgae with PEA at PPFD of actinic light 3000 and 750 μE m^{-2} s^{-1} as well as with a Water PAM fluorometer, the latter based on the procedure of fluorescence measurements different from those in PEA (see 'Materials and methods'). The ratio F_V/F_M estimated in *C. reinhardtii* was approximately the same for all measurements. In diatoms and dinoflagellates, F_V/F_M data showed the lowest values when measurements were carried out by PEA at 3000 μE m^{-2} s^{-1}. When PPFD was reduced to 750 μE m^{-2} s^{-1} the values of this parameter increased in all examined marine algae by 8–25%. Measurements provided by the PAM method gave values of F_V/F_M similar to those obtained with a PEA at PPFD 750 μE m^{-2} s^{-1}. This data suggests that to obtain correct evaluation of PS II photochemistry in diatoms and dinoflagellates using a standard PEA it is necessary to provide relatively low intensity of actinic light.

Analysis of OJIP transients in *T. weissflogii* during regeneration from N starvation

As known, the increase in algae biomass in batch cultures leads to the gradual consumption of nitrogen from the media. N deficiency may occur at the steady-state phase of culture growth causing the cease of cell division (Lomas and Glibert 2000; Young and Beardall 2003). The transfer of starved cells into N containing medium would lead to the restoration of the cell population until nitrogen is exhausted again. An investigation of photosynthesis recovery from

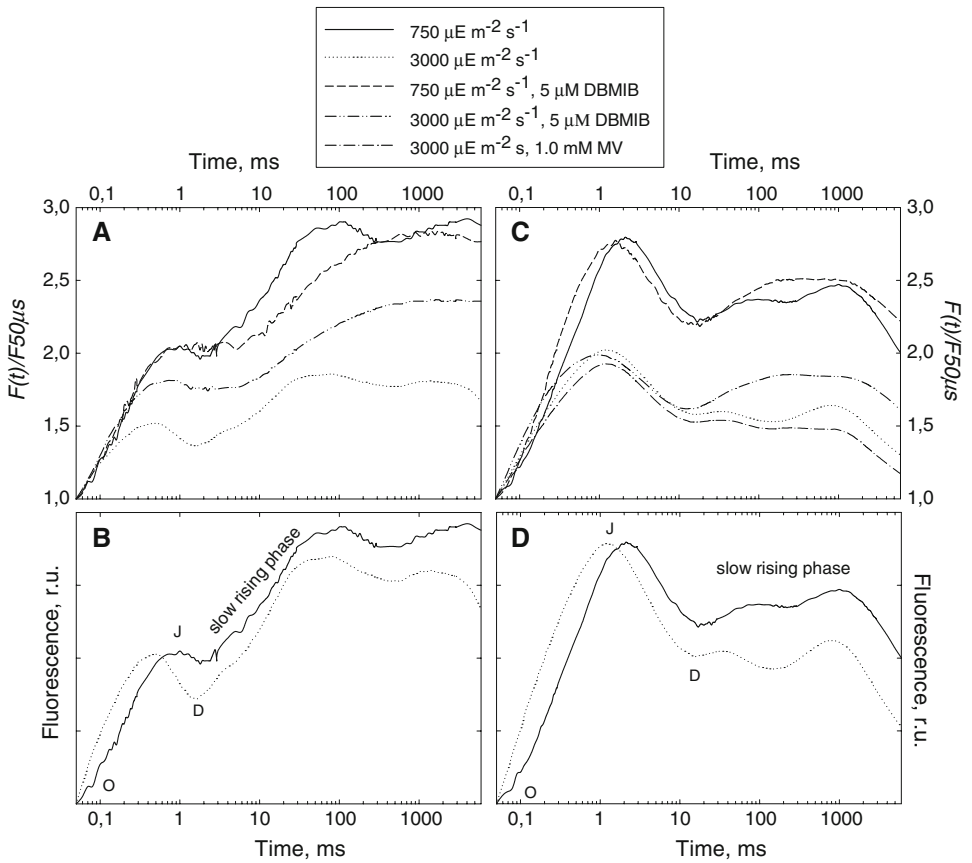

Fig. 3 OJIP transients measured in *P. minimum* (**a**, **b**) and *A. carterae* (**c**, **d**) with PEA at PPFD 750 and 3000 μE m^{-2} s^{-1}. Prior measurements cells were treated with 5 μM DBMIB or 1 mM methyl viologen (**a**, **c**). Fluorescence intensity is normalized to the value at the O point, $F_{50\mu s}$ (**a**, **c**) or to the amplitude of the OJ phase (**b**, **d**). Before measurements cells were placed in the dark for 15 min

Table 2 F_V/F_M calculated in *C. reinhardtii*, *T. weissflogii*, *C. mulleri*, *P. minimum*, and *A. carterae* using PEA and Water PAM fluorometers

	PEA (A)	PEA (B)	PAM
C. reinhardtii	0.73	0.72	0.73
T. weissflogii	0.64	0.71	0.72
C. mulleri	0.58	0.63	0.65
P. minimum	0.54	0.64	0.66
A. carterae	0.47	0.62	0.60

The intensity of saturating pulse in Water PAM fluorometer was 3000 μE m^{-2} s^{-1}. Measurements with PEA were carried out at PPFDs 3000 (A) and 750 μE m^{-2} s^{-1} (B). Before measurements cells were placed in the dark for 15 min

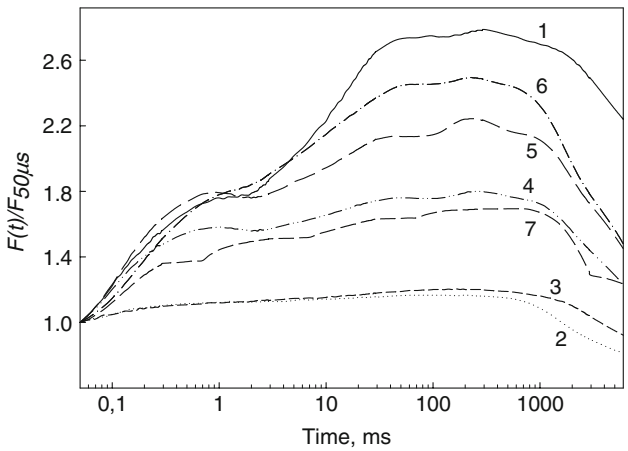

Fig. 4 OJIP transients recorded in *T. weissflogii* with PEA during regeneration of N-starved cells in the medium supplied with nitrate. Fluorescence intensity is normalized to the value at the O point, $F_{50\mu s}$. Curves 1 and 2: control and N-starved cells, respectively (see 'Materials and methods'). Curves 3, 4, 5, 6, and 7: 4 h, 1, 2, 12, and 28 days after transfer of N-starved cells to the N-replete medium. PPFD of actinic light was 3000 μE m^{-2} s^{-1}. Before measurements cells were placed in the dark for 15 min

N starvation in phytoplankton species can be used to obtain information on phytoplankton state in situ, e.g., during spontaneous influxes of nutrients to the upper ocean layers normally depleted of biogenic elements.

In order to characterize photosynthetic capacity of N-deprived *T. weissflogii* during the regeneration in N containing medium OJIP transients were recorded and analyzed at various periods of incubation (Fig. 4). As mentioned above, OJIP kinetics measured on *T. weissflogii* grown in the f/2 medium (control) demonstrated insignificantly reduced variable fluorescence, the rapid initial rise of fluorescence from the point O, and the reduced amplitude of the IP phase, as compared to *C. reinhardtii* (Figs. 1, 4, curve 1).

Fluorescence kinetics recorded on N-starved *T. weissflogii* were characterized by negligible variable fluorescence and by the indistinguishable J, I, and P steps (Fig. 4, curve 2). The reduced variable fluorescence was mainly due to the increase in the initial fluorescence yield, $F_{50\mu s}$ (data not shown) suggesting the appearance of the closed PS II centers and/or uncoupling of the PS II antennae components. F_V/F_M calculated by PAM method in starved cells was 28% of that of the control indicating photodamage of PS II centers as well as impairment of PS II reparation (Kolber et al. 1988, Plumley and Schmidt 1989, Berges et al. 1996). One day incubation of deprived cells in f/10 medium led to the essential increase in the variable fluorescence (F_V/F_M = 79% of control) and to the re-appearance of J, I and P steps (Fig. 4, curve 4). F_V/F_M reached 93% of control values after 12 days of incubation indicating almost complete recovery of PS II photochemistry. Cells concentration in suspension increased by about 3 times from 11×10^3 to 31×10^3 ml^{-1} during this period. The subsequent 16 days of incubation led to the decrease in F_V/F_M to 54% of control (Fig. 4, curve 7), while cells concentration increased insignificantly by 20%. This result can be explained by the re-establishment of N deficiency due to the consumption of nitrate from the f/10 medium containing limited amount of nutrients.

To obtain more information about the recovery of photosynthetic capacity the selected JIP-test parameters (M_O, S_M, and ψ_0) (see Table 1 for formulas and explanations) were calculated from the OJIP transients and the results are presented in Table 3. M_O is a measure of the slope of the initial fluorescence rise, which reflects the rate of Q_A reduction. The M_O value was about two times higher in N-deprived *T. weissflogii* than in control (Table 3), showing the decline from 208 to 117% within 12 days of incubation in N containing medium, followed by its increase to 158% during the subsequent 16 days. This dynamic was inversely related to changes of F_V/F_M indicating that M_O is related to the deprivation status of algae. This data agrees with the fact that nitrogen limited plants and sulphur deprived *C. reinhardtii* showed the elevated rate of the initial fluorescence rise (Strasser et al. 2005, Antal et al. 2007). As was shown, thylakoid preparations with destroyed OEC demonstrated the increased slope of the initial fluorescence rise (Strasser 1997). We suggest that the nutrient deprivation may induce damage of OEC accompanied by the increase in the initial fluorescence rise.

Parameters S_M and ψ_0 are proportional to the amount of PS II centers capable of moving electrons from Q_A to PQ pool via Q_B. Thus, these parameters reach minimal values, when electron transport between Q_A and Q_B is blocked by herbicides, e.g., by DCMU. Noteworthy, that photochemically active PS II showing high value of F_V/F_M and low values of S_M and ψ_0 provide the reduced contribution to the photosynthetic electron flow. As seen in Table 3, N-deprived *T. weissflogii* showed the decreased values of S_M and ψ_0 reaching 59 and 44% of those in control cells, respectively. During 12 days of incubation in nitrogen containing medium, both parameters gradually increased almost to 90%, followed by the decline to 60–70% during the subsequent 16 days of incubation.

According to data in Table 3, the dynamics of F_V/F_M, S_M, and ψ_0 showed obvious similarity suggesting close relationships between the amount of photochemically competent PS II (F_V/F_M) and the capability to transfer electrons between Q_A and PQs (S_M, ψ_0). Probably, the disturbance of electron transport at the acceptor side facilitates the damage of PS II under N deprivation.

Reactions of the light-induced energy dissipation in PS II antenna complex are important in a switching of photoprotecting processes under the excess of light. In *Chloroplastida* these reactions are regulated by trans-thylakoid proton gradient (ΔpH), xanthophylls cycle, and PsbS protein (Holt et al. 2004). Although diatoms share common components, they show some peculiarities, such as the absence of PsbS protein as well as different light-harvesting and xanthophyll cycle organization (Wilhelm et al. 2006). The analysis of the non-photochemical fluorescence quenching (NPQ) is a widely used method to

Table 3 JIP test and NPQ parameters estimated with PEA and PAM-2000 fluorometers in N-deprived *T. weissflogii* during regeneration in N-replete medium

Parameters	Control	0 h	4 h	1 day	2 days	12 days	28 days
M_O	1.2 (100)	2.5 (208)	2.5 (208)	2.4 (200)	2.1 (175)	1.4 (117)	1.89 (158)
S_M	42.1 (100)	24.8 (59)	25.2 (60)	27.8 (66)	33.5 (76)	38.1 (90)	29.3 (70)
ψ_0	0.57 (100)	0.25 (44)	0.27 (47)	0.27 (47)	0.36 (63)	0.49 (86)	0.34 (60)
NPQ_{PEA}	0.25 (100)	0.52 (208)	0.45 (180)	0.54 (216)	0.63 (252)	0.81 (324)	nd
NPQ_{PAM}	1.26 (100)	0.65 (52)	0.65 (52)	1.56 (124)	1.43 (113)	2.79 (221)	nd

JIP test and NPQ_{PEA} parameters were calculated from OJIP transients shown in Fig. 4. NPQ_{PAM} parameter was estimated with PAM-2000 at intensity of saturating pulse 540 μE m^{-2} s^{-1} and at the intensity of actinic light 300 μE m^{-2}. Numbers in parentheses show percentage of the control value

nd not determined due to a high statistical error

study photoprotective processes in higher plants and green algae. Thus, PAM fluorometers allow to study kinetics of NPQ light induction and dark relaxation in algae in a time domain from several minutes (xanthophylls cycle) to hours (photoinhibition-related component) (Schreiber et al. 1995). Fluorescence transients recorded in *T. weissflogii* showed a rapid decrease in the fluorescence yield after reaching maximal value, F_P (Fig. 5). This phase was completely abolished by the treatment with the ammonia chloride which removes ΔpH gradient (Fig. 5). This data suggests relation of the fluorescence yield declination phase to ΔpH-dependent NPQ. In order to assess quantitatively this rapid phase of NPQ we offered a formula: $NPQ_{PEA} = (F_P - F_{6s})/F_{6s}$, where F_{6s} is a fluorescence yield at 6 s in OJIP kinetics. This formulae is analogous to that used to calculate slower (minutes, hours) component of NPQ by PAM method: $NPQ_{PAM} = (F_M - F_M')/F_M'$. Table 3 demonstrates changes of the NPQ_{PEA} during the regeneration of N-starved *T. weissflogii* in the N-replete medium. The value of this parameter calculated in N-starved *T. weissflogii* was two times higher than in control. Two days of incubation of starved cells in N containing medium led to the increase in NPQ_{PEA} to 252% of control, which reached 324% after 12 days of incubation indicating the recovery of the dissipative processes in PS II antenna. Using PAM-2000 fluorometer, we also measured a minute component of NPQ, associated with ΔpH-dependent deepoxidation of diadinoxanthin into diatoxanthin (xanthophyll cycle) (Lavaud et al. 2004). To do it, cells were illuminated during 3 min by PPFD = 300 μE m^{-2} s^{-1} and NPQ_{PAM} was calculated according to formula: $(F_M - F_M')/F_M'$, where F_M and F_M' are the maximal fluorescence yield measured before and after 3 min exposure of dark-adapted cells to actinic light, respectively. As seen in Table 3, NPQ_{PAM}, estimated in starved cells attained 52% of control showing the increase by more than fourfolds up to 221% during 12 days of incubation in N containing medium. Therefore, both parameters NPQ_{PEA} and NPQ_{PAM} increased during the recovery period indicating the suppression of the capacity for the light-induced energy dissipation in the severely starved cells as compared to the recovered cells. However, changes of NPQ_{PEA} and NPQ_{PAM} were essentially different from each other that can be due to the: (1) different methods of measurements and different PPFD of actinic light used to induce NPQ, and (2) different components of NPQ associated with each of the parameters. Thus, NPQ_{PAM} reflects the component related to the xanthophyll cycle, while the mechanism underlying NPQ_{PEA} (seconds) in diatoms is still unclear.

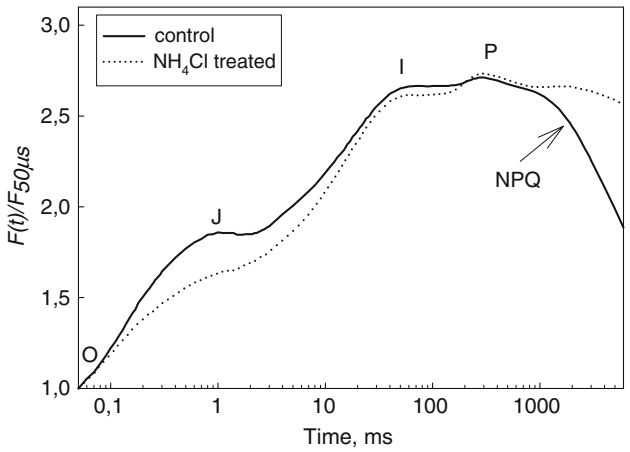

Fig. 5 OJIP transients recorded in *T. weissflogii* with PEA in the presence of 0.2 mM ammonia chloride. Fluorescence intensity is normalized to the value at point O, $F_{50\mu s}$. OJIP transients were induced by PPFD 3000 μE m^{-2} s^{-1}. Before measurements cells were placed in the dark for 15 min

Worth to note that recovered cells revealed much higher values of NPQ_{PEA} and NPQ_{PAM} than control ones suggesting the capability of N-starved *T. weissflogii* to maximize its photoprotection mechanism and to retain this capacity after the almost complete recovery of photosynthesis. This property may play an important role in ecological adaptation of diatoms to the intermittent nutrient supply in a turbulent aquatic environment.

The reduced amplitudes of NPQ in N-starved *T. weissflogii* as compared to those in recovered cells can be coupled to the reduced capacity to develop light driven ΔpH gradient. As known, the intensity of the millisecond component of the delayed light emission is modulated by transmembrane electrochemical potential that allows to use luminescence methods to test light induced changes in ΔpH gradient (Mayne and Clayton 1966). Figure 6 shows light-induced kinetics of the delayed light emission recorded in N-starved *T. weissflogii* before and after 12 days of incubation in N-replete medium. The signal measured on starved cells was two times lower than that observed in cells after the regeneration period. Moreover, it showed the essential suppression of the rising phase with a maximum at 1.2 s (see inset in the figure), which is ascribed to the generation of transmembrane ΔpH. This data indicates that severely starved cells have low capability to generate ΔpH that may limit their capacity to switch photosynthesis into the photoprotective mode.

Conclusions

In this work, OJIP transients were measured with a standard PEA on two diatoms (*T. weissflogii* and *C. mulleri*) and two dinoflagellates (*A. carterae* and *P. tricornutum*). Fluorescence kinetic curves showed the rapid non-sigmoid initial rise from the point O indicating the low connectivity between PS II units and high absorption cross-section of

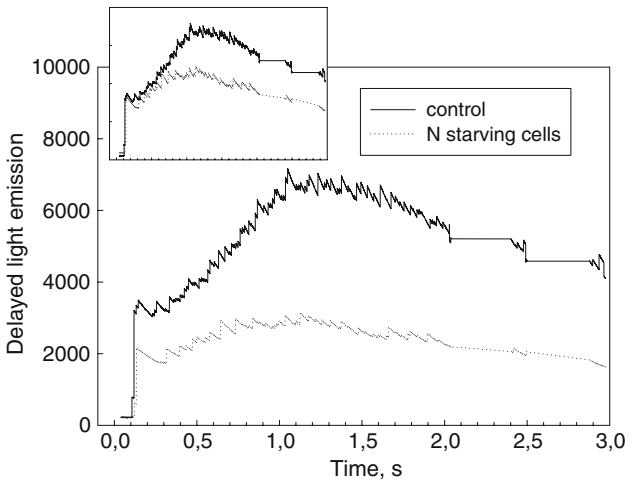

Fig. 6 Light-induced kinetics of delayed light emission recorded in control and N-starved *T. weissflogii*. Signal is normalized to F_O value. PPFD of actinic light was 300 μE m^{-2} s^{-1}. *Inset*: data are normalized to the initial value

PS II antenna. Dinoflagellates exhibited the dip after the J step suggesting the temporal separation between the oxidation and reduction of P_{680}. The analysis of the transients measured in the presence of DBMIB and methyl viologen showed that dark-adapted *A. carterae* and *P. tricornutum* exposed to the strong illumination maintain PS I-mediated PQ pool re-oxidation that may lead to the underestimation of F_M value ($F_P < F_M$).

Using selected parameters of the JIP test and formulas to calculate NPQ by PEA and PAM methods, we studied the recovery of photosynthetic reactions in *T. weissflogii* during incubation of N-starved cells in the medium supplied with nitrogen. N deprivation caused the impairment of electron transport between Q_A and PQs, accumulation of the closed PS II centers, as well as the reduced ability to generate transmembrane ΔpH upon illumination, which were practically fully restored during the regeneration period. Noteworthy that cells with recovered photosynthesis showed much higher values of NPQ than control cells suggesting the enhancement of photoprotective mechanisms in a population with a 'stress history.'

We showed that in OJIP transients recorded in phytoplanktonic algae by PEA the fluorescence yield at the point O ($F_{50\mu s}$) exceeds F_O level because Q_A has been already partly reduced at 50 μs after the onset of illumination. The low intensities of actinic light (<1000 μE m^{-2} s^{-1}) or detectors with rapid response are required to minimize this error.

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