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## **Delayed fluorescence: An *in vivo* method for electron transport studies and on-line applications in limnology.**

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**Abstract.** Delayed fluorescence (DF) only occurs in living plant cells. DF is a measure of the photosynthetic activity and it is the result of an electron hole recombination fluorescence (680–740 nm) at the reaction center P680 during dark-adaptation after light absorption by photosynthetically active pigments (chlorophylls, xanthophylls and phycobilins). DF can be observed over several minutes until a charge equilibrium between inner and outer side of the thylakoid membrane is reached. DF is an intrinsic fluorescence label of the efficiency of charge separation at P680 and a useful tool to investigate processes in photosynthesis, discriminating especially between processes occurring within the antenna or the electron transport chain.

The decay kinetic of the DF depends on the distribution of electrons and holes within the entire electron transport chain and the charging of the thylakoid membrane. Therefore, it is influenced e.g. by 1) electron blockers (herbicides), 2) the ratios of the pumping rates at PSI and PSII and 3) availability of CO<sub>2</sub>. The DF decay curve can be used to determine the concentration of photosynthetically active pigments and primary productivity.

Exciting a cell suspension (algal cultures, phytoplankton or chloroplasts) by monochromatic light (400–730 nm) DF action spectra can be measured and photosynthetically active pigments of different algal classes can be discriminated. DF excitation spectra change due to growth conditions (e.g. high light) which influence the exciton transfer within the antenna and the efficiency of charge separation at the reaction center P680.

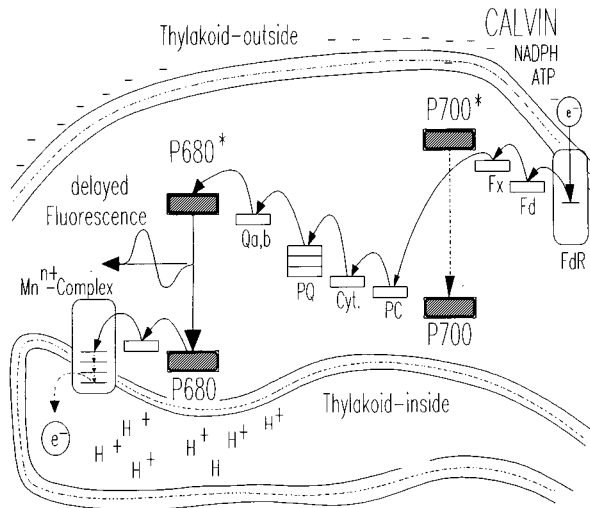
### **Introduction**

Delayed fluorescence (DF) only occurs in photosynthetically active material and is emitted from living cells with decay times from milliseconds to minutes. Research on the complex behavior of the DF (Strehler and Arnold 1951, Malkin 1977, Amesz and Gorkom 1978) have shown that DF decay kinetics can be used to determine *in vivo* the concentration of photosynthetically active pigments in freshwaters (Gerhardt et al. 1981, Krause et al. 1982, 1987). Herbicides can be detected by evaluating the difference in the decay kinetic of poisoned and unpoisoned algal cultures (Gerhardt and Putzger 1989). DF excitation spectroscopy is used to analyse the phytoplankton composition in freshwaters (Bodemer 1998, Bodemer et al. 2000).

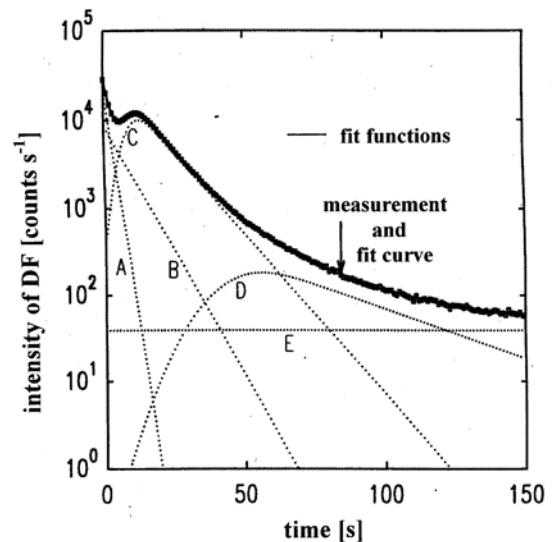
### **Materials and Methods**

The origin of DF is shown in Fig. 1. During photosynthesis charge separation at PSII and PSI starts due to light absorption. Electrons are transported through the electron transport chain to the Calvin cycle. Stopping illumination the processes of the light phase reverse: electrons on the electron transport chain flow back to the oxidized reaction center P680<sup>+</sup>, leading to an excited state P680\*. This excited state P680\* decays to the ground state emitting a delayed fluo-

rescence quant (680–720 nm). DF can be observed over several minutes until a charge equilibrium between donor side (water splitting complex, inside of the thylakoid membrane) and acceptor side (plastoquinone, photosystem I, outside of the thylakoid membrane) is reached by recombination. The resulting decay curve can be fitted well (Fig. 2): The fast decay (components A and B) are produced by recombination of electrons and holes which are located near  $P680^+$  after stopping illumination; the long-lasting decay components C and D are caused by those electron-hole pairs which are located at greater distances from  $P680^+$  (e.g. electrons near PSI together with holes at the  $Mn^{n+}$  complex, charges of the thylakoid membrane) (Krause et al. 1984, Kretsch and Gerhardt 1987).



**Fig. 1.** Genesis of delayed fluorescence (DF) – reversion of photosynthesis: Back reaction of electrons leads to DF at the reaction center P680.



**Fig. 2.** Decay curve of the DF (semi-logarithmic). The measurement can be fitted by four components A-D; E describes the constant dark rate of the photomultiplier.

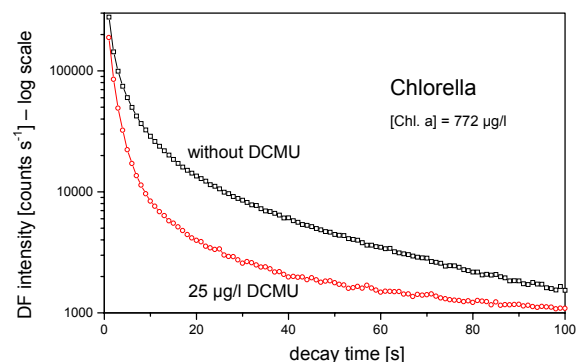
Details of the devices to measure DF decay kinetic and DF excitation spectra are described in Gerhardt and Bodemer (1999; 2000).

## Results and Discussion

**Electron blockers.** Electron transport is blocked by DCMU (or Atrazin) between  $Q_a$  and  $Q_b$ . Therefore, all  $Q_a$  are reduced during illumination. Stopping illumination leads to a high DF signal during the first seconds (Fig. 3, steep decay, ○) because all – and only – electrons from  $Q_a^-$  recombine at  $P680^+$ .

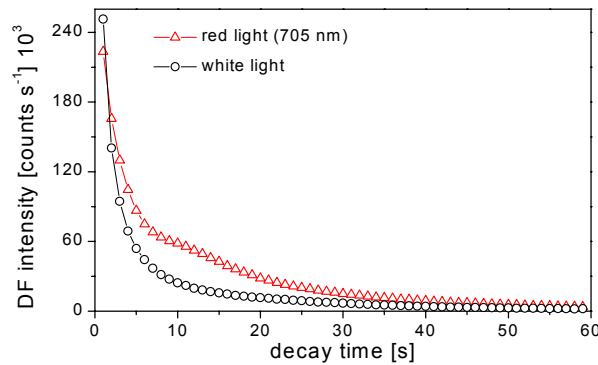
**PSII and PSI pumping rates.** The decay kinetic of *Chlorella* spp. cultures excited by white light (Fig. 4, ○, both PSII and PSI are pumped) and red light (Fig. 4, ▲, 705 nm interference filter pumping mainly PSI) differs. The long-lasting decay components C and D (compare Fig. 2) increase when mainly PSI is excited.

**Availability of CO<sub>2</sub>.** The decay kinetic of the DF is also influenced by metabolic processes in the Calvin cycle, e.g. availability of CO<sub>2</sub> (Fig. 5). The thylakoid membrane cannot be dis-

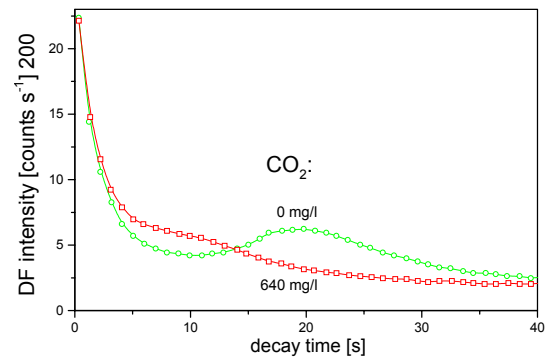


**Fig. 3.** DF decay curve of a *Chlorella* spp. culture poisoned by DCMU compared to an unpoisoned culture (semi-logarithmic).

charged by the Calvin cycle due to a lack of  $\text{CO}_2$ . Electrons remain within the electron transport chain (Fx, Fd, FdR) and flow back causing enhanced DF at P680 after illumination is stopped (○ in Fig. 5). Adding  $\text{CO}_2$  leads to an increased uptake of electrons by the Calvin cycle.



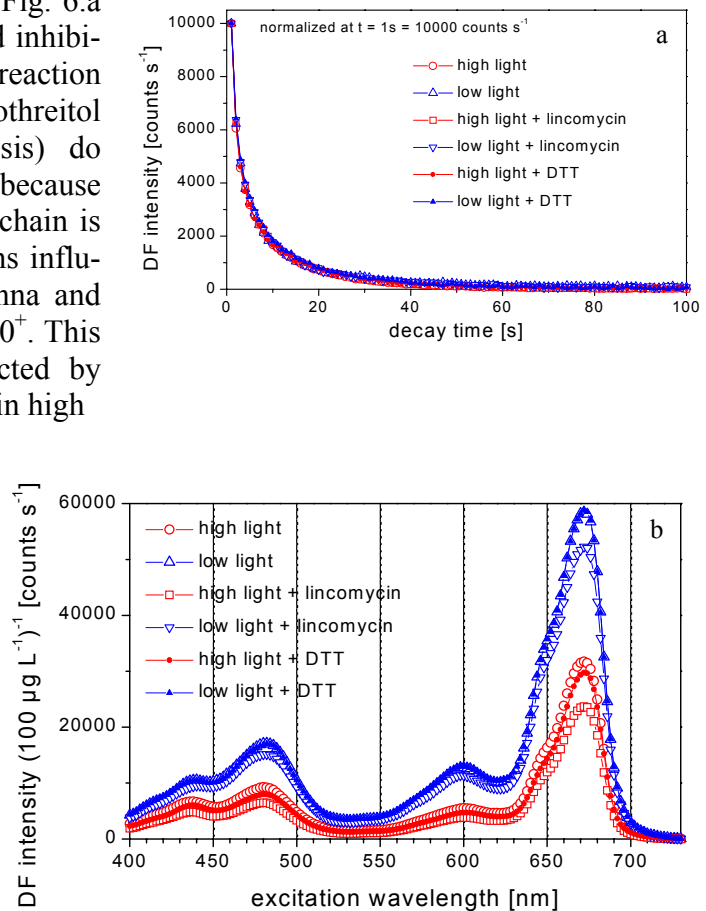
**Fig. 4.** Different DF decay kinetics in *Chlorella* spp. cultures excited by white and red light (705 nm).



**Fig. 5.** Different DF decay kinetics in *Chlamydomonas reinhardtii* cultures with and without  $\text{CO}_2$ .

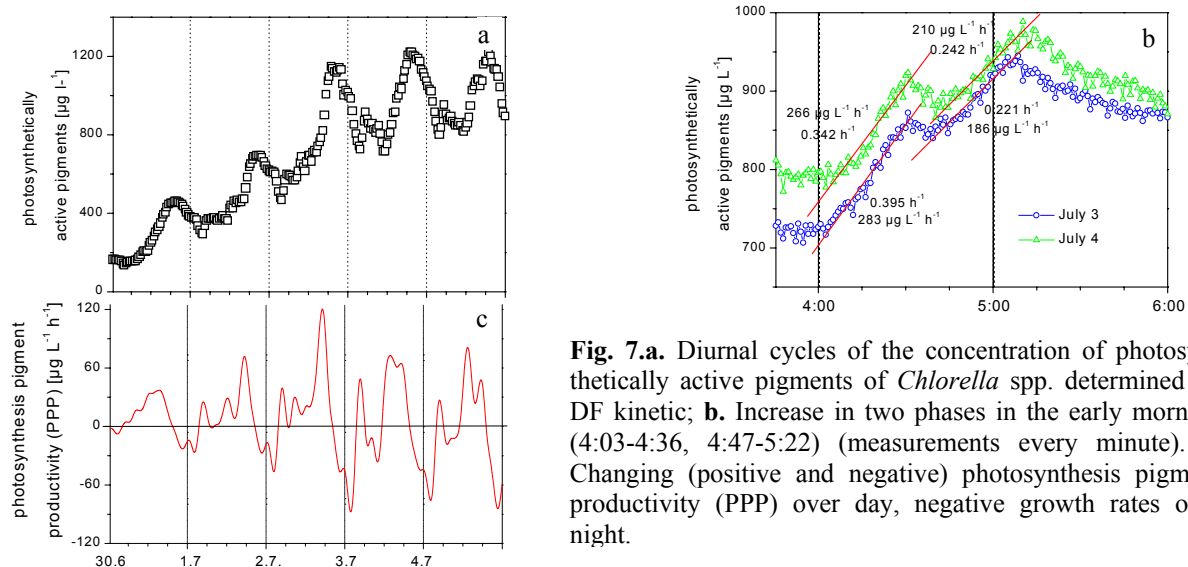
*Processes within the antenna of PSII.* Fig. 6.a shows that growth light conditions and inhibitors, e.g. lincomycin (inhibition of reaction center D1 protein synthesis) or dithiothreitol (DTT, inhibitor of zeaxanthin synthesis) do not influence the DF decay kinetics because the structure of the electron transport chain is not changed. However, these conditions influence exciton transfer within the antenna and the efficiency of charge transfer at  $\text{P680}^+$ . This efficiency is reduced and can be detected by DF excitation spectra which decreases in high light grown *Chlorella* spp. cultures, in lincomycin-treated high and low light grown *Chlorella* spp. cultures and in DTT-treated high light grown *Chlorella* spp. cultures (Fig. 6.b) (Bodemer 2001).

**Fig. 6.** *Chlorella* spp. cultures grown in constant high light ( $285 \mu\text{Einst m}^{-2} \text{s}^{-1}$ ) and low light ( $30 \mu\text{Einst m}^{-2} \text{s}^{-1}$ ); 10 ml 1mM solution of lincomycin or DTT was added to 750 ml culture of  $100 \mu\text{g L}^{-1}$  [chl*a*]; illumination 1 h. **a.** Decay curves of the DF. **b.** DF excitation spectra normalized to  $100 \mu\text{g L}^{-1}$  [chl*a*] at  $21^\circ\text{C}$ .



*Photosynthesis pigment productivity (PPP).* Measuring the DF decay every minute growth rates of phytoplankton can be determined within 15 minutes: The integral of the DF decay curve is calibrated by parallel determination of chlorophyll *a* concentration (standard extraction method (Gerhardt and Bodemer 2000)). Fig. 7.a presents an example of a daylight-synchronized *Chlorella* spp. culture. The concentration of photosynthetically active pigments ( $\mu\text{g L}^{-1}$ ) shows a pronounced diurnal cycle: Photosynthesis starts with the first light in the mor-

ning with high reproduciveness from day to day (Fig. 7.b); PPP ( $\mu\text{g L}^{-1} \text{h}^{-1}$ ) is changing over day due to varying light intensity; in the night PPP is negative (Fig. 7.c). In contrast to prompt fluorescence (PF) DF is proportional to the fraction of absorbed light that leads to the charging of the thylakoid membrane. Therefore, DF may have a strong correlation to primary productivity of the cell.



**Fig. 7.a.** Diurnal cycles of the concentration of photosynthetically active pigments of *Chlorella* spp. determined by DF kinetic; **b.** Increase in two phases in the early morning (4:03-4:36, 4:47-5:22) (measurements every minute). **c.** Changing (positive and negative) photosynthesis pigment productivity (PPP) over day, negative growth rates over night.

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