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Photosynthesis in Microalgae as Measured with Delayed Fluorescence Technique

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1. Introduction

Two to three percent of the absorbed sun energy is re-emitted from the pigment systems as fluorescence. Delayed fluorescence (DF) represents only 0.03 % of that emission (Jursinic, 1986). But although DF reflects an insignificant loss of the total energy stored by photosynthesis, it is a sensitive indicator of the many steps in photosynthesis processes (Jursinic, 1986). This sensitivity makes DF an extremely complex phenomenon, however with awareness and control of the variables, DF becomes an important intrinsic probe (Jursinic, 1986).

Delayed fluorescence, also termed delayed luminescence or delayed light emission, is a long-lived light emission by plants, algae and cyanobacteria after being illuminated with light and placed in darkness (Strehler & Arnold, 1951). It can last from milliseconds to several minutes, which is quite a long time in a nanosecond world of classical fluorescence.

The main source of DF is the photosystem II (PSII) (Jursinic, 1986), whereas the photosystem I (PSI) contributes much less to the emission. Delayed fluorescence emission spectrum resembles the fluorescence emission spectrum of chlorophyll a (Arnold & Davidson, 1954; Jursinic, 1986; Van Wijk et al., 1999). The main difference from prompt fluorescence is in the origin of the excited single state of the emitting pigment molecule (Jursinic, 1986). Delayed fluorescence originates from the repopulation of excited states of chlorophyll from the stored energy after charge separation (Jursinic, 1986), whereas prompt fluorescence reflects the radiative de-excitation of excited chlorophyll molecules before charge separation. This is why delayed and prompt fluorescence contain information about different fundamental processes of the photosynthetic apparatus (Goltsev et al., 2003).

An important feature of DF is that it is emitted only by a functionally active chlorophyll – in other words, when photosynthesis is active (Bertsch, 1962). The emission depends on the number of PSII centers and the rate of back reactions in the photosynthetic chain, which are influenced by the membrane potential and pH gradient (Avron & Schreiber, 1979; Joliot & Joliot, 1980).

Delayed fluorescence is affected by many chemical and physical variables, such as ATP (Avron & Schreiber, 1979), proton gradient in the thylakoids (Wraight & Crofts, 1971), chill stress (Melcarek & Brown, 1977), different xenobiotics (Berden-Zrimec et al., 2007; Drinovec

et al., 2004a; Katsumata et al., 2006), excitation light spectral and intensity characteristics (Wang et al., 2004; Zrimec et al., 2005), cell culture growth stage (Berden-Zrimec et al., 2008b; Monti et al., 2005), and nutrient status (Berden-Zrimec et al., 2008a). The changes in chemical and physical parameters affect the reduction state of the plastoquinone pool or its coupling with PSII by modulating the reversed electron flow (Avron & Schreiber, 1979; Mellvig & Tillberg, 1986).

In the field studies, the intensity of delayed fluorescence is used as a measure of photosynthetic activity and living algal biomass (Berden-Zrimec et al., 2009; Krause & Gerhardt, 1984; Kurzbaum et al., 2007; Schneckenburger & Schmidt, 1996). Additionally, DF excitation spectra can be utilized for the analysis of taxonomical changes in the algal communities (Greisberger & Teubner, 2007; Hakanson et al., 2003; Istvanovics et al., 2005; Yacobi et al., 1998) (Figure 1).

2. Basic characteristics of delayed fluorescence decay kinetics

Delayed fluorescence shows monotonic decay kinetics in the first seconds, sometimes followed by a more or less pronounced transient peak (Bertsch & Azzi, 1965). The emission is composed of several components, characterized by different decay rates (Bjorn, 1971; Desai et al., 1983). The faster decaying components (first few seconds) provide information about the fate of energy absorbed by PSII (Desai et al., 1983). The slow components (from few seconds to minutes or hours) originate in back reactions in the photosynthetic chain as well as between the S2 and S3 states of the oxygen evolving complex (OEC) and quinones Q_A and Q_B (Joliot et al., 1971). OEC reacts with quinone molecules and their reduction state is in the equilibrium with PQ. The increase of PQH_2 concentration induces reverse electron flow, producing Q_A^- and Q_B^- states (Joliot & Joliot, 1980), which contribute to DF. The

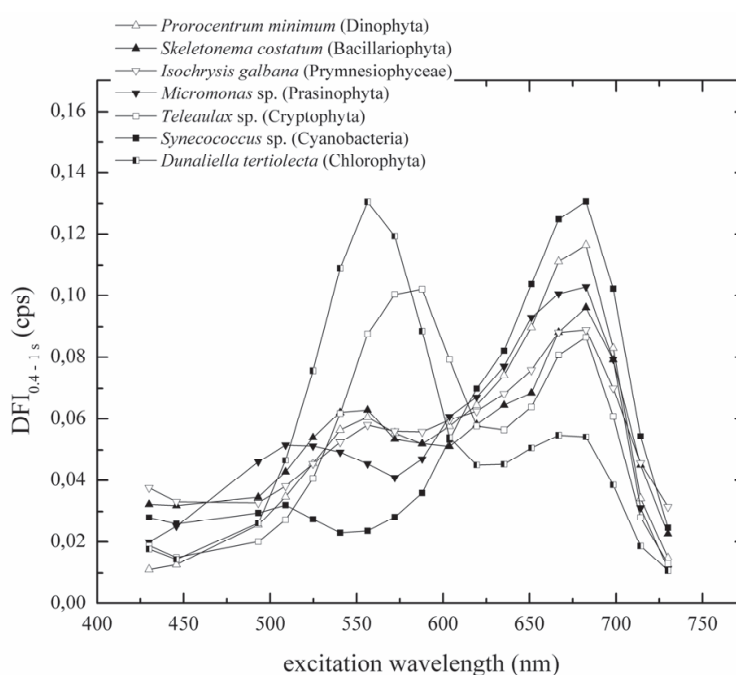


Fig. 1. Delayed fluorescence excitation spectra of marine phytoplankton species. cps – counts per second, $DFI_{0.4-1s}$ – delayed fluorescence intensity in the interval 0.4 – 1 s after sample illumination (Berden-Zrimec et al., 2010).

reduction state of PQ pool is influenced by many reactions including electron transport in PSI and dark reactions of photosynthesis.

The reaction rate is additionally influenced by changes in pH gradient and electric field in thylakoid membrane of chloroplasts. In the case of monotonous DF decay kinetics (without the transition peak), both electric field and pH gradient decay slowly after the initial increase caused by single light pulse excitation.

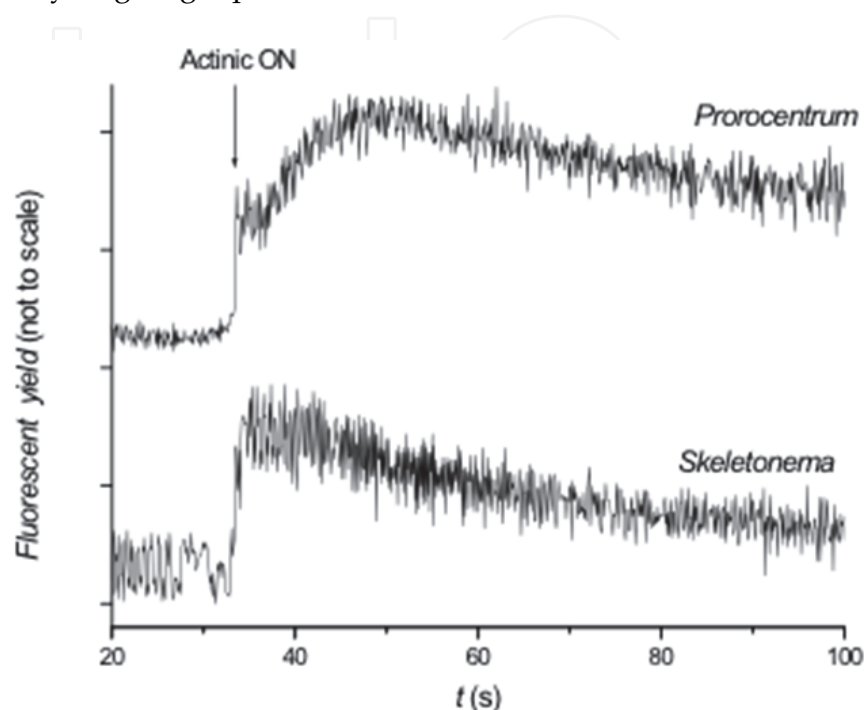


Fig. 2. Slow fluorescence induction kinetics of two marine algae (*Prorocentrum minimum* and *Skeletonema costatum*) after switching on actinic light (Drinovec et al., 2004b).

The slow components probably provide information on temporary energy storage during the photosynthetic electron transport (Desai et al., 1983), because they depend on electron distribution in the plastoquinone pool (PQ) and photosystem I (PSI) (Katsumata et al., 2006). By a measurement of the fluorescent yield it is possible to monitor the reduction state of quinone Q_A in PSII. We measured slow fluorescence induction kinetics by switching on an actinic source of the same intensity as was used for illumination in the DF measurement (Drinovec et al., 2004b). The fluorescence induction curves of marine dinoflagellate *Prorocentrum minimum* had a distinct transient peak and in marine diatom *Skeletonema costatum* a monotonous decay kinetics (Figure 2). In *S. costatum*, a slow decay of fluorescent yield after the initial sharp increase was observed. This is a consequence of progressive oxidation of quinones as Calvin cycle is initiated. *P. minimum* produces a maximum fluorescent yield about 15 seconds after the start of actinic illumination. At this point, the reduction state of the quinones is at the maximum and starts decreasing slowly. This experiment showed the occurrence of transient peaks represents an important physiological parameter for investigation of photosynthetic processes.

The occurrence of the transient peak in DF decay kinetics probably depends on the rates of back reactions and possibly the organization of the thylakoid membrane (Desai et al., 1983). The exact physiological interpretation of transient peaks is quite difficult due to complex electron pathways and their interactions, however they appear to be formed when the

metabolic conditions affect the redox status of Q_A and Q_B directly or indirectly. It has been reported that ATP and NADPH can reduce quinones (Joliot & Joliot, 1980). Degradation of starch to PGA begins immediately after switching off the light. ATP and NADPH thus formed may enhance the reduction of quinones and induce the formation of charge pairs with higher S states as long as they exist (Mellvig & Tillberg, 1986). ATP concentration oscillates after switching cells from light to darkness which is a consequence of feedback mechanisms in the reactions of photosynthesis. A strong coupling of biochemical reactions in thylakoid membrane is also an essential prerequisite for hyperbolic decay.

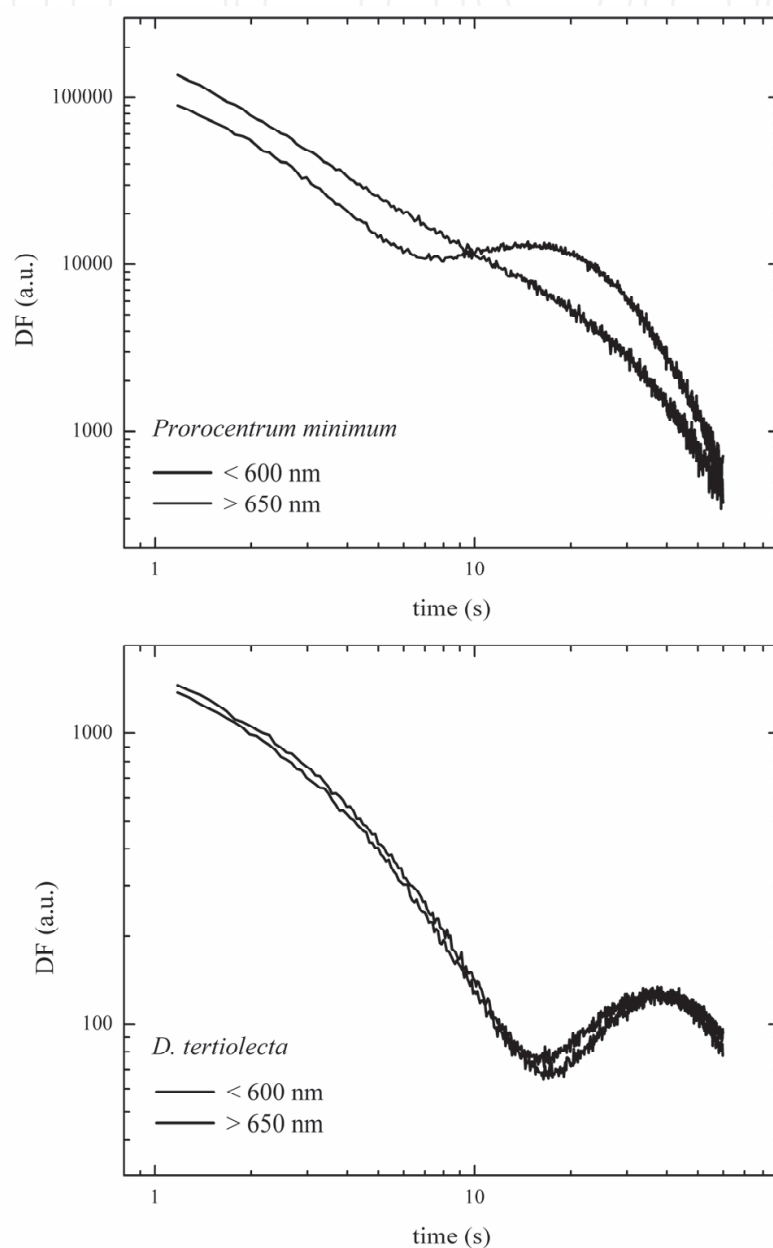


Fig. 3. Delayed fluorescence decay kinetics after illumination with two different wavelengths. a) *Prorocentrum minimum*, b) *Dunaliella tertiolecta*. a.u. – arbitrary units.

The transient peak is preferentially stimulated by far-red excitation (Desai et al., 1983; Hideg et al., 1991), but in some species it can also be induced by shorter wavelengths

(Berden-Zrimec et al., 2008a; Zrimec et al., 2005) (Figure 3). In our experiments, *Dunaliella tertiolecta* Butcher (Chlorophyta) exhibited a peak at the illuminations below 600 nm and above 650 nm (Zrimec et al., 2005) (Figure 3b), whereas *Desmodesmus* (*Scenedesmus*) *subspicatus* Chodat 1926 (Chlorophyta) did not exhibit the peak at all (Berden-Zrimec et al., 2007). *Prorocentrum minimum* (Pavillard) Schiller (Dinophyta) exhibited a peak only when illuminated with wavelengths above 650 nm (Figure 3a). Bertsch (1962) observed the peak in *Chlorella* sp. (Chlorophyta) at the illumination wavelength of 700 nm, but not at 650 nm.

The presence of a peak in DF decay curves after a pulse of light of longer wavelengths indicates PSI involvement in DF generation (Bertsch, 1962; Desai et al., 1983; Hideg et al., 1991; Mellvig & Tillberg, 1986), because far-red light is predominantly absorbed by PSI. If cyclic electron flow produces excess ATP over NADPH, back electron flow from PSI can be generated, resulting in the transient peak from a few to tens of seconds after their being illuminated (Mellvig & Tillberg, 1986).

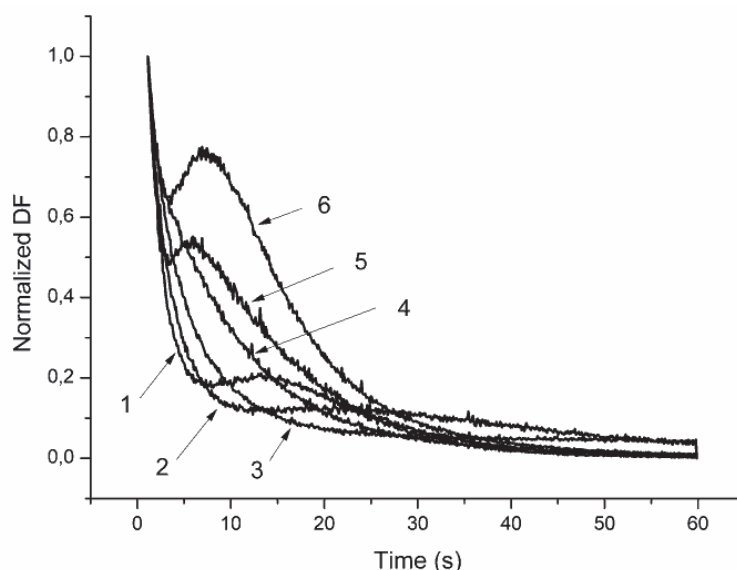


Fig. 4. Normalized delayed fluorescence decay curves of different algal species after a 3 s white-light illumination pulse. (1) *Prorocentrum minimum* (Dinophyta), (2) *Scrippsiella trochoidea* (Dinophyta), (3) *Gyrodinium* sp. (Dinophyta), (4) *Skeletonema costatum* (Bacillariophyceae), (5) *Pyrenomonas* sp. (Chryptophyta), (6) *Isochrysis galbana* (Chrysophyta). (Berden-Zrimec et al., 2010).

The peak position varies greatly between species (Figure 4). In algae, the peak is usually positioned in first minute after the illumination (Figure 4) (Mellvig & Tillberg, 1986), whereas in higher plants it lies in the range of minutes after excitation (Desai et al., 1983). DF decay kinetics can differ even among strains (Berden-Zrimec et al., 2008b; Monti et al., 2005), due to the kinetic rate constants of the electron back reactions depending on the physiological and organizational state of the entire photosynthetic apparatus. Several peaks appear when algae are put in low CO₂ conditions or as a consequence of phosphorous starvation (Mellvig & Tillberg, 1986). In both cases, dark reactions of photosynthesis are affected.

3. Modelling of delayed fluorescence kinetics

There are several proposed phenomenological models of long-term DF decay kinetics: the “multiexponential models” (Schmidt & Schneckenburger, 1992), the “hyperbolic models” (Lavorel & Dennery, 1982; Scordino et al., 1993), the “coherence models” (Yan et al., 2005). Recently, Qiang Li and co-workers presented a very interesting mathematical-physical analysis where they modeled the electron reflux for photosynthetic electron transport chain (Li et al., 2007). Unfortunately, all the presently published models fail to include all the experimental data available in the literature. Especially problematic is the modeling of DF decay kinetics with the transient peaks.

The monotonous decay kinetics (Figure 5) is simply described as a hyperbolic decay using a function $I=I_0/(t+t_0)^m$ (Scordino et al., 1996) (Figure 6). In usual decay processes the relaxation kinetics is exponential. A hyperbolic decay kinetics was reported to be a sign of a coupled system (Lavorel & Dennery, 1982).

We modeled the non-monotonous relaxation kinetics of DF – the decay curves with the transient peak – as a multiexponential relaxation among three pools of electrons in metastable states. The first pool are the excited electrons, stabilized on quinones, $q(t)$, that relaxes as delayed fluorescence emission or by transfer to the plastoquinone pool, $p(t)$, which in turn preferably relaxes further to the slower reactions, $d(t)$, or back to the quinones (Eqs. 1, 2 and 3):

$$q'(t) = -k_1q(t) + k_2p(t), \tag{1}$$

$$p'(t) = -k_2p(t) + k_3d(t), \tag{2}$$

$$d'(t) = -k_3d(t), \tag{3}$$

where t is time in seconds, and k_i are the kinetic constants, and we assume only the pool of electrons that eventually relaxes back to the ground state by emitting delayed fluorescence.

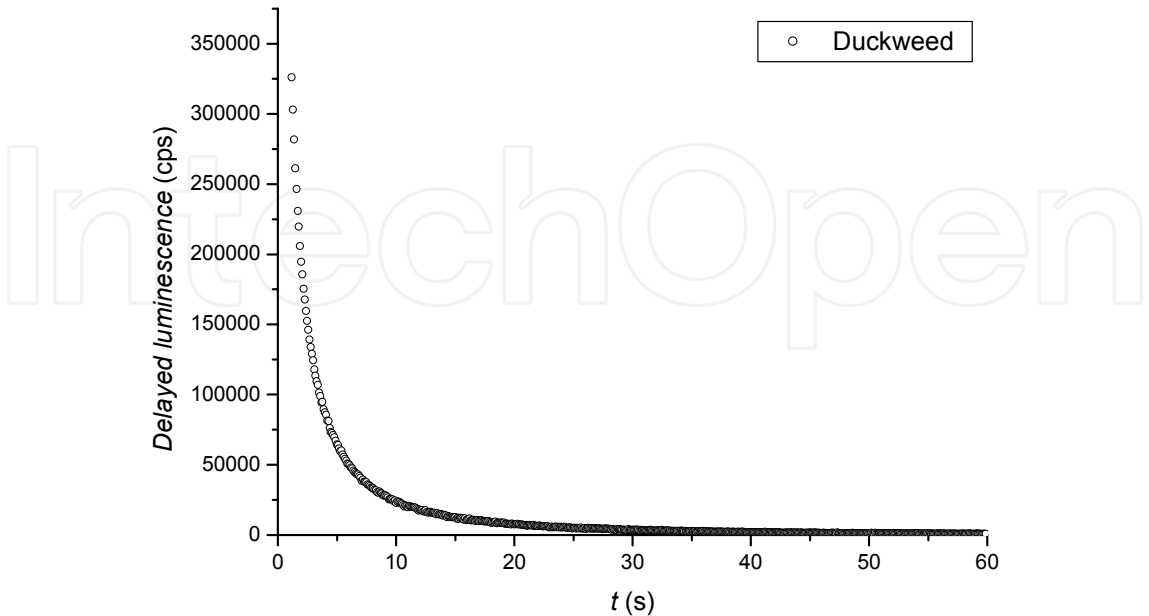


Fig. 5. A monotonous delayed fluorescence of duckweed (*Lemna minor*). cps – counts per second.

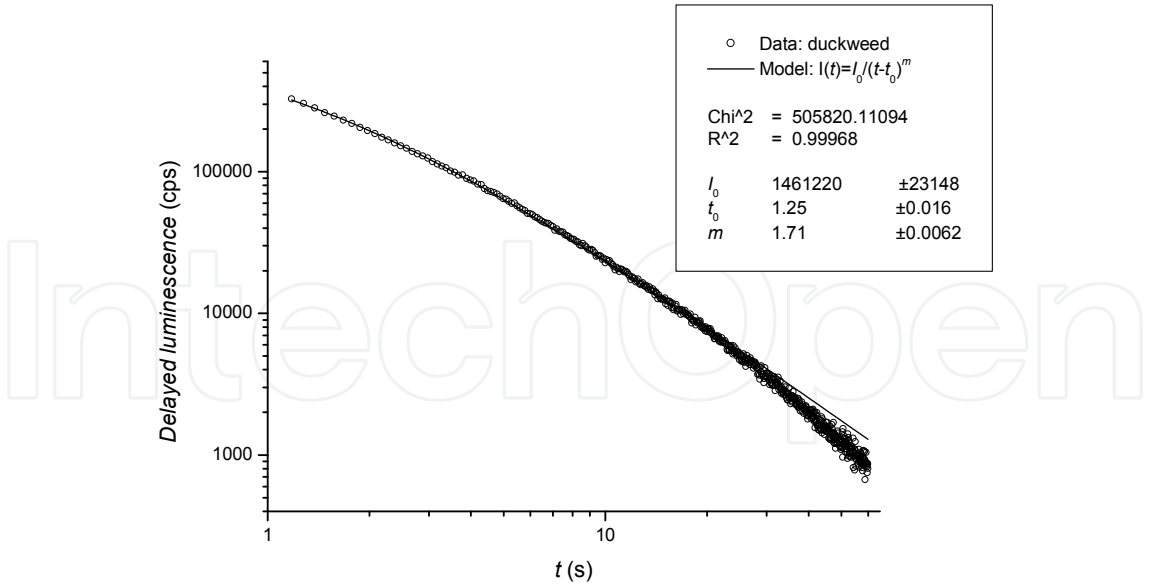


Fig. 6. Delayed fluorescence kinetics of duckweed in log- log scale. A hyperbolic curve was fitted to the measured data. cps – counts per second (Drinovec et al., 2004b).

To model the different positions and amplitudes of the transient peak, we introduced a variable parameter, a , that continuously variates the different equation parameters. The variation in the peak amplitude (Figure 7) is best ascribed to the different pumping of the system, therefore a variates the initial distribution of excited electrons among the three pools – where $a=1$ when the quinone pool is maximized. The location of the peak (Figure 8) depends on the reactions rate, namely the kinetic constants – where $a=1$ when the k_i are maximized. The combined effect of both, the pumping rate and the relaxation rate, best models the variations in temperature dependence of DF (Figure 9). In this case, the initial distribution of electrons among the three pools as well as the kinetic constants variate in parallel. Higher temperatures are modeled with higher values of the parameter a .

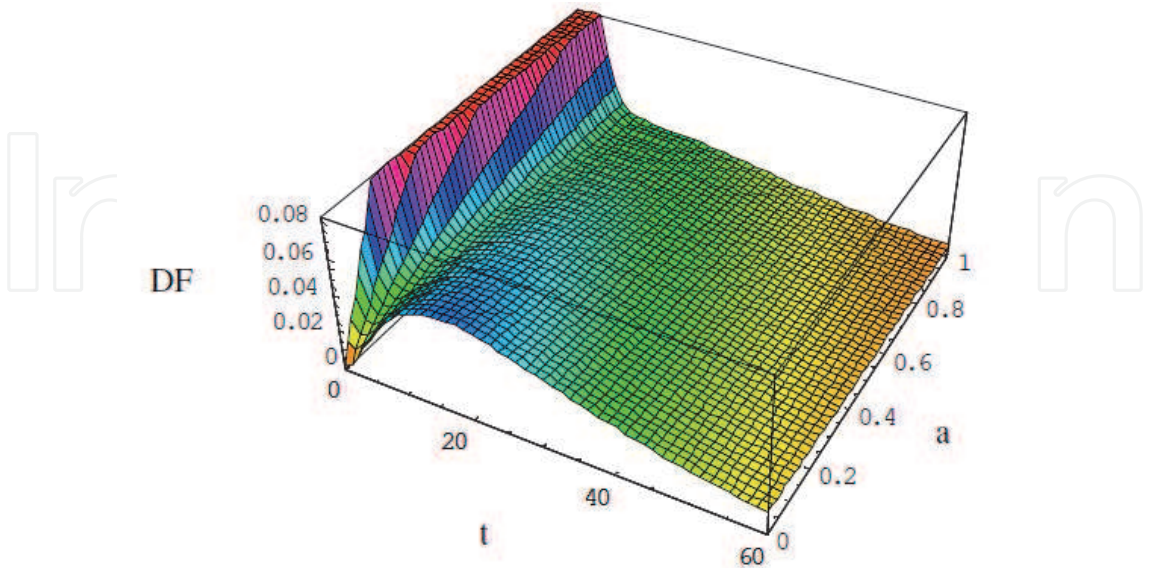


Fig. 7. Dependence of the transient peak amplitude on the initial pumping of the electron pools. DF is in relative units, t is in seconds, and a is the variable parameter, where $a=1$ when the quinone pool is maximized.

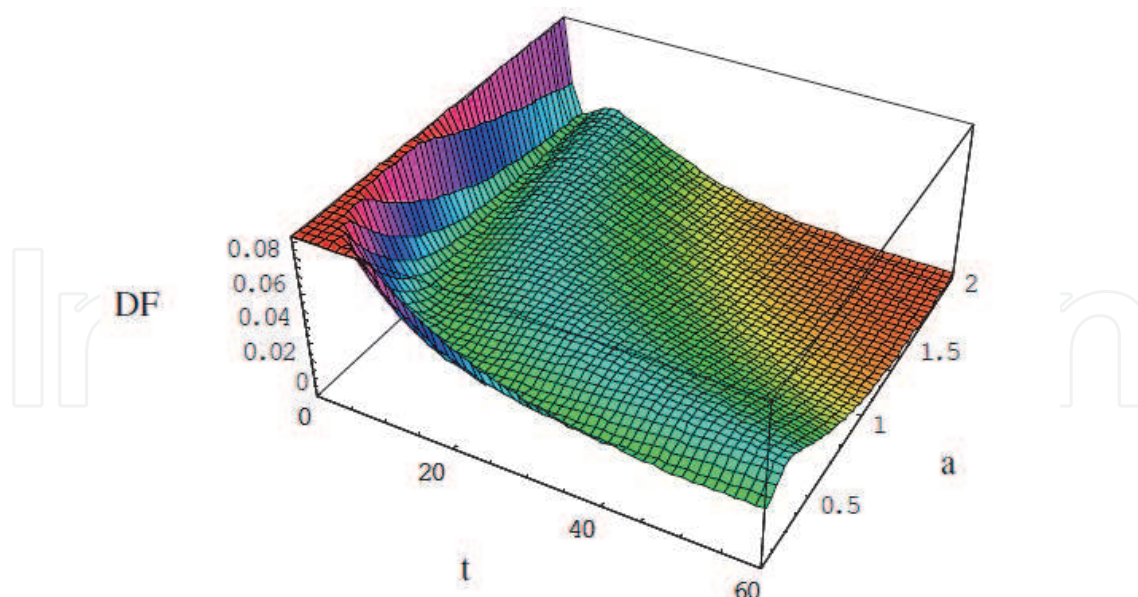


Fig. 8. Dependence of the transient peak position on the reaction rates. DF is in relative units, t is in seconds, and a is the variable parameter, where $a=1$ when the kinetic constants are maximized.

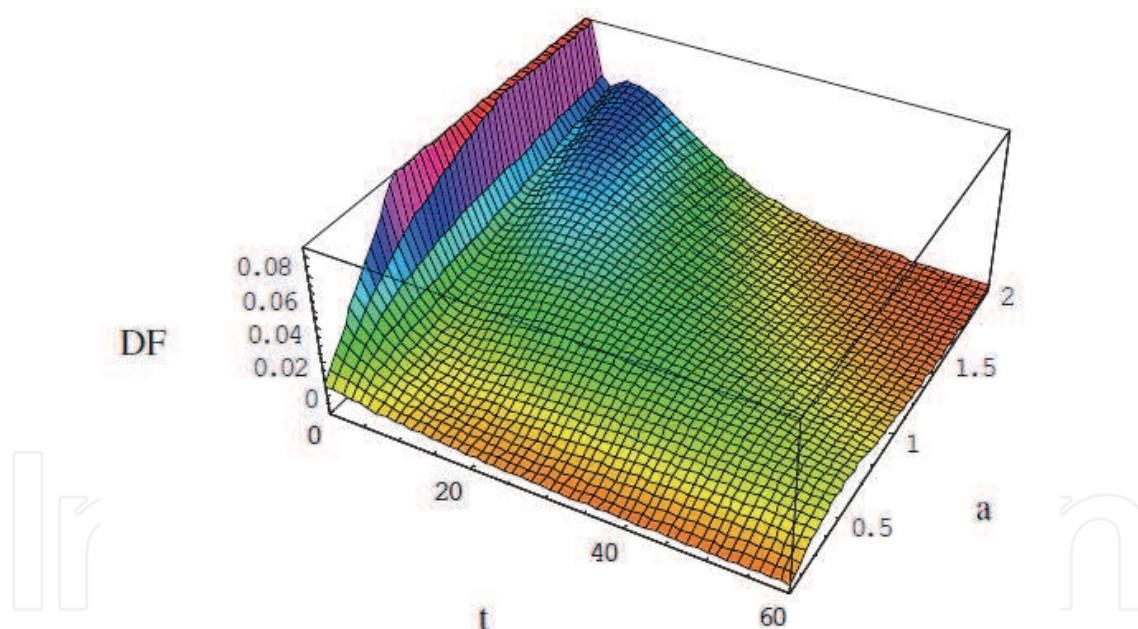


Fig. 9. Dependence of the transient peak on temperature. Both, peak position and amplitude change with temperature, with higher a standing for higher temperatures. DF is in relative units, t is in seconds.

4. Physiology

In delayed fluorescence, specific changes of physiological state are reflected in its intensity and kinetics. Delayed fluorescence intensity (DFI) is represented by an integral under the DF decay curve. In many cases, DFI can be utilized as a measure of living cell concentration (Berden-Zrimec et al., 2009). It also reflects the number of PSII centers, the fluorescence yield, and the

rate of back reactions, which are influenced by the membrane potential and pH gradient (Avron & Schreiber, 1979; Joliot et al., 1971; Joliot & Joliot, 1980; Wraight & Crofts, 1971). In DF kinetics, the changes are most obvious when observing the position and intensity of the transient peak. The peak is culture-state dependent – the peak position and intensity change during culture growth (Berden-Zrimec et al., 2008a; Monti et al., 2005). The results presented here were acquired by a 3 seconds long illumination and a sensor with a red-light-sensitive photomultiplier tube (Hamamatsu R1104) with a Hamamatsu C3866 Photon Counting Unit for signal conditioning and amplification (Monti et al., 2005; Zrimec et al., 2005).

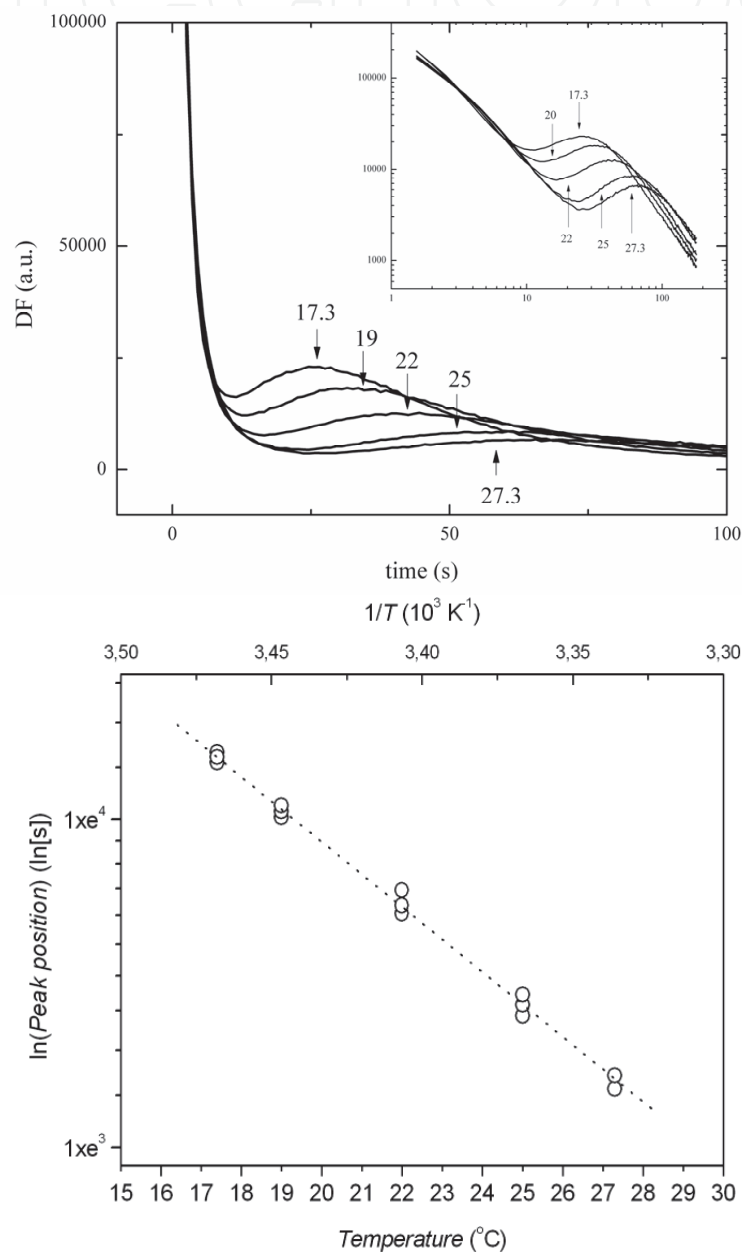


Fig. 10. Temperature dependence of delayed fluorescence. a) Delayed fluorescence decay kinetics at different temperatures in *Prorocentrum minimum* (Dinophyta); inset: log-log scale of DF decay kinetics, a.u. – arbitrary units; b) temperature dependence of the peak position: the line represents a linear fit.

4.1 Temperature and illumination intensity dependence

The temperature and illumination intensity strongly influence the transient peak position and the intensity. With increasing temperature, peak position is moving towards the beginning of the relaxation curve (Figure 10a) and average delayed fluorescence intensity increases until a maximum around 28 - 30°C (Wang et al., 2004; Yan et al., 2005; Zrimec et al., 2005). Due to changes in the kinetics, the temperature dependence of DFI is more complicated because it also depends on the time interval on which it is averaged.

The peak position has a typical temperature dependency for metabolic biochemical reactions (Zrimec et al., 2005). In the Arrhenius plot the natural logarithm of the peak position is linearly dependent on temperature (Figure 10b). Q10 of 2.6 and the activation energy of 71.5 kJ/mol calculated from the plot are in the expected range of plastoquinone-PSII reactions (Zrimec et al., 2005).

The illumination intensity profoundly influences only the peak intensity and less the peak position (Figure 11). Delayed emission can already be observed at relatively low illumination intensities. In the experiment with *Dunaliella tertiolecta* Butcher (Chlorophyta), DF was saturated already by a 3 s excitation pulse of 15 $\mu\text{mol m}^{-2} \text{s}^{-1}$ PAR (Zrimec et al., 2005), which is even lower than obtained by Wang et al. (2004) for isolated spinach chloroplasts. At the excitation light intensity of 3.75 $\mu\text{mol m}^{-2} \text{s}^{-1}$ PAR, DF showed slight differences in decay kinetics in the region of the transient peak, probably due to the changed oxidation state of the plastoquinone pool (Zrimec et al., 2005). DFI at 3.75 $\mu\text{mol m}^{-2} \text{s}^{-1}$ PAR was only approximately 3% lower than at 15 and 60 $\mu\text{mol m}^{-2} \text{s}^{-1}$ PAR (Zrimec et al., 2005). The maximal peak intensity was observed at the light intensity which was used for growing of batch cultures.

Dependence on light intensity can be explained by its influence on the ratio of the light captured by PSI and PSII: at low light intensity more light is absorbed by PSI, because its absorption spectrum has a maximum at longer wavelengths compared to the PSII. Thus cyclic electron transport producing only ATP is stronger than linear transport and an excess of ATP over NADPH is produced. The electron flow through PSI also causes oxidation of the plastoquinone pool. At higher light intensities the PSI get saturated and the ratio of the light absorbed by PSI compared to PSII is decreased.

4.2 Salinity

Changes in salinity influence photosynthesis in several ways. Increased salinity studies in the red alga *Porphyra perforata* showed there are at least three sites in the photosynthetic apparatus that are affected (Sato et al., 1983). The first site, photoactivation and dark-inactivation of electron flow on the reducing side of PSI, was completely inhibited at high salinity. The second site, electron flow on the oxidizing side (water side) of PSII, was inhibited as was the re-oxidation of Q in the presence of 3-(3,4-dichlorophenyl)-1,1-dimethylurea. The third site affected by high salinity was the transfer of light energy probably from pigment system II to I. High salinity also reduced the amount of light energy that reached the reaction centers of PSII.

Photosynthetic activity was reduced by lowered salinity in two brown algae *Ascophyllum nodosum* and *Fucus vesiculosus* (Connan & Stengel, 2011). Chlorophyll and phycobiliprotein concentrations were lower in changed salinity conditions in red alga *Gelidium coulteri* (Macler, 1988).

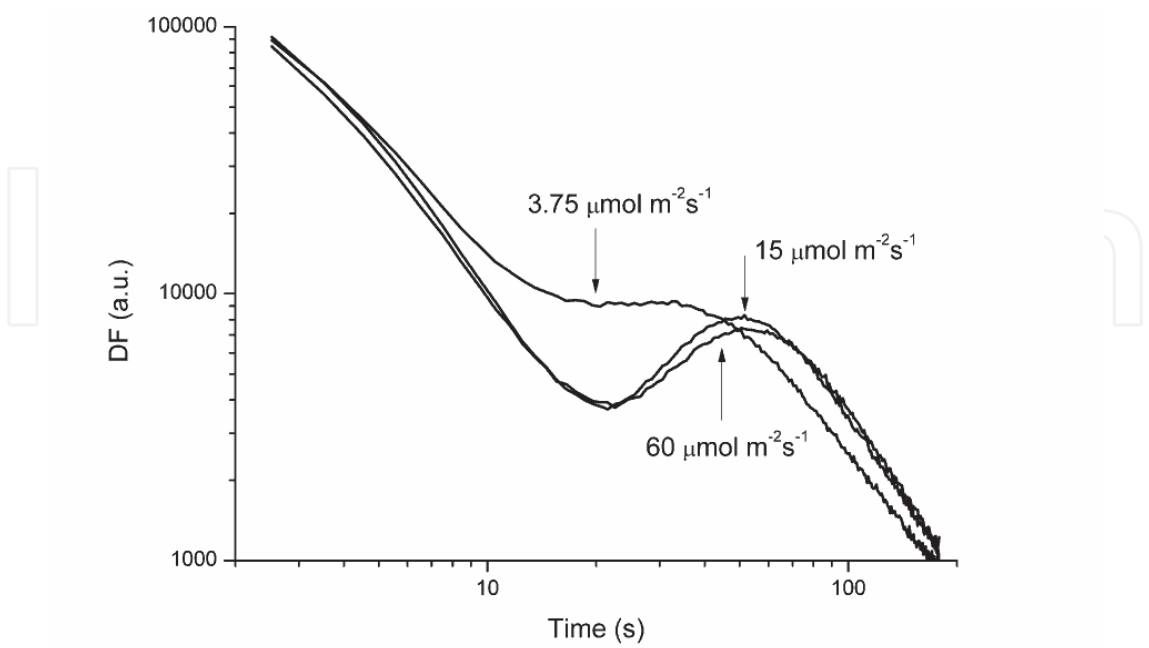
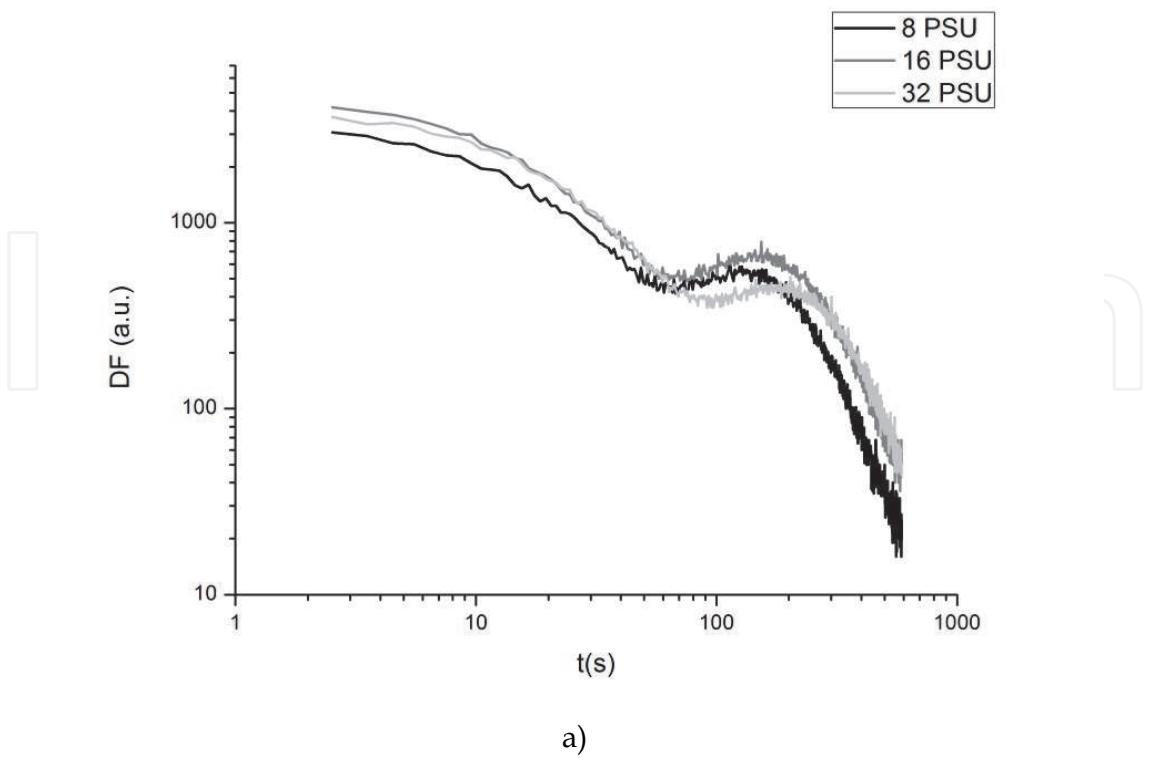


Fig. 11. Delayed fluorescence decay kinetics of *Dunaliella tertiolecta* after 3 s illumination with white light of different intensities.



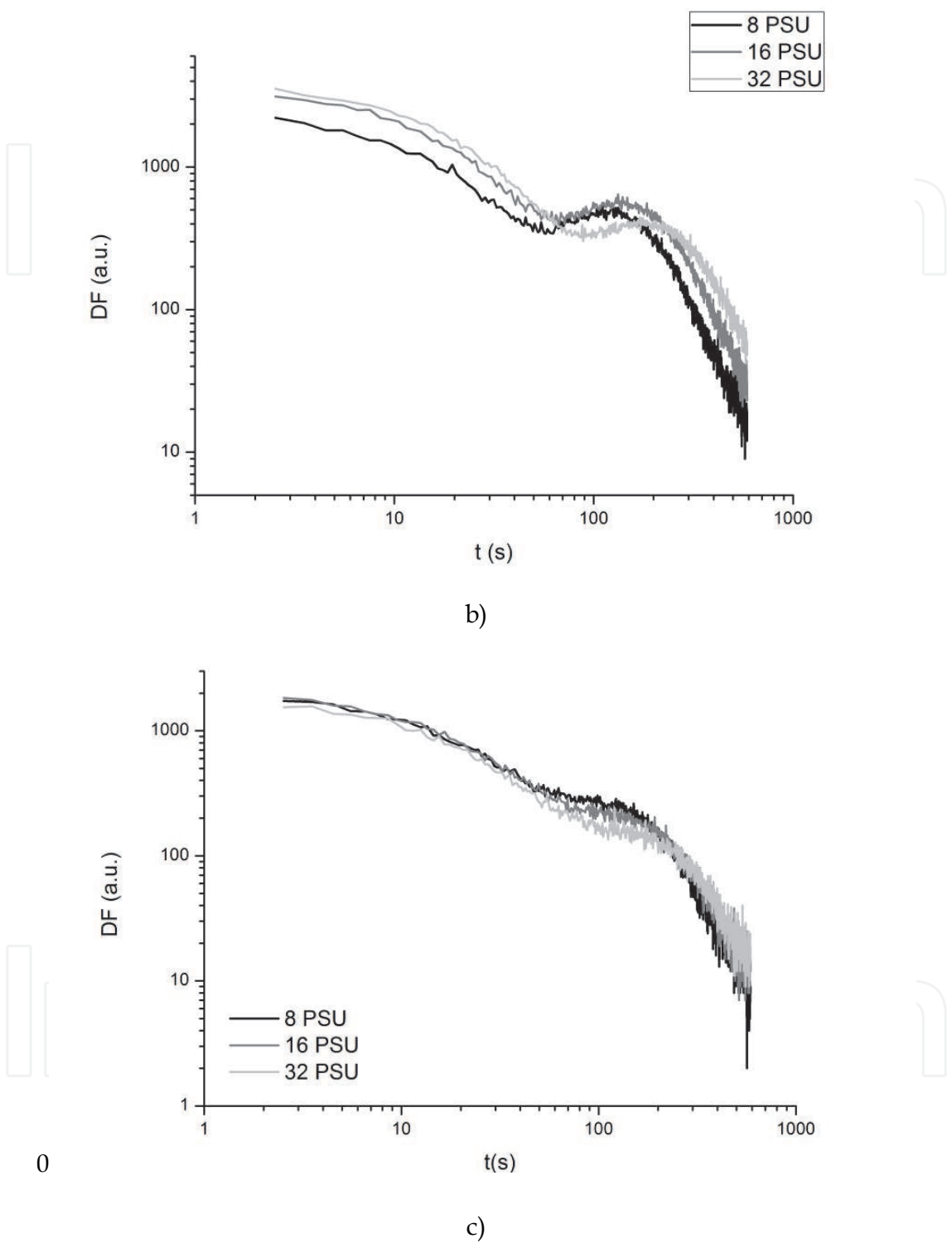
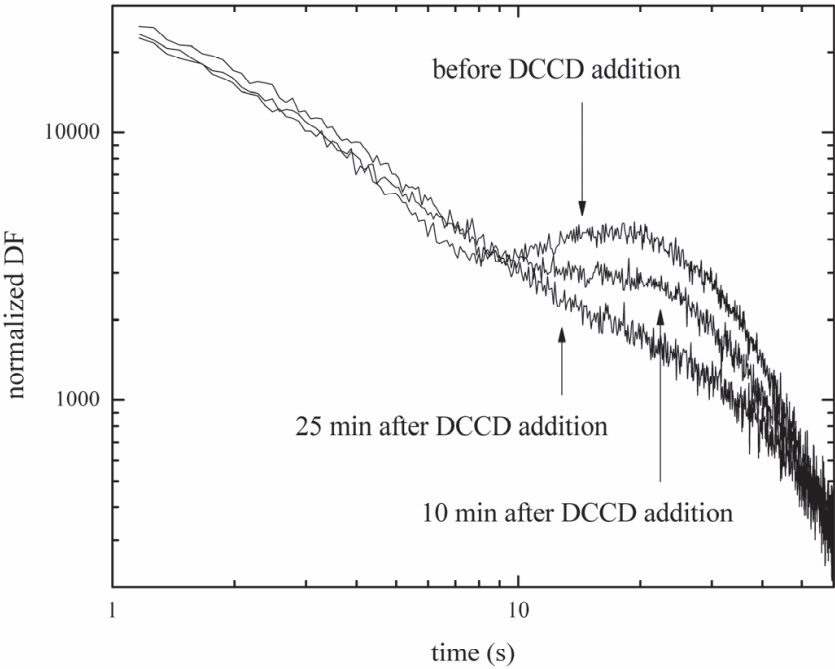
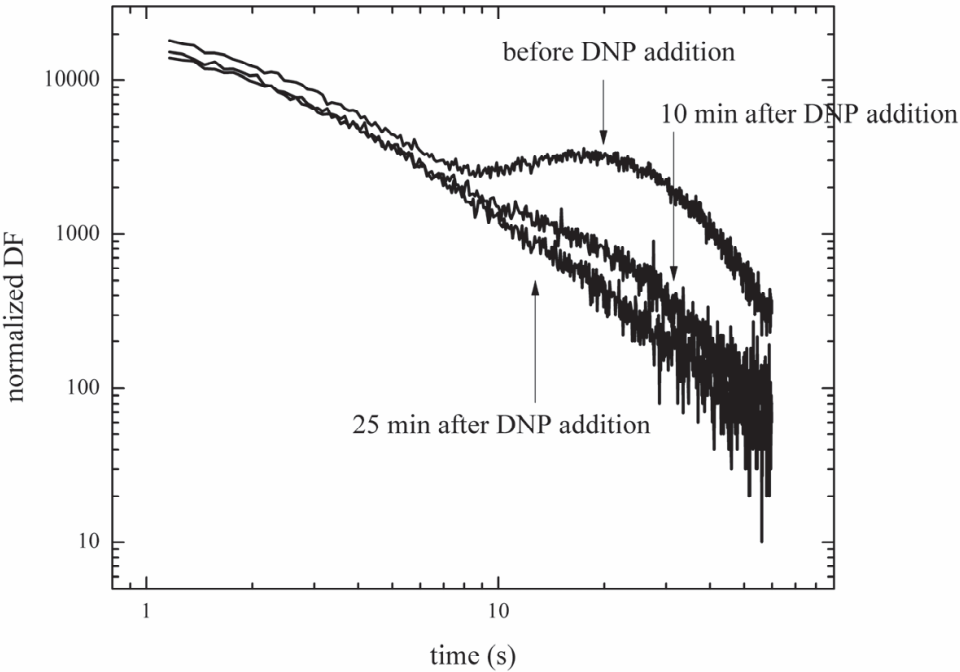


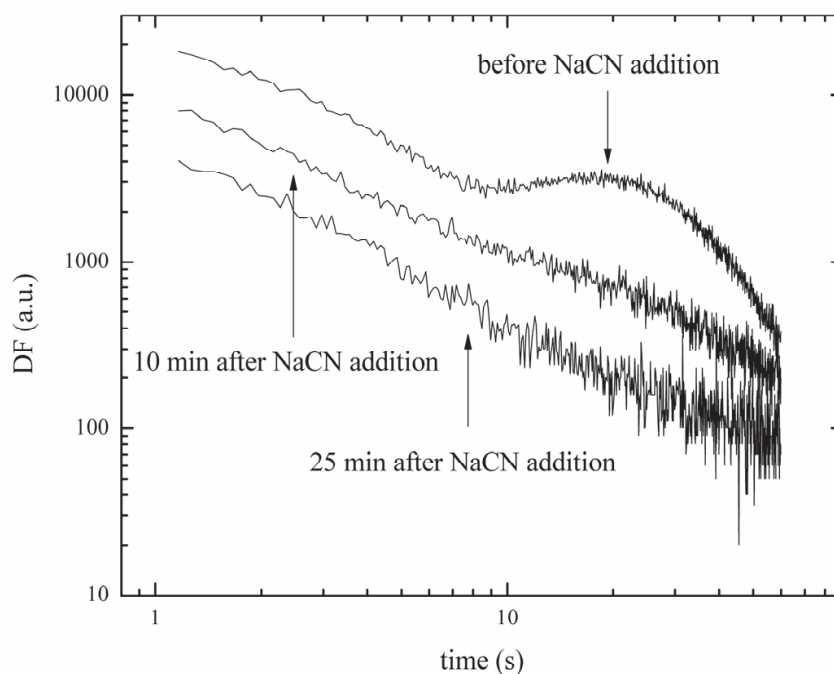
Fig. 12. Delayed fluorescence decay kinetics at different salinities in three strains of *Prorocentrum minimum*. a) strain from the Adriatic Sea, original salinity 32 PSU; b) strain from the Chesapeake Bay, USA, original salinity 16 PSU; c) strain from the Baltic Sea, original salinity 8 PSU. a.u. – arbitrary units.



a)



b)



c)

Fig. 13. Influence of photosynthetic inhibitors on *P. minimum* DF decay kinetics: a) 15 μ M dicyclohexylcarbodiimide (DCCD) – inhibition of ATP synthesis inhibition; b) 2 μ M dinitrophenol (DNP) – destruction of proton gradient and inhibition of electron flow in thylakoid membrane; c) 30 mM NaCN – inhibition of respiration and photosynthesis.

In DF kinetics, higher salinity delayed the transient peak in three strains of *Prorocentrum minimum* (Figure 12). The strains were originally growing at different salinities, namely the Baltic strain (BAL) at 8 PSU, the Chesapeake Bay strain (D5) at 16 PSU, and the Adriatic strain (PmK) at 32 PSU (Monti et al., 2005). In our study, all strains were incubated in all three salinities for their whole growth period. BAL had in general earliest peak onset and PmK the latest in all salinities. The transient peak ceased at the end of the growth period, disappearing sooner at 32 PSU than in the lower salinities.

4.3 Influence of toxins

Toxic effects of photosynthesis inhibitors can be measured by DF already after a few minutes of incubation (Figure 13) (Berden-Zrimec et al., 2007). The transient peak can disappear soon after the addition of photosynthetic inhibitors like ATP synthesis inhibitor dicyclohexylcarbodiimide (DCCD) – (Figure 13a), dinitrophenol (DNP), which destroys the proton gradient and inhibits electron flow in thylakoid membranes (Figure 13b), or NaCN, an inhibitor of respiration and photosynthesis (Figure 13c) (Berden-Zrimec et al., 2010). These toxins influence photosynthesis in different ways, but at the end they affect the reduction state of the plastoquinone pool or its coupling with PSII by inhibiting the reversed electron flow and thus DF (Wang et al., 2004).

Delayed fluorescence response to toxins is dose-dependent (Figure 14), like in the case of herbicide diuron (DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea), which competes with

plastoquinone and plastoquinol for the Q_B binding site, preventing the electron flow between PSII and the plastoquinone pool, or 3,5-dichlorophenol (3,5-DCP), which is used as an unspecific reference toxicant in toxicity tests (Berden-Zrimec et al., 2007). DNP locks the ATP-ase in open state, thus allowing H^+ ions to pass freely. The backreactions in PSII are enhanced by pH gradient in thylakoid membrane and there is a report that a permanent pH gradient in thylakoid membrane is present even in the dark (Joliot and Joliot 1980). The reason for the reduction of DF intensity in the region before peak formation is most likely the collapse of pH gradient caused by DNP. The disappearance of transient peak after 10 minutes of DNP action confirms the idea that pH gradient is directly or indirectly responsible for peak formation.

DF is a very good parameter in rapid toxicity tests (Berden-Zrimec et al., 2007; Katsumata et al., 2006). DFI was equally or more sensitive to the tested toxicants compared with the cell concentration and absorbance, which are standard parameters in algal growth inhibition tests (Berden-Zrimec et al., 2007). The advantage of the delayed fluorescence is that only living cells are measured, the sensitivity in toxicity tests thus being increased. Additionally, minimal disturbance to the cells, small sample volumes enabling homogenous illumination of all samples, and short test duration minimize a negative influence of changing physico-chemical properties of the medium on the results, being the most important features for the quality toxicity tests.

4.4 Nutrients

The nutrient status of algal cells markedly influences delayed fluorescence decay kinetics (Berden-Zrimec et al., 2008a; Burger & Schmidt, 1988; Mellvig & Tillberg, 1986). Phosphorus starvation can induce one or several transient peaks (Mellvig & Tillberg, 1986) or change the peak position (Berden-Zrimec et al., 2008a) (Figure 15). Nitrogen limitation also influences the peak position as well as causes the peak cessation (Figure 15) (Burger & Schmidt, 1988). DFI per cell significantly increases due to both, phosphorus and nitrogen limitation (Figure 16).

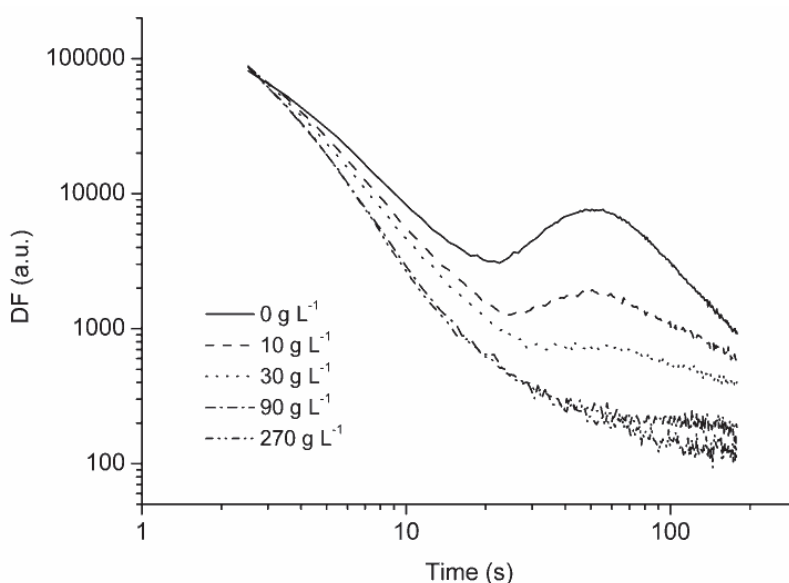


Fig. 14. The response of delayed fluorescence decay kinetics to geometrical series of diuron (DCMU) concentrations.

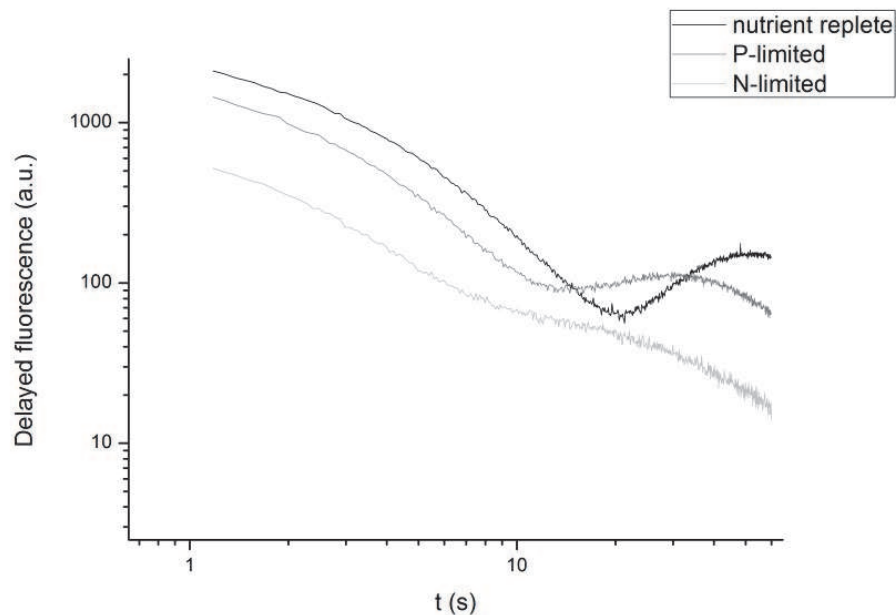


Fig. 15. Peak position dependence on nutrient status in *Dunaliella tertiolecta* cells. P – phosphorus, N – nitrogen, a.u. – arbitrary units.

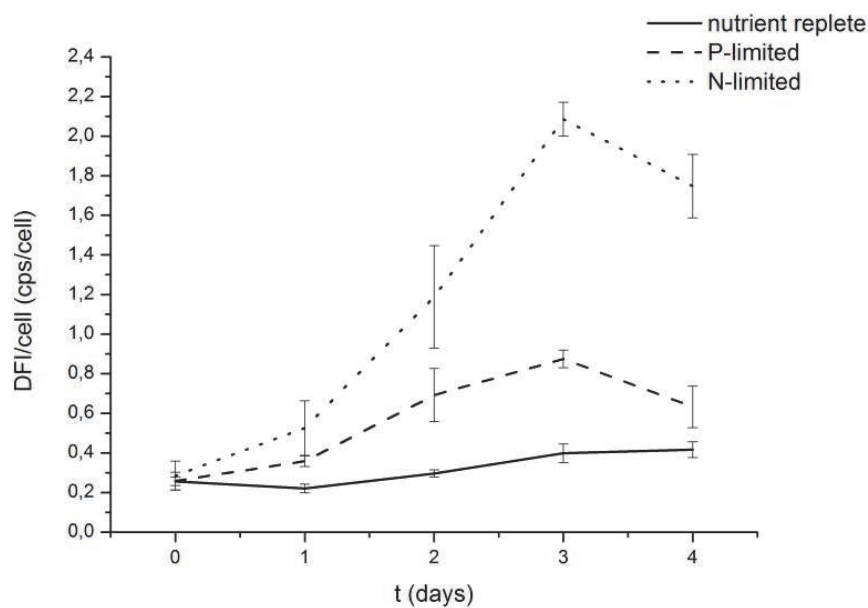


Fig. 16. DFI per cell dependence on nutrient status in *Dunaliella tertiolecta* cells. P – phosphorus, N – nitrogen, a.u. – arbitrary units.

Nitrogen and phosphorus starvation influence DF decay kinetics by changing the back reactions rates in the electron backflow. Nitrogen starvation causes reduction in the number of active PSII reactive centers and linear electron flow, but does not influence active PSI leading to relatively higher rates of cyclic photophosphorylation (Berges et al., 1996). Nitrogen starvation also influences thylakoid organization, facilitating trapped energy transfer from PSII to PSI (Berges et al., 1996). In marine phytoplankton, nitrogen limitation affects photosynthesis by reducing the efficiency of energy collection due to loss of

chlorophyll a and increases non-photochemically active carotenoid pigments (Berges et al., 1996; Geider et al., 1998). Phosphate availability is connected to regulation of Calvin cycle activity or by interdependence of light and dark reactions via ATP/ADP, with consequent reductions in the efficiency of photosynthetic electron transfer (Geider et al., 1998).

DF decay kinetics is influenced differently by nitrogen and phosphorus starvation, making it a potential method of discriminating various nutrient conditions. Such a discriminating technique is still missing in the monitoring of oceanic phytoplankton population changes.

5. Conclusions

Delayed fluorescence has been used for researching photosynthesis since 1951. Nevertheless, not many publications can be found in the literature, mostly due to lack of commercially available measuring devices. Delayed fluorescence provides different information about photosynthesis as prompt fluorescence. It is emitted only from living cells therefore the problems with fluorescent background in field samples are omitted. The measurements can be successfully utilized in toxicity tests, biomass monitoring, primary productivity measurements and following changes in phytoplankton composition. Some more research, however, must be done on better understanding of the complex processes influencing the delayed fluorescence kinetics.

6. Acknowledgements

Our colleague Luka Drinovec has assembled the delayed fluorescence measuring apparatus and prepared the software. Alfred Beran has provided us with the algal cultures. Lidija Berden revised the language. The work was financed by the Slovenian Research Agency (grants #P1-0237, L4-6222, and V4-0106).

7. References

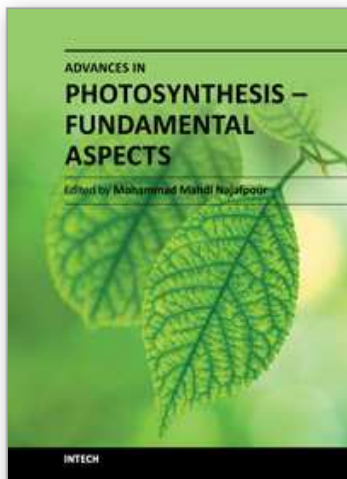
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