

Chapter 11

Pulse-Amplitude-Modulation (PAM) Fluorometry and Saturation Pulse Method: An Overview

Ulrich Schreiber*

Lehrstuhl Botanik I, Julius-von-Sachs Institut für Biowissenschaften,
Universität Würzburg, D-97082 Würzburg, Germany

Summary	280
I. Introduction	280
II. Principle of Pulse-Amplitude-Modulation	282
III. Information Carried by Chlorophyll Fluorescence Yield	284
A. Rapid Fluorescence Induction Kinetics	284
B. Slow Chlorophyll Fluorescence Changes	286
IV. Saturation Pulse Method of Quenching Analysis	287
A. Principle of the Method	287
B. Examples of Some Typical Applications	288
C. Contribution of Photosystem (PS) I Fluorescence and Other Background Signals	292
D. Heterogeneity of Fluorescence Signal	292
V. Assessment of Quantum Yield and Relative Electron Transport Rate	294
A. Relationship Between Fluorescence Yield and PS II Quantum Yield	294
1. Simple Derivation Based on Law of Energy Conservation	294
2. Experimental Evidence for Close Correspondence	294
3. Possible Deviation from Linearity due to Cyclic PS II	295
B. Relative Electron Transport Rate and Light Response Curves	296
VI. Intrinsic Heterogeneity of Variable Chlorophyll Fluorescence	297
A. Polyphasic Rise of Fluorescence Yield upon Onset of Saturating Light	298
B. I_1 (or J)-level and $F_{50\mu s}$ Measured by the Pump-and-Probe Method	300
C. Quenching Related to PS II Charge Recombination and Q_B -quenching	302
VII. Pulse Amplitude Modulation (PAM) Fluorometry for Special Applications	306
A. Phytoplankton Analysis by 4-Wavelength Excitation Technique	306
B. Systems for Assessment of Photosynthesis at Single Cell Level	307
1. Epifluorescence Microscope System	308
2. Microfiber System	308
C. Imaging of Photosynthetic Activity	309
Acknowledgments	312
References	312

*Email: ulrichschreiber@gmx.de

Summary

Chlorophyll (Chl) *a* fluorescence originates in close vicinity to the sites where light energy is transformed into chemically fixed energy. The same excitation states that give rise to fluorescence emission also participate in photochemical energy conversion. These features render Chl fluorescence a unique indicator of photosynthesis. During the past 15 years there has been remarkable progress in Chl fluorescence research. In practical applications, Pulse-Amplitude-Modulation (PAM) fluorometry in conjunction with the saturation pulse method has been particularly successful. This chapter outlines the principles of PAM fluorometry and saturation pulse method. Some examples of typical applications are given. Also the limits of the method are outlined, with emphasis on the fact that *absolute* assessment of photosynthetic parameters is complicated by various factors, while *relative* changes can be assessed with high reliability. Particular attention is given to the theoretical foundation of the method, at a level, which can be also understood by non-specialists and students. A pivotal role in the determination of Photosystem (PS) II quantum yield by fluorescence measurements is played by the maximal fluorescence yield, F_m , proper measurement of which has been a matter of controversy. This is related to a large intrinsic heterogeneity of variable Chl fluorescence, which is revealed in the polyphasic rise of fluorescence yield upon the onset of saturating light. Arguments are put forward in favor of F_m determination after full reduction of the plastoquinone pool, including the secondary quinone acceptor of PS II, Q_B , the oxidized form of which can support in my view a particular type of nonphotochemical quenching, when the primary quinone acceptor, Q_A , is reduced. Possible mechanisms and consequences of this ' Q_B -quenching' are discussed. Some PAM fluorometers for special applications are described, including systems for phytoplankton analysis, for investigations at the single cell level (epifluorescence microscopy and microfiber technique) and for imaging of photosynthetic activity.

I. Introduction

Chl fluorescence is not only 'red and beautiful' (Govindjee, 1995), but also provides an exceptionally large signal that, when properly measured and analyzed, can give detailed information on what is going on inside a photosynthetic organism. (For an early book on this topic, see Govindjee et al., 1986). Chl fluorescence originates where the fuel for all other life processes is generated. Hence, it provides information on the efficiency of primary energy

conversion, reliable measurement of which is a task of outstanding importance.

The analytical potential of Chl fluorescence was first recognized by Hans Kautsky and co-workers, starting with the discovery of the dark/light induction phenomenon ('Kautsky effect'; Kautsky and Hirsch, 1931), culminating in a series of publications with Ulrich Franck (Kautsky and Franck, 1943) and ending with the remarkable study of Kautsky et al. (1960). The latter study, as well as that by Govindjee et al. (1960), anticipated some of the conclusions

Abbreviations: AC – alternating current; ADRY – Acceleration of the Deactivation Reactions of the water splitting enzyme system Y; ANT-2p – 2-(3-chloro-4-trifluoromethyl)-anilino-3,5-dinitrothiophene; BBY – Berthold-Babcock-Yocum; CCCP – carbonylcyanide-m-chlorophenylhydrazone; Chl – chlorophyll; D1, D2 – Photosystem II reaction center proteins; DCMU – 3-(3,4-dichlorophenyl)-1,1-dimethylurea; ETR – relative electron transport rate; F – chlorophyll *a* fluorescence yield; $F_{50\mu s}$ – chlorophyll *a* fluorescence yield measured 50 μs after pump flash; F_m – maximal chlorophyll *a* fluorescence yield after dark adaptation; F_m' – maximal chlorophyll *a* fluorescence yield in illuminated state; F_0 – minimal chlorophyll *a* fluorescence yield after dark adaptation; F_0' – minimal chlorophyll *a* fluorescence yield in illuminated state; F_v – variable chlorophyll *a* fluorescence, F_m-F_0 ; F_v/F_m – maximal PS II quantum yield calculated from chlorophyll *a* fluorescence after dark adaptation; I_1 – first intermediate chlorophyll *a* fluorescence level (equivalent to J); I_2 – second intermediate chlorophyll *a* fluorescence level (equivalent to I); I_k – light intensity in light response curve characteristic for beginning of saturation; LED – light emitting diode; NPQ – expression for nonphotochemical quenching of chlorophyll *a* fluorescence; P680 – primary electron donor of Photosystem II; PAM – pulse amplitude modulation; Pheo – pheophytin molecule in Photosystem II; P-I – rate of photosynthesis versus light intensity; P_{max} – maximal rate of photosynthetic electron flow in a light response curve; PQ – plastoquinone; PS – photosystem; Q_1 , Q_2 – hypothetical subfractions Q_A ; Q_A – primary quinone acceptor of Photosystem II; Q_B – secondary quinone acceptor of Photosystem II; qI – coefficient of photoinhibitory nonphotochemical quenching of chlorophyll *a* fluorescence; qN – coefficient of overall nonphotochemical quenching of chlorophyll *a* fluorescence; qP – coefficient of overall photochemical quenching of chlorophyll *a* fluorescence; Y_z – electron donor to oxidized P680; α – initial slope in light response curve, reflecting maximal quantum yield of photosynthesis; $\Delta F/F_m'$ – effective PS II quantum yield in illuminated state calculated from chlorophyll *a* fluorescence; Φ_{II} – quantum yield of Photosystem II; σ_{PSII} – functional absorption cross-section of Photosystem II, m^2 quanta $^{-1}$

later drawn by Duysens and Sweers (1963) on the control of the fluorescence yield by a quenching acceptor molecule and two light reactions in series. The tradition of Kautsky's work was carried on by Ulrich Franck and co-workers (Franck et al., 1969; Schreiber et al., 1971).

When looking back on the history of Chl fluorescence, now 73 years since Kautsky's discovery, the years between 1960 and the mid-1980s were dominated by biophysically oriented basic research (for reviews see Govindjee and Papageorgiou, 1971; Papageorgiou, 1975; Lavorel and Etienne, 1977; Butler, 1978; Govindjee and Jursinic, 1979). During this period, Chl fluorescence had been a pioneering tool in the elucidation of basic reaction mechanisms of photosynthesis. On the other hand, up to then the practical use of this tool in applied research (like plant stress physiology, ecophysiology and phytopathology) had been quite limited, due to a lack of appropriate instrumentation and methodology. Also the phenomenology of whole leaf fluorescence appeared by far too complex to be reliably interpreted. Thus, for a long time there was a gap between basic and applied research in Chl fluorescence. During the past 20 years, the situation has been continuously changing, since it became clear that despite its complexity, the fluorescence signal does carry reliable quantitative information, which can be gathered with relatively simple instrumentation (for reviews see Briantais et al., 1986; Renger and Schreiber, 1986; Krause and Weis, 1991; Horton and Bowyer, 1990; Schreiber and Bilger, 1993; Schreiber et al., 1994; Govindjee, 1995; Joshi and Mohanty, 1995; Mohammed et al., 1995; Lazar, 1999).

As already pointed out by van Kooten and Snel (1990) in their editorial note to a special issue of *Photosynthesis Research* devoted to Chl fluorescence, there has been a very fruitful dynamic interaction between Basic Research, Applied Research and Instrument Development (driven by the Technical Progress), which brought about a 'renaissance' of interest in Chl fluorescence. Actually, the interacting fields in this dynamic process also include Teaching, which offers a strong incentive for leaving the 'ivory tower' of pure basic research and linking biophysical knowledge with practical applications of general relevance (Schreiber, 1997).

The technical progress in optoelectronics and photonics has been particularly important for the development of the Chl fluorescence instrumentation described in this chapter. Pulse-Amplitude-Modula-

tion (PAM) fluorometry is closely linked with the availability of strong, compact light sources for rapid pulse-modulated excitation and of sensitive detector systems. Current examples are the recent progress in light-emitting diode (LED) and charge-coupled-device (CCD) technologies, which opened the way for the development of powerful fluorescence imaging systems, with which the two-dimensional distribution of photosynthetic activity can be analyzed (see Section VII.C and Chapters 14, Nedbal and Whitmarsh; 15, Oxborough; 17, Allen and Mullineaux; and 28, Lichtenthaler and Babani).

In principle, the technical possibilities for developing Chl fluorometers for applied research already existed since the early 1970s, when solid-state optoelectronic components, such as LEDs and fast photodiodes, became generally available. The first portable fluorometer for field studies (Schreiber et al., 1975) was already introduced 10 years before the development of the first PAM fluorometer. However, this instrument was restricted to the recording of dark-light induction curves, without the possibility of further analysis of the obtained data. At that time the interactions mentioned above were not yet strong enough to stimulate further development. Decisive stimulation leading to progress in instrumentation came from the physiological work of Briantais et al. (1979), Bradbury and Baker (1981), Krause et al. (1982), Horton (1983) and Walker et al. (1983). Quick and Horton (1984) and Dietz et al. (1985) applied chopper-modulated fluorometers for distinguishing different types of fluorescence quenching. The first portable fluorometers featuring modulated LEDs were introduced by Ögren and Baker (1985) and Schreiber (1986). As these fluorometers became commercially available, they triggered a boom of practical applications in plant physiology and ecophysiology that led to important new insights, particularly with respect to the regulated dissipation of excess light energy (see reviews by Demmig-Adams and Adams, 1992; Schreiber and Bilger, 1993; Björkman and Demmig-Adams, 1994; Pfundel and Bilger, 1994; see also Chapters 10, Kramer et al.; 18, Krause and Jahns; 20, Golan et al.; 22, Adams and Demmig-Adams; and 21, Gilmore).

Most of this chapter is not written for specialists in Chl fluorescence and photosynthesis, but rather for persons who are not yet familiar with this tool, i.e., students as well as researchers interested in applying PAM fluorometry in adjacent fields of research, like ecophysiology, agriculture, plant molecular biology,

plant pathology, limnology and oceanography. It is not intended to present a review on the vast amount of data collected with PAM fluorometry during the past 18 years. Only a very limited amount of original data will be presented. It is rather intended to give an introduction to the principles of Chl fluorescence measurements with PAM fluorometers, to outline the various types of information, which can be gained and to describe a number of devices for special applications. Special attention will be given to the theoretical basis of the saturation pulse method of quenching analysis and to an intrinsic heterogeneity of variable Chl fluorescence, the mechanistic cause of which has been discussed controversially in recent years (for a review, see Samson et al., 1999; see also Chapter 6, Vredenberg and Chapter 19, Bruce and Vasil'ev).

II. Principle of Pulse-Amplitude-Modulation

For understanding the principle of Pulse-Amplitude-Modulation (PAM), it is important to realize the difference between fluorescence *intensity* and fluorescence *yield*. Depending on the light conditions, fluorescence *intensity* may vary by several orders of magnitude. On the other hand, in most practical applications fluorescence *yield* does not vary by more than a factor of 5–6. It is the fluorescence *yield* that carries information on photosynthesis. The ideal Chl fluorometer is capable of measuring the fluorescence yield in all physiologically relevant situations without changing the state of the sample, i.e., in a non-intrusive way. In practice this means:

- the measuring light (i.e., exciting light) intensity has to be very low for assessment of the fluorescence yield of a dark-adapted sample;
- the detection system has to be extremely selective to distinguish between the fluorescence excited by the measuring light and the much stronger signals caused by ambient and actinic light, e.g., full sun light in field studies or saturating light pulses for assessment of maximal fluorescence yield (quenching analysis); and
- the measuring system must display a fast time response in order to resolve the rapid changes in fluorescence yield upon dark-light and light-dark transitions.

The challenge consists in realizing the combination of all of these properties in one instrument. In any fluorescence measuring system, there has to be efficient separation of the fluorescence from the much stronger excitation light. This generally is achieved by the use of appropriate optical filters, i.e., short-pass for excitation and long-pass in front of the detector (Schreiber, 1983; Renger and Schreiber, 1986; Horton and Bowyer, 1990). In conventional Chl fluorometers the excitation light, at the same time, serves for driving the photosynthetic reactions (actinic light). However, such fluorometers are not suited for *in situ* studies. In order to distinguish between fluorescence and ambient light, fluorescence excitation has to be modulated (either mechanically or electronically) and the fluorescence amplifier has to be highly selective for the modulated signal. While modulation fluorometers featuring mechanical choppers and lock-in amplifiers had been playing an important role in basic photosynthesis research for a long time (Duysens and Sweers, 1962; Bonaventura and Myers, 1969), the first measuring system fulfilling all of the above requirements was the PAM-101 Chlorophyll Fluorometer (Schreiber, 1986; Schreiber et al., 1986). A chopper-modulated precursor of this measuring system was used by Dietz et al. (1985).

The term Pulse-Amplitude-Modulation (PAM) was coined for a novel modulation technique specially developed for Chl fluorometers with the above stated properties. The technical details are described in a patent publication (Schreiber and Schliwa, 1987a). Essential features of this technique are:

- the fluorescence measuring light consists of very short (μs -range) pulses, which can be applied repetitively at different frequencies;
- when applied at low frequency, the intensity of each μs -measuring pulse can be quite high without inducing a significant increase of fluorescence yield;
- the resulting μs -fluorescence pulses are detected by a photodiode detector featuring fast response and large linearity range (up to 10^9);
- the pulse-preamplifier is AC-coupled, such that background signals are rejected;
- the preamplified signal is further processed by a selective-window-amplifier, which amplifies the

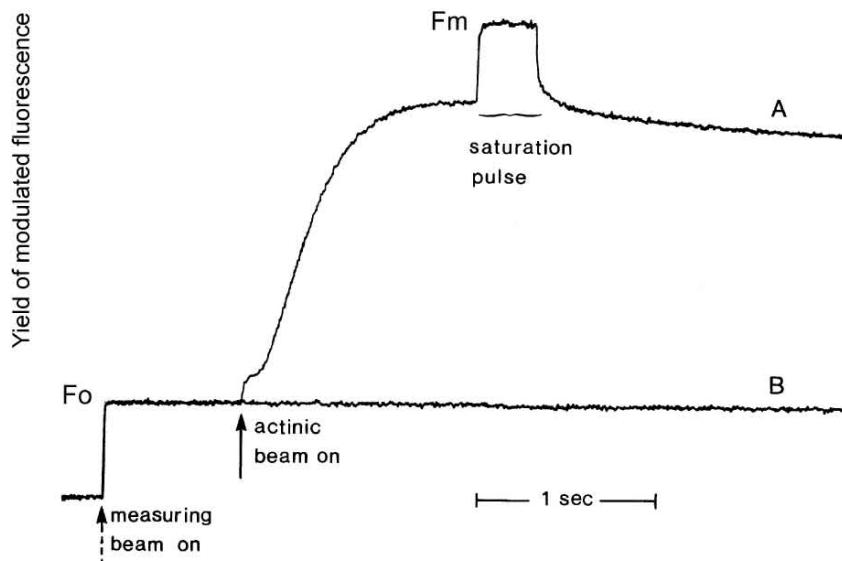


Fig. 1. Selective detection of light induced changes of chlorophyll fluorescence yield. Comparison of intact leaf (A) with ethanolic chlorophyll extract (B). Chlorophyll concentration of extract adjusted to give same signal as leaf. Modified from Schreiber et al. (1986). See text for further explanations.

difference between the signal during the excitation pulse and the signal a few μs after it; in principle, the difference signal can be ‘disturbed’ only by very rapid changes between the two μs -window periods;

- the switching on/off of actinic light sources as well as the triggering of discharge flashes is synchronized to be in the middle of the dark periods between measuring light pulses (i.e., outside the ‘measuring window’), such that the switching-on/off artifacts found with conventional lock-in amplifiers are avoided; and
- the measuring light frequency can be automatically increased upon triggering of actinic illumination, such that rapid induction and relaxation kinetics can be followed.

The reliability of PAM measurements may be judged from the experiment in Fig. 1 where the responses to actinic illumination of a bean leaf and a Chl solution are compared. The quasi-dark fluorescence signals, F_0 , of the two samples were adjusted to the same level. Vastly different light intensities were applied for measuring light ($0.05 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$), actinic light ($80 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$) and a saturating light pulse ($8,000 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$).

The measuring light is sufficiently weak to monitor the dark-adapted fluorescence level, F_0 . When the 1,600 times stronger actinic light and the 160,000 times stronger saturating light pulse are applied, the fluorescence yield of the Chl solution does not change, whereas the fluorescence yield of the bean leaf displays the expected induction phenomenon. This demonstrates the selectivity of the PAM measurement for the modulated signal representing Chl fluorescence *yield*. While the 160,000 stronger, nonmodulated saturation pulse induces much higher fluorescence *intensity* than the modulated measuring light, the nonmodulated fluorescence signal caused by the saturation pulse is completely rejected by the amplifier system. The same is true for any other nonmodulated signal including ambient daylight and even full sunlight reflected from a leaf into the detector system. Actually, it is even possible to apply far-red background light that can pass freely the filters in front of the detector.

The pulse-modulated measuring light can be generated either by a light-emitting diode (LED, in most PAM fluorometers) or a flash discharge lamp (e.g., Xe-PAM). LEDs can be modulated at high frequencies (MHz range). They also have the advantages of low cost and being easy to handle. Flash lamps can provide extremely strong measuring light, which can be particularly helpful in analyzing low Chl samples,

e.g., in oceanography. Regarding the optical set-up, it is important that all light qualities (i.e., measuring, actinic, saturation pulse, far-red background light and flash light) are evenly distributed over the sample. For this purpose, most PAM fluorometers employ multibranched fiberscopes, with the fibers being randomly mixed. One branch of the fiberscopes normally is used for carrying the fluorescence to the detector, while the other branches serve for guiding the various light beams to the sample. Such fiberscopes systems are particularly suited for portable devices in fieldwork. The distance and angle between the joint end of the fiberscopes and the investigated sample can be adjusted such, that on the one hand the ambient light conditions are not disturbed and on the other hand a sufficiently high signal is obtained (Bilger et al., 1995).

Most PAM fluorometers apply photodiodes as fluorescence detectors, which not only are linear over a large range of light intensities, but also maintain a low noise level even at extremely high signal levels. This is not the case with photomultipliers, where the noise increases with light intensity, thus causing problems in experiments with strong actinic light and saturation pulses. Recently, however, special PAM fluorometers were developed, in which such problems are avoided by pulse modulating not only the measuring light, but actinic light and saturation pulses as well (Schreiber, 1998). While this technique allows extremely sensitive measurements (e.g., with phytoplankton at less than $1\mu\text{g Chl l}^{-1}$ and even with single cells), it requires full instrument control of all types of applied light and, hence, cannot be used in natural light under field conditions.

Besides Chl fluorescence measurements, the PAM technique has also been successfully applied for related measurements of other photosynthetic parameters. P700 absorbance changes can be measured with the same PAM-101 system as Chl fluorescence using special emitter-detector units (Schreiber et al., 1988; Klughammer and Schreiber, 1994, 1998; Chapter 12, Strasser et al.). The same is true for P515 absorbance changes (Klughammer et al., 1998) and NADPH fluorescence changes (Mi et al., 2000). A 16-channel LED-array spectrophotometer for measurement of time resolved difference spectra in the 530–600 nm wavelength region was developed for assessment of absorbance changes of cytochromes (Cyt *f*, Cyt *b*-563 and Cyt *b*-559) (Klughammer et al., 1990). Furthermore, a computer-controlled pulse modulation system for the analysis of photoacoustic signals

(Kolbowski et al., 1990) has been applied for the study of pulse-modulated heat release, O₂-evolution and CO₂-uptake associated with stroma alkalization (Reising and Schreiber, 1992). In principle, all of these instruments can be linked via fiberscopes to the same sample, thus offering a vast analytical potential for the investigation of photosynthesis.

III. Information Carried by Chlorophyll Fluorescence Yield

PAM fluorometers measure the relative quantum yield of Chl fluorescence by applying measuring light with constant pulse amplitude. Each excitation pulse hitting a sample probes the probability of absorbed light energy being re-emitted in the form of fluorescence. Fluorescence emission competes with a number of other de-excitation pathways (see Chapter 4, Clegg). The mechanisms governing fluorescence yield may be dealt with in a more or less detailed and sophisticated way. (For a discussion of fluorescence lifetime measurements that provide absolute quantum yields of fluorescence, see Chapters of 19, Bruce and Vasil'ev; and 21, Gilmore; such measurements become essential when 'state' changes occur: see Chapter 17, Allen and Mullineaux). Here a simple way is chosen in an attempt to concentrate on aspects of practical relevance. For a first step in understanding fluorescence yield, it suffices to consider competition with photochemical energy conversion at the reaction centers, in particular when dealing with rapid fluorescence changes. In a second step, also competition with nonradiative energy dissipation into heat has to be considered, particularly when slow fluorescence changes are involved.

A. Rapid Fluorescence Induction Kinetics

Considering the competition of fluorescence with photochemical energy conversion, two extreme situations are possible (assuming the yield of heat dissipation is constant). All reaction centers can be open (normal dark-adapted state) or closed (e.g., in saturating light), with fluorescence yield being minimal (F_o) or maximal (F_m), respectively. The difference between F_o and F_m is called 'variable fluorescence,' F_v. Plant leaves typically show F_m/F_o ratios of around 5 (corresponding to a F_v/F_m value around 0.8), which vary depending on the particular plant and the physiological conditions. Lower values

are found in most algae. When a dark-adapted sample suddenly is illuminated with sufficiently strong continuous light, fluorescence yield rises within fractions of a second from F_0 to F_m . The kinetics of the fluorescence rise provides information on various steps of photosynthetic electron transport (see Chapter 12, Strasser et al.).

The interpretation of light induced changes in fluorescence yield is greatly facilitated by the empirical fact that *variable* fluorescence at room temperature originates from Photosystem II (PS II). There is, however, a contribution of *non-variable* PS I fluorescence, which can be of considerable practical relevance, as it lowers the measured F_v/F_m (Pfündel, 1998; Section IV.C; Chapters 9, Itoh and Sugiura; and 21, Gilmore).

Light absorbed by PS II pigments results in Chl excitation, which is funneled by a special transfer mechanism into the reaction centers, where energy conversion by charge separation takes place (see a simple scheme in Fig. 2). The reaction center Chl (P680), and pheophytin (Pheo) function as primary donor and acceptor molecules of PS II, respectively. The ‘energy trapping’ process is completed when the separated charges are ‘stabilized’ by electron transfer from reduced Pheo to Q_A^- on the acceptor side, and from Y_Z^- to oxidized P680 on the donor side. (For a complete description of electron transport in photosynthesis, see Ke, 2001). Under normal physiological conditions, the rate of energy conversion at PS II reaction centers is acceptor side limited and, hence, the fluorescence yield is controlled by the ‘quencher’ Q_A^- (Duysens and Sweers, 1963). Upon onset of illumination, Q_A^- as well as the secondary acceptor Q_B^- will accumulate in their reduced forms, while at the same time sufficient electrons are available from H_2O -splitting (oxygen-evolving-complex, $(Mn)_4$) for Y_Z^- and P680 re-reduction. This does not hold for the rapid induction kinetics in very strong light, which also reveal donor side limitations (Section VI.A).

The relationship between Q_A^- and fluorescence yield is nonlinear (Joliot and Joliot, 1964), due to energy transfer between PS II reaction centers via common antenna domains. When a fraction of the reaction centers is closed, this does not lead to a proportional increase of fluorescence yield, if neighboring open centers can use the excitation energy. Therefore, a fluorescence change close to F_0 may reflect considerably larger changes in Q_A^- than the same change close to F_m . The extent of nonlinearity increases with the

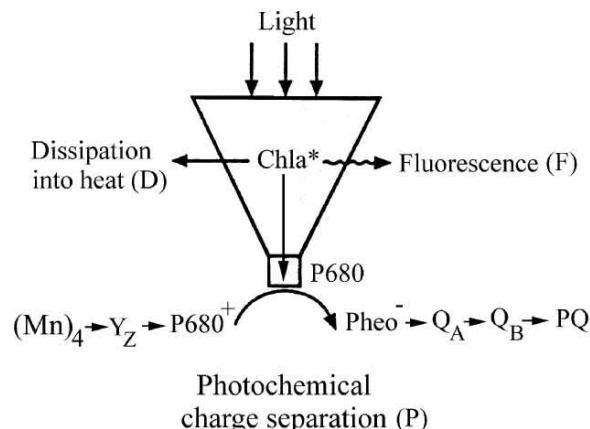


Fig. 2. Competition between the yields of fluorescence emission (F), photochemical energy conversion in Photosystem II (P) and heat dissipation (D). See text for further explanations.

degree of ‘connectivity’ between PS II units (also see Chapter 12, Strasser et al.).

At moderate light intensities, upon onset of continuous illumination the fluorescence yield rises in two steps to a peak level. A rapid initial rise is separated by an intermediate level from a major slower phase (typical example in Fig. 3a). In the presence of 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU), which prevents oxidation of Q_A^- by occupying the Q_B^- binding site in the reaction center complex, the rapid phase is strongly increased at the cost of the slow phase. In both cases the fluorescence rise reflects the exhaustion of the available PS II acceptor pool. A measure of the acceptor pool size is given by the area between induction curve and the F_m -line (Malkin and Kok, 1966; Murata et al., 1966). The area in presence of DCMU corresponds to one electron per center, which can be stored on Q_A^- , while the area in the control corresponds to approximately 15 electrons per center, mainly reflecting the PQ-pool. The acceptor pool size is larger in sun plants than in shade plants. The rise from F_0 to the intermediate level partially reflects charge accumulation at the two-electron gate Q_B^- (Velthuys and Amesz, 1974). Illumination initially causes accumulation of the semiquinone anion Q_B^- in equilibrium with some Q_A^- , before Q_B^- can be formed, which after protonation is released into the PQ-pool. Part of the initial fluorescence rise appears to be due to a fraction of PS II centers that are not connected to the PQ-pool (‘inactive PS II’; Chylla and Whitmarsh, 1990; Cao and Govindjee, 1990; Lavergne and Lec, 1993; Lavergne and Briantais, 1996).

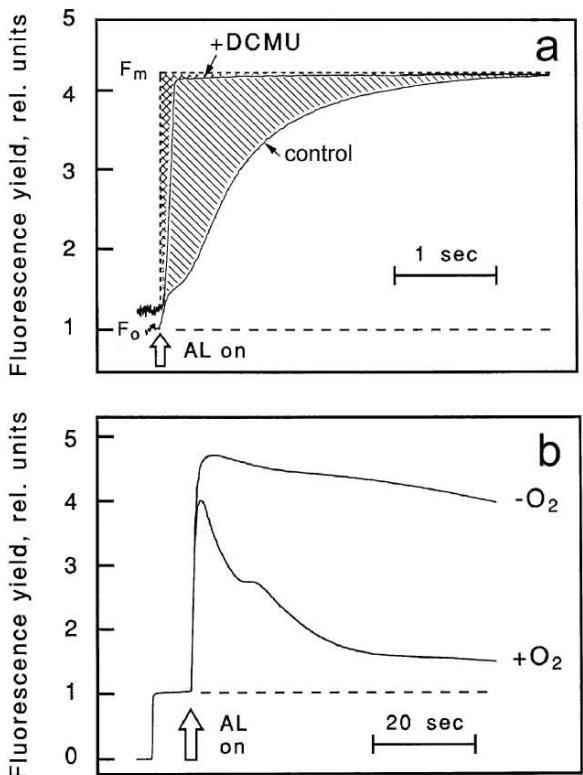


Fig. 3. Induction kinetics of chlorophyll fluorescence upon onset of actinic illumination (AL). (a) Rapid induction kinetics of spinach chloroplasts in the absence and presence of 10^{-5} M 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU). Hatched areas, bound by the broken lines and the two curves, correspond to the size of the PS II acceptor pools. F_0 , fluorescence yield measured in the absence of DCMU before onset of actinic illumination; F_m , maximal fluorescence yield reached during actinic illumination; actinic intensity $250 \mu\text{mol photons m}^{-2} \text{s}^{-1}$. Modified from Schreiber et al. (1994). (b) Slow induction kinetics of spinach leaf in the presence and absence of oxygen. Actinic intensity $250 \mu\text{mol m}^{-2} \text{s}^{-1}$. Data from Schreiber et al. (1994).

B. Slow Chlorophyll Fluorescence Changes

With dark-adapted samples, after onset of illumination the initial rapid fluorescence increase is followed by a slower decline with complex kinetics (see Fig. 3b). This decline reflects the activation of photosynthetic electron transport, involving various regulatory processes. It has been known for a long time that the secondary fluorescence decline requires the presence of oxygen (Kautsky and Franck, 1943; Munday and Govindjee, 1969; Schreiber et al., 1971) and that photoreduction of O_2 can prime CO_2 assimilation (Radmer and Kok, 1976). Further research has revealed a particular role of ascorbate peroxidase

in O_2 -dependent electron flow in vivo (Asada and Badger, 1984; Schreiber and Neubauer, 1990; Schreiber et al., 1995c; Asada, 1999). Calvin-Benson cycle activity requires proton gradient formation and stroma alkalinization for enzyme activation and ATP formation. It is the O_2 -dependent electron flow, i.e., O_2 -reduction and the subsequent reduction of the formed H_2O_2 , which is mainly responsible for ΔpH formation during the induction period and in other situations when CO_2 reduction is limiting. The rate of the decline of fluorescence yield, following the initial peak, is much higher in algae than in higher plant leaves. It is also higher in mosses and ferns. As the O_2 -dependent fluorescence decline is preceded by a rise to a peak, one may conclude that also O_2 -reduction requires activation, which appears to be faster in lower than in higher plants.

Slow changes in fluorescence yield cannot be interpreted by considering changes in the Q_A -redox state alone. Once a ΔpH is formed, this not only leads to ATP formation and activation of assimilatory electron transport, but also to complex changes at the level of pigment protein complexes and the reaction centers (review by Krause and Weis, 1991; Chapter 10, Kramer et al.). Acidification of the thylakoid internal space not only leads to the de-epoxidation of violaxanthin to zeaxanthin, which can dissipate excitation energy in the antenna (reviewed by Demmig-Adams, 1990; also see Chapters 19, Bruce and Vasil'ev; 20, Golan et al.; 21, Gilmore; and 22, Adams and Demmig-Adams), but also slows down electron donation from the water splitting enzyme system to the oxidized primary donor P680, thus favoring energy dissipation by charge recombination (Schreiber and Neubauer, 1989; Crofts and Horton, 1991; Krieger and Weis, 1993). Furthermore, also the distribution of absorbed light energy between PS II and PS I displays slow changes, which are related to ΔpH formation as well as to the redox state of the PQ-pool (review by Allen 1992; see also Chapters 17 by Allen and Mullineaux, and 26 by Papageorgiou and Stamatakis). Hence, when dealing with an illuminated sample that has developed a transthalakoid ΔpH , any change in fluorescence yield could be caused either by a change of photochemical energy utilization or of nonphotochemical energy dissipation or by a change of energy distribution between the two photosystems, with very different functional implications. For example, an increase of fluorescence could either reflect accumulation of Q_A^- , associated with inhibition of electron transport, or a decrease in

ΔpH , paralleled by stimulation of electron transport (uncoupling), or a relative increase of PS II excitation (state 1 formation). This fundamental ambiguity had to be overcome before reliable information from slow fluorescence changes could be obtained.

IV. Saturation Pulse Method of Quenching Analysis

A. Principle of the Method

The term ‘fluorescence quenching’ assumes that for every investigated sample a maximal fluorescence yield can be defined, which is observed when various potentially quenching, alternative deexcitation pathways are minimized. The latter may be divided into two basic categories, namely photochemical energy utilization by charge separation at the reaction centers of PS II, causing ‘photochemical quenching’ and nonradiative energy dissipation into heat, causing ‘nonphotochemical quenching.’ Under normal physiological conditions, the photosynthetic apparatus of most plants reaches a relatively stable state after dark-adaptation, which is characterized by a fully oxidized state of the PS II acceptor, Q_A , and absence of a thylakoid proton gradient. Hence, in the dark-adapted state photochemical quenching is maximal and nonphotochemical quenching minimal. A prerequisite for evaluation of fluorescence quenching during illumination is reliable determination of the minimal and maximal fluorescence yields after dark adaptation, F_0 and F_m , respectively, which can be readily carried out by PAM-fluorometry. Due to the extremely low measuring light intensity, F_0 can be monitored continuously, without affecting the dark state, and a short pulse of saturating light can be applied for full reduction of Q_A and assessment of F_m . These values of F_0 and F_m obtained in the dark state serve as reference values for the evaluation of photochemical and nonphotochemical quenching in an illuminated sample by the saturation pulse method.

The rationale of the saturation pulse method is simple. In any given state of illumination, Q_A can be fully reduced by a saturation pulse, such that photochemical quenching is completely suppressed. During the saturation pulse, a maximal fluorescence yield, F'_m , is reached, which generally is lower than the dark reference value, F_m . Assuming that nonphotochemical quenching does not change during a short

saturation pulse, the lowering of F_m is a selective measure of nonphotochemical quenching.

In order to quantify photochemical and nonphotochemical quenching, originally the quenching coefficients q_Q (control by redox state of acceptor Q_A) and q_E (control by energy state) were introduced (Dietz et al., 1985; Schreiber et al., 1986). These quenching coefficients define the two types of quenching in terms of the observed fluorescence lowering relative to total variable fluorescence. They can vary between 0 and 1. It was first assumed that only variable fluorescence could be quenched nonphotochemically. However, later it was shown that also the dark fluorescence level, F_0 , can be lowered by strong energy-dependent quenching (Bilger and Schreiber, 1986). In Fig. 4 the principle of quenching analysis by the saturation pulse method is outlined following the nomenclature proposed by van Kooten and Snel (1990). This nomenclature, which was agreed on by researchers at a workshop on practical applications of Chl fluorescence in Doorwerth, Netherlands, August 1989, is widely accepted and has greatly facilitated the communication of fluorescence data.

For determination of F'_m in the light state, the sample has to be transiently darkened and it has to be assured that Q_A is quickly and fully oxidized, before there is substantial relaxation of nonphotochemical quenching. Far-red illumination can be applied for selective excitation of PS I in order to enhance oxidation of the intersystem electron transport chain. In some practical applications, however, F'_m -determination may be problematic, as e.g., in the field, when a sample cannot be transiently darkened. In this case, the extent of nonphotochemical energy dissipation can be described by the NPQ-parameter introduced by Bilger and Björkman (1990; see Fig. 4), which does not require knowledge of F'_m . Definition of nonphotochemical quenching in terms of NPQ assumes the existence of traps for nonradiative energy dissipation (like zeaxanthin) in an antenna pigment matrix (see reviews by Lavorel and Etienne, 1977; Butler, 1978). A close correlation between NPQ and the relative content of zeaxanthin was reported (Bilger and Björkman, 1990; Demmig-Adams, 1990).

The saturation pulse method not only allows us to assess photochemical and nonphotochemical quenching parameters, but also to obtain a good estimate of the quantum yield of energy conversion in PS II. The optimal quantum yield, which normally is observed after dark-adaptation, is well described by the fluorescence parameter $(F_m - F_0)/F_m = F_v/F_m$.

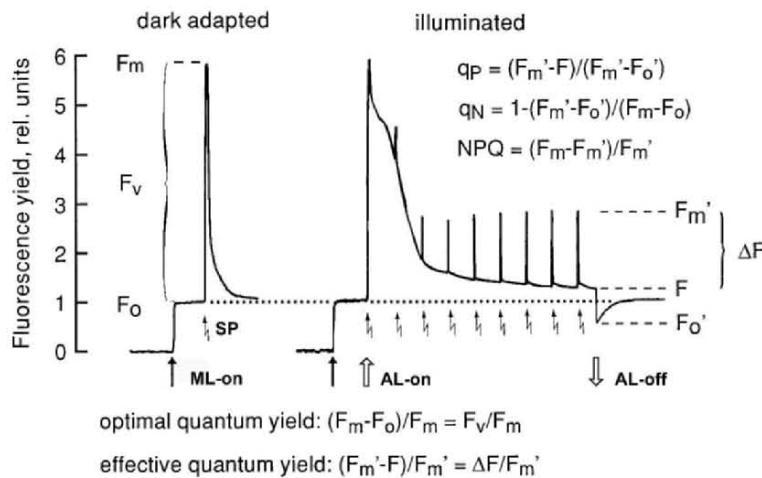


Fig. 4. Fluorescence quenching analysis by the saturation pulse method. Typical fluorescence levels and definition of basic fluorescence parameters. F_o , fluorescence yield of dark-adapted sample; F_m , maximal fluorescence yield of dark-adapted sample, reached during a saturation pulse; F_v , increase of fluorescence yield during saturation pulse; F , fluorescence yield observed at any time during illumination; F_m' , maximal fluorescence yield of illuminated sample, reached during a saturation pulse; F_o' , minimal fluorescence yield observed shortly after darkening of illuminated sample and reoxidation of the PS II acceptor side; q_P , coefficient of photochemical quenching; q_N , coefficient of nonphotochemical quenching; NPQ, nonphotochemical quenching parameter; ML, measuring light; AL, actinic light; SP, saturation pulse. For further explanations, see text. From Schreiber et al. (1998).

During illumination, the PS II quantum yield is lowered by closure of reaction centers (decreased photochemical quenching) and by stimulated heat dissipation (increased nonphotochemical quenching). The resulting ‘effective quantum yield of PS II’ was shown to correspond to the fluorescence parameter $(F_m' - F)/F_m' = \Delta F/F_m'$ (Genty et al., 1989). Notably, assessment of $\Delta F/F_m'$ does not require knowledge of F_o' . The relationship between these fluorescence parameters and PS II quantum yield is discussed in more detail in Section V; also see Chapters 3, Baker and Oxborough; and 18, Krause and Jahns.

B. Examples of Some Typical Applications

Saturation pulses can be applied repetitively without substantially affecting the state of a sample. Therefore, the derived fluorescence parameters can be monitored quasi-continuously, with the obtained information largely exceeding that of fluorescence yield as such. For example, Fig. 5 shows dark-light induction curves of a spinach leaf in the presence and absence of CO_2 . Saturation pulses are applied every 10 s for the determination of F_m and F_m' . While the induction kinetics of fluorescence yield, F (Kautsky effect) are very similar with and without CO_2 , completely different induction kinetics of F_m'

(and, hence, of nonphotochemical quenching) are observed. Both samples first show the same decline of F_m' , reflecting the light induced formation of a trans-thylakoid ΔpH . But then, ca. 1 min after onset of illumination, in the presence of CO_2 there is a remarkable increase of F_m' , whereas in the absence of CO_2 the decline of F_m' continues. When ca. 4 min after onset of illumination CO_2 is added, also the F_m' of the originally CO_2 -free sample increases. Notably, these pronounced changes of F_m' are paralleled by very small changes in the fluorescence yield, F . The obtained information relates to the activation of CO_2 -fixation in the Calvin-Benson cycle. In the given example, ca. 1 min after onset of illumination Calvin-Benson cycle is sufficiently activated to assimilate the offered CO_2 and to consume ATP, with the consequence that ADP becomes available and the light-induced ΔpH now can be used for ATP-synthesis. Hence, while fluorescence yield as such monitors energy conversion at PS II centers, in conjunction with the saturation pulse method it also provides important information on reactions at the level of enzymatic dark reactions. Such information, which is immediately apparent in the induction kinetics of F_m' , can be quantified by calculation of various fluorescence parameters, like q_P , q_N , NPQ and $\Delta F/F_m'$ (see Fig. 4). The last mentioned param-

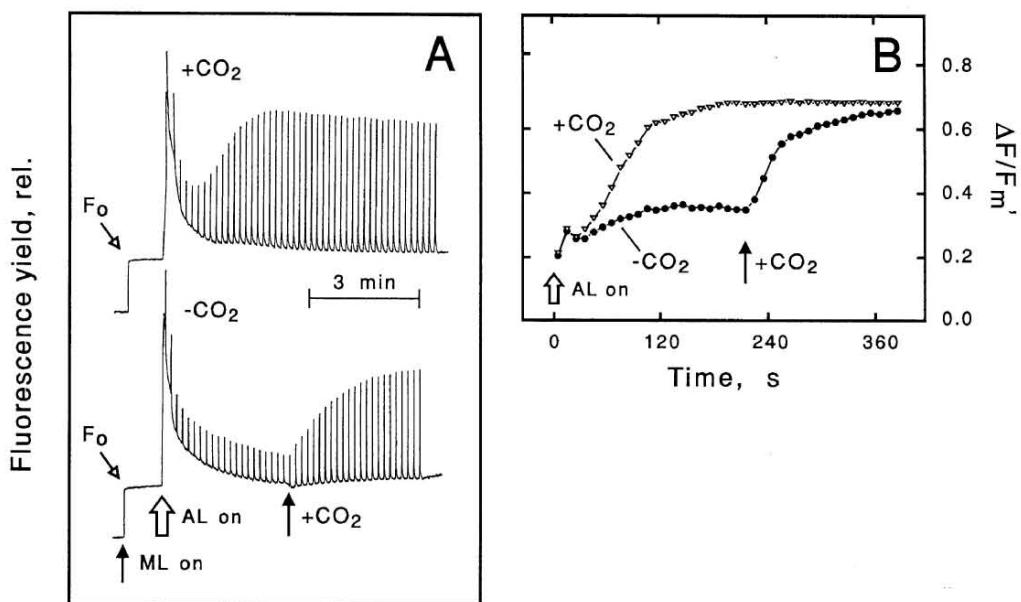


Fig. 5. Dark-light induction kinetics of fluorescence with repetitive application of saturation pulses for quenching analysis. Spinach leaf in the absence and presence of CO₂. A. Original recordings. B. Derived kinetics for the effective PS II quantum yield, $\Delta F/F_m'$. AL = actinic light; ML = measuring light. See text for explanations. From Schreiber et al. (1994).

eter, $\Delta F/F_m'$, is plotted in Fig. 5B. It may be noted that in the given example ca. 50% of the effective steady-state quantum yield observed in the presence of CO₂ is found even in the absence of CO₂. Obviously, the remaining electron transport is capable of creating a large ΔpH . As mentioned above (section III.B), O₂-dependent electron transport involving the Mehler-Ascorbate-Peroxidase cycle is responsible for this type of non-assimilatory electron flow.

Modulated Chl fluorescence can be readily measured in parallel with photosynthetic gas exchange using infrared gas analysis or oxygen polarography. In Fig. 6 an example of simultaneous measurements of a dark-light induction response of a spinach leaf with PAM fluorometry and O₂-exchange using an O₂-electrode is shown. The oxygen signal is presented as the rate of O₂-evolution. The quenching coefficient qP is calculated for every saturation pulse applied at 20 s intervals. It is apparent that both signals show very similar induction transients. Obviously, under the given conditions, the rate of oxygen evolution is closely correlated with the ‘openness’ of PS II reaction centers. There are other situations, where such close correlation does not hold (e.g., after certain stress treatments). Comparative measurements of fluorescence with gas exchange generally are informative; possible deviations may allow insights into

reactions, which otherwise are difficult to assess, as e.g., cyclic electron flow around PS II (Schreiber et al., 1989; Section V.A.3).

Dark-light induction curves with repetitively applied saturation pulses, as depicted in Figs. 5 and 6, have proven particularly useful for assessment of stress-induced damage of photosynthetic organisms. A typical example is presented in Fig. 7 that shows the effect of relatively mild heat stress on leaves of *Arbutus unedo*. It is apparent that already 5 min pretreatment at 36 °C causes a substantial slowdown of the rise of Fm' following the initial decline, suggesting that Calvin-Benson cycle activity is affected at a very early stage by heat stress. The same treatment does not yet lead to an increase of F₀, which would reflect damage in PS II (Schreiber and Berry, 1977; Bilger et al., 1987). Very similar changes are induced by water stress in *Arbutus unedo* (Schreiber and Bilger, 1987). Again, the onset of Calvin-Benson cycle activity is affected before any effect on PS II is observed. Obviously, the saturation pulse induction kinetics is sensitive to a very early type of stress induced damage. Plants that have experienced such primary damage normally continue to be exposed to natural daylight, which then is likely to cause secondary damage by photoinhibition. The same light intensity, a plant can cope with when Calvin-Benson cycle is still

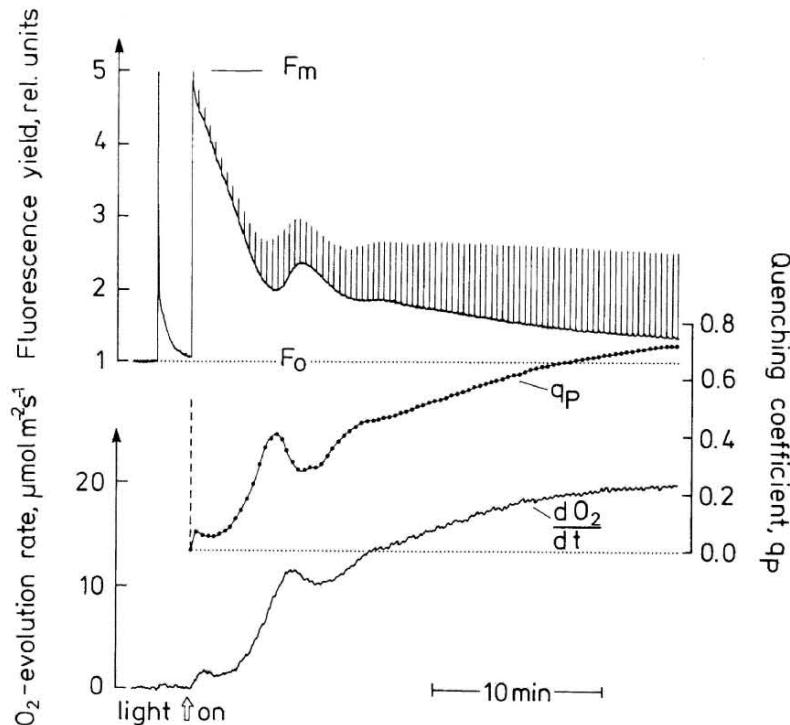


Fig. 6. Simultaneous recordings of Chl fluorescence and rate of oxygen evolution during dark-light induction of spinach leaf. Repetitive application of saturation pulses for assessment of photochemical quenching. Data from Bolhar-Nordenkampf et al. (1989).

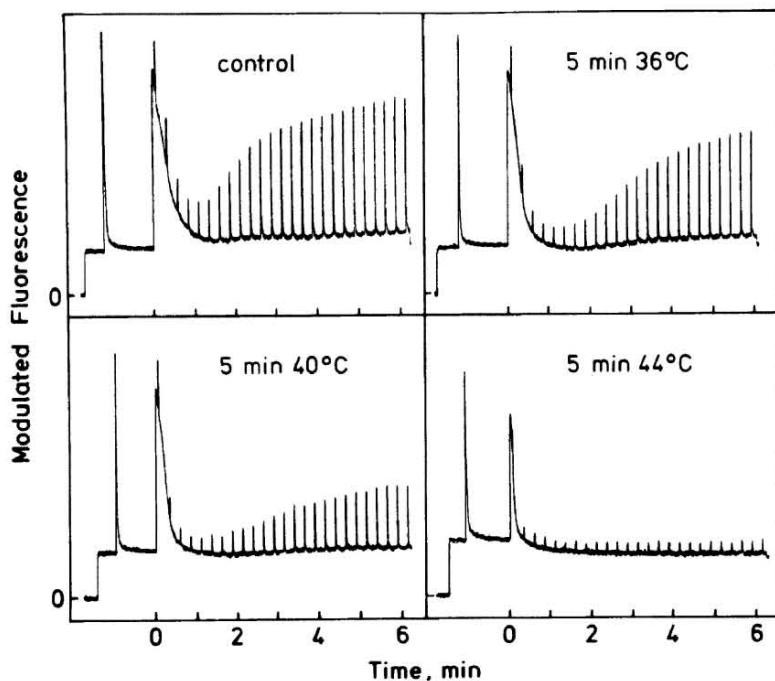


Fig. 7. Effect of heat-pretreatment on dark-light induction kinetics of Chl fluorescence with repetitive application of saturation pulses of *Arbutus unedo*. 5 min treatment at indicated temperatures and 5 min recovery at 22 °C before measurement. Actinic intensity, 150 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$. Data from Schreiber and Bilger (1987).

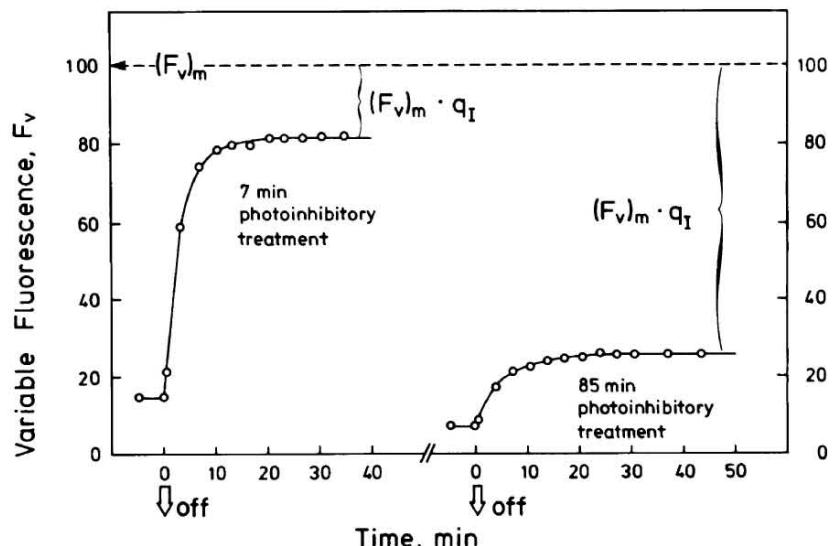


Fig. 8. Relaxation of nonphotochemical quenching in *Arbutus unedo* following illumination at $1,700 \mu\text{mol photons m}^{-2}\text{s}^{-1}$ for 7 and 85 min. Determination of coefficient of photoinhibitory nonphotochemical quenching, q_I . $(F_v)_m$, maximal variable Chl fluorescence observed with dark-adapted leaf before photoinhibitory illumination or 45 min after 5 min illumination at $500 \mu\text{mol photons m}^{-2}\text{s}^{-1}$ (non-photoinhibitory) (control, data not shown).

intact, will become excessive when CO_2 -assimilation becomes limiting. Photoinhibitory damage will occur when the capacity of protective reactions, like the Mehler-Ascorbate-Peroxidase cycle (Schreiber and Neubauer, 1990; Schreiber et al., 1995c; Asada, 1999) and the xanthophyll cycle (Demmig-Adams, 1990; Demmig-Adams and Adams, 1992; Pfundel and Bilger, 1994) become exhausted.

Assessment of photoinhibition has become a widespread application of PAM fluorometry and the saturation pulse method. It had been known for a long time that photoinhibition causes a decrease of variable fluorescence, F_v (Björkman, 1987). Before introduction of PAM fluorometry, F_v used to be determined by measuring the light-induced fluorescence increase at liquid nitrogen temperature. Besides the fact that PAM measurements are much more easily carried out, they also have the decisive advantage of being nonintrusive (in contrast to the low temperature method), so that measurements can be repeated on the same sample over extended periods of time. In this way, development and recovery of photoinhibition can be studied.

For determination of maximal variable fluorescence, $(F_v)_m$, a sample must be dark adapted and the reversible part of nonphotochemical quenching, most of which is related to ΔpH -dependent membrane energization (Schreiber and Bilger, 1987; Horton and Hague, 1988; Quick and Stitt, 1989), must be fully re-

laxed. Following a non-photoinhibitory illumination, variable fluorescence, F_v , increases with biphasic kinetics, reaching $(F_v)_m$ within 40 min (data not shown). In contrast, following photoinhibitory illumination, even after prolonged darkness full recovery of $(F_v)_m$ is not observed (see examples of recovery kinetics in Fig. 8 for two different photoinhibitory treatments). The nonphotochemical quenching persisting after ca. 40 min darkness may be considered photoinhibitory. It is quantified by the quenching coefficient q_I , with the quenched fraction corresponding to $(F_v)_m \cdot q_I$ (Schreiber and Bilger, 1987). The extent of photoinhibition may be overestimated, if the dark intervals between saturation pulses are too short, so that energy dependent quenching is partially maintained. Furthermore, under certain conditions, the saturation pulses may support a shift from pigment state 1 to 2, resulting in an increase of the fraction of energy distributed to PS I, which causes state-dependent nonphotochemical fluorescence quenching (Horton and Hague, 1988; Quick and Stitt, 1989; Schreiber et al., 1995b; Bukhov et al., 1996; Chapter 17, Allen and Mullineaux). In general, the intrusiveness of saturation pulses increases with their intensity, length and frequency, whereas it decreases with the intensity of ambient light. Saturation pulses at intensities exceeding $5,000 \mu\text{mol photons m}^{-2}\text{s}^{-1}$ even may cause significant photodamage (Shen et al., 1996; Apostol et al., 2001).

C. Contribution of Photosystem (PS) I Fluorescence and Other Background Signals

For the sake of simplicity, in most fluorescence studies the tacit assumption is made that at room temperature the measured fluorescence originates exclusively from PS II. In reality, however, this is only true for variable fluorescence, while depending on the organism and measuring conditions, PS I may contribute a more or less large background signal (Adams et al., 1990a; Genty et al., 1990; Pfundel, 1998; Gilmore et al., 2001; Chapter 9, Itoh and Sugiura). Normally, PS I is donor-side limited, and variable PS I fluorescence is either quenched photochemically (when P700 is reduced) or nonphotochemically (when P700 is oxidized). Knowledge of the PS I background signal is essential for quantitative determination of $\Delta F/Fm'$ and NPQ, but not for qP and qN. Pfundel (1998) estimated the PS I contribution to total Fo at wavelengths greater than 700 nm to be about 30% and 50% in C3 and C4 plants, respectively. The corresponding values for Fm were 6% and 12%. It is generally assumed that the contribution of PS I to fluorescence at wavelengths below 700 nm is negligibly small. In principle, it is possible to apply blue measuring light and to specifically assess PS II fluorescence at wavelengths below 700 nm by placing a short-pass filter in front of the fluorescence detector. However, in particular when dealing with leaves, short-wavelength fluorescence is weak due to strong reabsorption, such that a considerable part of the signal is sacrificed when excluding long wavelength fluorescence.

Genty et al. (1990) suggested that the part of Fo, which is originating from PS I cannot be quenched nonphotochemically and developed a simple extrapolation method for its determination. Using this method these authors estimated a PS I contribution of 30% and 50% to Fo in barley and maize, respectively. Once the PS I contribution is known, it can be subtracted from Fm' in order to calculate the correct $\Delta F/Fm'$. With increasing PS I contribution, the apparent $\Delta F/Fm'$ is decreased.

The apparent $\Delta F/Fm'$ is decreased not only by PS I fluorescence, but by any non-PS II-Chl background signal, which trivially may originate from the measuring system (e.g., fluorescence of optical components) or more seriously from non-PS II-Chl fluorescence of the investigated sample. For example, in cyanobacteria besides the fluorescence from PSI Chl, allophycocyanin fluorescence also contributes to the background signal, which explains the relatively small

extent of variable fluorescence generally observed in these organisms. The contribution of a background signal has to be taken into account, if a quantitative assessment of $\Delta F/Fm'$ (or NPQ) is essential. In most practical applications, however, only relative changes are of interest. While the background signal due to optical components and to PS I fluorescence of green plants normally can be considered constant, this is not always true for cyanobacteria. Allophycocyanin fluorescence is stimulated upon functional detachment of phycobilisomes (Schreiber, 1979, 1980). Furthermore, cyanobacteria show pronounced dark-light-dark changes of energy distribution between the two photosystems, leading to large variations in the ratio of PS II/PS I fluorescence yields (review by Allen, 1992; Chapter 17, Allen and Mullineaux). Such changes can be studied without interference of photochemical quenching in the presence of DCMU. As an example Papageorgiou and co-workers have shown osmotic regulation of phycobilisome-sensitized Chl *a* fluorescence in cyanobacteria (Papageorgiou and Alygizaki-Zorba, 1997; Papageorgiou et al., 1999; Stamatakis and Papageorgiou, 2001; Chapter 29, Papageorgiou and Stamatakis). The latter work is a good example of an application of PAM fluorometry in the study of physiological changes going beyond the usual assessment of photosynthetic activity.

D. Heterogeneity of Fluorescence Signal

The saturation pulse method, as outlined above, is of outstanding simplicity, a feature that has greatly contributed to its success during the past 18 years. Standard PAM fluorometry integrates over large sample areas containing millions of cells and the obtained fluorescence parameters represent the average response of the sample. This approach makes sense from a practical point of view, as it assures a high signal/noise ratio and high reproducibility. On the other hand, it should be realized that the overall fluorescence signal is composed of a number of components that may display quite different properties.

Functional heterogeneity of PS II is primarily based on structural differences at the level of PS II acceptor and donor sides as well as PS II interactions with other components of the thylakoid membrane. There is a vast amount of literature on PS II heterogeneity with numerous types of postulated 'fluorescence quenchers' (for reviews, see Black et al., 1986; Govindjee, 1990; Melis, 1991; Lavergne and Briantais, 1996). Somewhat surprisingly, a substantial fraction of PS II

centers has been characterized as being ‘inactive’ with respect to linear electron transport (Chylla et al., 1987; Chylla and Whitmarsh, 1990; Cao and Govindjee, 1990; Lavergne and Leci, 1993). Such heterogeneities have been an important topic of basic photosynthesis research, with Chl fluorescence being the most informative tool. Detection and quantification of heterogeneous populations of PS II, like α and β centers (Melis and Homann, 1976; Melis and Duysens, 1979; Melis and Schreiber, 1979), normally involves measurements of dark-light induction kinetics, often in the presence of PS II inhibitors, in order to avoid interference of electron transport beyond PS II. Hence, the assessed heterogeneity is characteristic for the dark-adapted and inhibited state that may differ considerably from the regulated steady state during continuous illumination, which is relevant for photosynthetic performance of a plant *in vivo*. In view of the fact that measured quantum yields of photosynthesis *in vivo* are close to the maximum theoretical quantum yield (Graan and Ort, 1986; Björkman and Demmig, 1987; Long et al., 1993), there is no room for substantial PS II inefficiency under normal physiological conditions. On the other hand, heterogeneous populations of PS II may well be expected in view of the known topography of the thylakoid membrane of green plants (for review see Melis, 1991), with 70–80% of PS II being localized in the stacked grana region and the remaining PS II found in the stroma-exposed region of the thylakoid membrane. Considering that PS I as well as the reversible ATP-ase are found only in the latter region, functional PS II heterogeneity may be expected. Physiologically regulated changes in the distribution of the pigment-protein complexes within the membrane (for review see Allen, 1992; see Chapter 17, Allen and Mullineaux) will give rise to dynamic changes of PS II heterogeneity. While the saturation pulse method does not distinguish between different PS II populations, the general rationale of quantum yield assessment applies to all types of PS II (Section V.A.1).

A very basic aspect of Chl fluorescence heterogeneity was addressed by Schreiber and Krieger (1996), who argued on the basis of the reversible radical pair model of PS II (Schatz et al., 1988) that variable Chl fluorescence consists of two different fractions, which are affected differently by nonradiative radical pair recombination at PS II reaction centers. This aspect bears considerable consequences for the interpretation of fluorescence data measured

under conditions favoring a high quantum yield of nonradiative radical pair recombination and will be dealt with in more detail in Section VI.C.

Besides the functional heterogeneity of Chl fluorescence, sample heterogeneity must also be considered. There is a gradient of intensities of measuring and actinic light across a leaf that depends on wavelength as well as on absorbance and scattering properties of the leaf (for review see Vogelmann, 1993). The overall fluorescence signal originates from different chloroplast layers, displaying different photosynthetic activities. The relative contribution of deeper layers can be minimized by using red or blue measuring light, which is strongly absorbed in the uppermost cell layers, and by measuring short wavelength fluorescence (< 700 nm), the detected part of which is unlikely to originate in deeper layers because of its strong reabsorption. For assessment of photosynthetic performance in deeper tissue layers special microfiber techniques were developed (Section VII.B.2). There can also be lateral heterogeneities of fluorescence yield and photosynthetic activity over the surface of a leaf, which are not ‘differentiated’ by standard PAM fluorometry. In recent years, considerable progress has been made in the development of instrumentation for fluorescence imaging (Section VII.C and Chapters 14, Nedbal and Whitmarsh; 15, Oxborough; and 28, Lichtenthaler and Babani). Alternatively, heterogeneities over the surface of a sample can be also studied by standard PAM fluorometry, combined with epifluorescence microscopy (Section VII.B.1).

Largely heterogeneous fluorescence responses are found in natural surface water samples with mixed phytoplankton populations. While fluorescence measurements have proven very useful for assessment of phytoplankton biomass even when mixed responses were analyzed (Chapters 16, Moya and Cerovic; 30, Falkowski et al.; and 31, Raven and Maberly), special techniques were developed, which can differentiate between the responses of the major groups of phytoplankton on the basis of their differences in Chl fluorescence excitation spectra (Section VII.A).

In view of the numerous types of potential heterogeneities in fluorescence responses, it is clear that determination of *absolute values* of fluorescence parameters is problematic. Actually, in most practical applications *relative changes* of fluorescence parameters are of primary interest (see, however, discussions in Chapters 17, Allen and Mullineaux; 19, Bruce and Vasil’ev; and 21, Gilmore).

V. Assessment of Quantum Yield and Relative Electron Transport Rate

A. Relationship Between Fluorescence Yield and PS II Quantum Yield

1. Simple Derivation Based on Law of Energy Conservation

The relationship between fluorescence yield and quantum yield of PS II is of fundamental importance for all practical applications of Chl fluorescence. Theoretical derivations are based on the competition of fluorescence emission with other pathways of exciton de-excitation, the details of which depend on the assumed model of exciton dynamics in the antenna system and of trapping at the reaction centers (see review by Dau, 1994). For the uninitiated, such theoretical derivations often are difficult to follow, with the consequence that uncertainty and even confusion may remain. Therefore, here, a well-known and well-established very general approach will be presented, which does not require any specific model. The following derivation essentially is based on the law of energy conservation and the assumption that during a saturation pulse the ratio of the rate constants of fluorescence emission and nonradiative deexcitation does not change.

(1) The sum of the quantum yields (Φ) of photochemistry (P), fluorescence (F) and nonradiative de-excitation (D) is unity:

$$\Phi_p + \Phi_F + \Phi_D = 1 \text{ or } \Phi_p = 1 - \Phi_F - \Phi_D \quad (1)$$

(2) Upon application of a saturation pulse, the photochemical quantum yield, Φ_p , becomes zero, while the remaining yields assume maximal values:

$$\Phi_{Fm} + \Phi_{Dm} = 1 \text{ or } \Phi_{Dm} = 1 - \Phi_{Fm} \quad (2)$$

(3) It is assumed that the ratio between fluorescence and nonradiative de-excitation does not change during a short saturation pulse:

$$\Phi_{Dm} / \Phi_{Fm} = \Phi_D / \Phi_F \quad (3)$$

(4) By combining equations 2 and 3, Φ_D can be expressed in terms of fluorescence yield:

$$\Phi_D = \Phi_F / \Phi_{Fm} - \Phi_F \quad (4)$$

(5) By combining equations 1 and 4, Φ_p can be expressed in terms of fluorescence yield:

$$\Phi_p = 1 - \Phi_F - \Phi_F / \Phi_{Fm} + \Phi_F = (\Phi_{Fm} - \Phi_F) / \Phi_{Fm} \quad (5)$$

(6) In practice, the photochemical quantum yield of PS II (with the definition $\Phi_{II} = \Phi_p$) can be either assessed after dark-adaptation or during illumination. After dark-adaptation, the fluorescence yield is defined as F_0 and the maximal fluorescence yield induced by a saturation pulse is F_m . During illumination the fluorescence yield is defined as F , with the maximal yield induced by a saturation pulse being F_m' (see Fig. 4). The photochemical quantum yield after dark adaptation normally corresponds to the 'optimal quantum yield', which is lowered by illumination to an 'effective quantum yield'. Hence, the following fluorescence expressions for determination of PS II quantum yield are obtained:

Optimal quantum yield:
 $(\Phi_{II})_{max} = (F_m - F_0)/F_m = F_v/F_m \quad (6)$

Effective quantum yield:
 $\Phi_{II} = (F_m' - F)/F_m' = \Delta F/F_m' \quad (7)$

A thorough theoretical derivation of the relationship between the quantum yields of PS II photochemistry and fluorescence was presented by Lavergne and Trissl (1995), which was based on a detailed model of exciton dynamics derived from picosecond fluorescence lifetime measurements (Schatz et al., 1988; Roelofs et al., 1992). It may be considered reassuring that the expression derived by these authors was quite similar, although not identical, to the simple expressions derived above, with the possible deviation being estimated to be rather small (ca. 14% underestimation of Φ_{II}). Actually, in practice larger deviations may be caused by other factors (see below).

2. Experimental Evidence for Close Correspondence

Numerous laboratories have presented experimental evidence for a close relationship between Φ_{II} and F_v/F_m (as well as $\Delta F/F_m'$) for a number of different plant species under a variety of physiological conditions (Genty et al., 1989; Seaton and Walker, 1990; Krall and Edwards, 1991; Oberhuber et al., 1993; Hormann et al., 1994; Schreiber et al., 1995a; Earl

and Tollenaar, 1998; Gilbert et al., 2000; Chapter 3, Baker and Oxborough; Chapter 30, Falkowski et al.). While the original data of Genty et al. (1989) showed a linear relationship between Φ_{II} and $\Delta F/Fm'$, later work revealed nonlinearity in the range of high Φ_{II} values, i.e., low photon flux densities (Seaton and Walker, 1990). The observed nonlinearity was particularly pronounced in C₃ plants at elevated O₂-concentration, whereas C₄ plants showed a close to linear relationship independent of O₂-concentration (Krall and Edwards, 1990). These findings suggested that photorespiratory electron flow or the Mehler-Ascorbate-Peroxidase cycle are likely to cause at least part of the nonlinearity.

However, a pronounced deviation from linearity at high Φ_{II} values was also observed with isolated chloroplasts using ferricyanide or methylviologen as electron acceptor (Hormann et al., 1994; Schreiber et al., 1995a). As shown in Fig. 9, there is a quite robust basic relationship under a variety of experimental conditions. A linear relationship holds over a wide range of Φ_{II} values. In the coupled state, significant deviation from linearity occurs only at light intensities below approximately 30 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ (photosynthetically active radiation, PAR). While in vitro measurements with isolated chloroplasts give reliable results even in this low intensity range, in vivo measurements of electron transport by gas exchange are problematic, not only because of a low signal/noise, but also because of overlapping respiratory gas exchange (Oberhuber et al., 1993; Schreiber et al., 1995a). Furthermore, at low light intensities cyclic electron flow around PS II may compete with linear electron flow. With decreasing quantum flux densities and, hence, increasing times between S-state advancement, the probability for S-state deactivation by the PS II acceptor side increases (Renger, 1973). (For a discussion of the relationship of PS II reactions with Chl fluorescence, see Chapter 8, Shinkarev.)

3. Possible Deviation from Linearity due to Cyclic PS II

The potential role of cyclic PS II in affecting the relationship between Φ_{II} and $\Delta F/Fm'$ at low PAR-values can be judged from experiments with ADRY-reagents (Acceleration of the Deactivation Reactions of the watersplitting enzyme system Y; Renger, 1973) like carbonylcyanide-m-chlorophenylhydrazone (CCCP) or 2-(3-chloro-4-trifluoromethyl)-anilino-3,5-dinitro-thiophene (ANT-2p), which artificially enhance cyclic

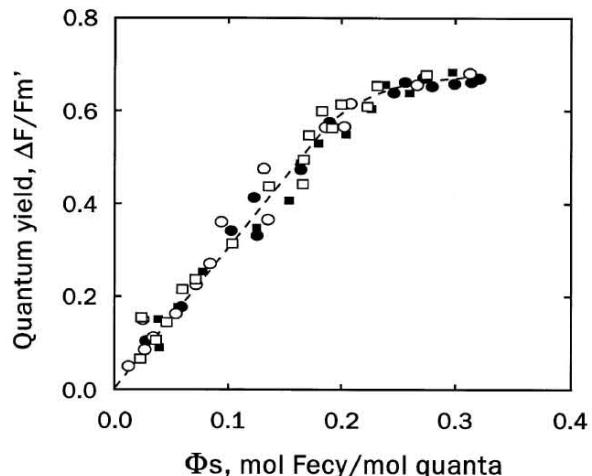


Fig. 9. Single curvilinear relationship between apparent quantum yield of PS II, $\Delta F/Fm'$, and quantum yield of ferricyanide reduction in spinach thylakoids under a variety of conditions. The quantum yield was varied by light intensity (7 to 3080 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$) in the coupled and uncoupled state (0.5 μM nigericin or 0.5 μM nigericin + 0.5 μM valinomycin) or by addition of increasing DCMU concentrations ranging from 15 nM to 1 μM at 16 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$. Ferricyanide (Fecy) concentration, 0.25 mM. Φ_s , quantum yield of ferricyanide reduction (mol Fecy per mol photons). Modified from Hormann et al. (1994).

PS II flux (Heber et al., 1979; Schreiber et al., 1989; Heimann, 1998). On the one hand, an ADRY-reagent, like ANT-2p, inhibits linear electron transport and on the other hand it substantially stimulates photochemical fluorescence quenching and the effective quantum yield, $\Delta F/Fm'$. This paradoxical behavior does not question the basic relationship between Φ_{II} and $\Delta F/Fm'$ derived above (Section V.A.1). It is clear that fluorescence cannot distinguish between primary energy conversion leading to linear or cyclic electron transport. The question is whether the same or a similar pathway does play a significant role under physiological conditions. As suggested above, it may well occur at low light intensities. On the other hand, Paul Falkowski and co-workers reported evidence for cyclic PS II at high quantum flux densities (Falkowski et al., 1986a; Prasil et al., 1996) on the basis of simultaneous measurements of fluorescence yield and O₂-evolution. At PAR-values above 200 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ these authors observed a decline of O₂-evolution yield to zero, without a corresponding decline of Φ_{II} calculated from fluorescence parameters, which remained constant at 20–40% of its maximal value. For an understanding of this surprising observation, which is not confirmed by corresponding PAM-data

(see e.g., Fig. 9), it is important to note that Falkowski and co-workers apply a different technique for determination of maximal fluorescence yield, on which calculation of Φ_{II} is based (Sections VI.B and VI.C; Chapter 30, Falkowski et al.).

B. Relative Electron Transport Rate and Light Response Curves

As outlined above, the fluorescence parameter $\Delta F/Fm'$ has been experimentally proven to be a reliable measure of PS II quantum yield for a variety of plants. Using PAM fluorometry and the saturation pulse method, $\Delta F/Fm'$ can be readily measured with a plant *in situ* under natural light conditions. For evaluation of the obtained information, knowledge of the ambient quantum flux density (PAR) and of temperature is essential. If, for example, a sample shows a low $\Delta F/Fm'$, this could be due to an intrinsic deficiency (e.g., stress induced damage) or to high ambient PAR or to low ambient temperature. Therefore, special microquantum-temperature sensors were developed, which assess incident PAR (in $\mu\text{mol photons m}^{-2} \text{s}^{-1}$) and temperature (in $^{\circ}\text{C}$) at the very site of a sample where fluorescence is measured (Bilger et al., 1995). For estimating the relative electron transport rate, ETR, the PAR apportioned to PS II is decisive. As normally information on energy distribution is not available, it is assumed that photons are evenly distributed between the two photosystems. This assumption may be considered reasonable during steady state illumination, when the turnover rates of PS I and PS II are equal. Possible cyclic electron transport around PS I or PS II would cause deviations. It also has to be considered that only part of the incident light is absorbed. If no information on absorbance is available, it may be assumed that an average leaf absorbs about 84% of incident PAR (Björkman and Demmig, 1987). Hence, the following expression may serve for an estimate of the relative electron transport rate in leaves:

$$\begin{aligned} \text{Rel. electron transport rate} &= \text{ETR} \\ &= 0.84 \cdot 0.5 \cdot \text{PAR} \cdot \Delta F/Fm' \\ &= 0.42 \cdot \text{PAR} \cdot \Delta F/Fm' \end{aligned} \quad (8)$$

Even if, as often is the case in practice, the *absolute* values of PS II absorbance and PS II quantum yield are uncertain, the *relative* changes of ETR with environmental parameters can be very informative. This is particularly true for the change of ETR with PAR.

Plots of ETR versus PAR give light response curves. In analogy to corresponding curves obtained from gas exchange measurements (so-called P-I curves, with P standing for the rate of photosynthesis and I for the quantum flux of photosynthetically active radiation, PAR), the slope at low PAR reflects maximal photosynthetic quantum yield (parameter α) and the plateau reached at light saturation is a measure of photosynthetic capacity (ETR_{max}, equivalent to the parameter P_{max}, defined as maximal gas exchange rate in P-I curves). The parameter $I_k = \alpha/P_{max}$ is a convenient measure of the PAR-value above which saturation becomes dominant. While healthy leaves are characterized by similar maximal quantum yields (0.80–0.85), they may display very different photosynthetic capacity, depending on their long-term light adaptation state during growth. Typical examples of light response curves of a sun leaf of *Helianthus tuberosus* and a shade leaf of *Fagus sylvatica* are shown in Fig. 10. The potential rates at maximal quantum yields are defined by the initial slope lines. The deviation of the measured ETR from the potential ETR (i.e., if maximal quantum yield were effective at given PAR) is a measure of ‘intrinsic limitation,’ which increases with PAR, and can be quantified by the expression $a/(a + b)$ (see Fig. 10). This ratio (without dimensions) can be also considered a measure of ‘excessive light intensity’ (Demmig and Winter, 1988; Björkman and Demmig-Adams, 1994; Schreiber et al., 1994). In the examples of Fig. 10, ‘intrinsic limitation values’ of 0.54 and 0.84 can be calculated for the sun leaf of *Helianthus tuberosus* and the shade leaf of *Fagus sylvatica*, respectively, at an irradiance of 1,250 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$. (For a further discussion on differences between leaves of sun and shade plants see Chapter 28, Lichtenhaller and Babani.)

In the case of leaves a quantitative comparison of *absolute* rates (e.g., as measured by gas exchange) and estimated rates based on PAM fluorometry is difficult due to the fact that the measured fluorescence primarily originates from the surface, whereas gas exchange also includes deeper layers. Hence large heterogeneities of effective light intensity have to be considered and a quantification of *absorbed* PAR is problematic. A more quantitative comparison is possible with dilute suspensions using a cuvette with defined optical geometry that allows homogenous illumination and determination of the absorbed PAR. Gilbert et al. (2000) compared light response curves of various phytoplankton species measured simul-

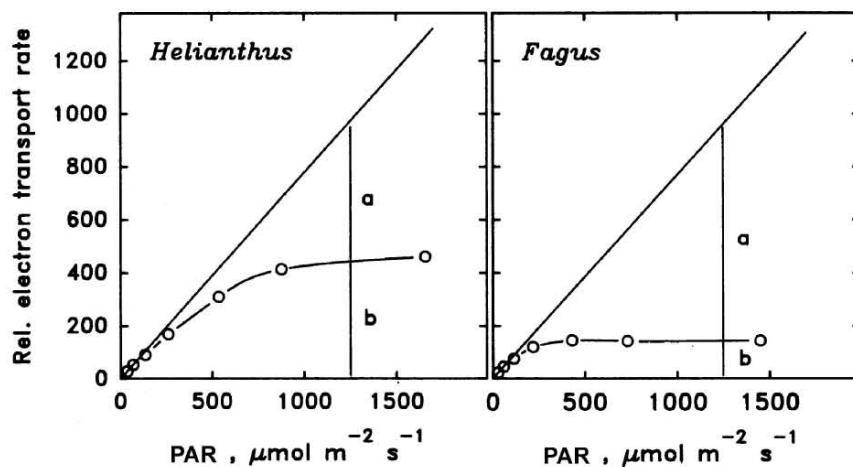


Fig. 10. Comparison of light response curves of sun leaf of *Helianthus tuberosus* and shade leaf of *Fagus sylvatica*. The potential rates at maximal PS II quantum yield are defined by the initial slope lines. The expression $a/(a+b)$ is a measure for rate limitation at a given light intensity. The abscissa scale ($\text{PAR } \mu\text{mol m}^{-2}\text{s}^{-1}$) refers to the photon flux density of photosynthetically active radiation. Data from

taneously by PAM fluorometry and O_2 -polarography. They reported a surprisingly close correspondence on an absolute scale of the light response curve fit parameters α , I_k and P_{\max} (see definitions above). This was particularly true for diatoms. With green algae the fluorescence derived maximal rate (P_{\max}) was ca. 20% higher than measured gross O_2 -evolution, probably due to the Mehler-Ascorbate-Peroxidase cycle (Schreiber et al., 1995c). In the case of cyanobacteria larger deviations were observed (up to 100%), presumably caused by respiratory O_2 -uptake, or by dynamic changes in PS II optical cross section (via reversible state 1-state 2 transitions; Chapter 17, Allen and Mullineaux).

In principle, in order to be comparable with conventional P-I curves measured by gas exchange, at each PAR-value of a light response curve, sufficient time should be given to the sample to reach steady state during illumination. In practice, however, this often is neither possible nor desired. Actually, even with illumination intervals as short as 10 s, relevant information on the saturation characteristics of electron transport can be obtained (Schreiber et al., 1997; White and Critchley, 1999; Rascher et al., 2000). Such ‘rapid light curves’ depend strongly on the momentary state of light adaptation of a sample and, hence, vary considerably during the course of a day. The maximal relative electron transport rate, ETR_{\max} , reached during rapid light curves generally increases during the morning, shows a dip at noon (mid-day depression) and increases again towards the evening (Ralph et al., 1999). Diurnal changes

in ETR_{\max} reflect the photosynthetic performance of a plant in its natural environment. The obtained information exceeds that of $\Delta F/F_m'$ measured under ambient light conditions, as with each rapid light curve the performance over a wide range of light intensities is assessed and maximal capacity is determined. This approach is particularly useful under rapidly changing light conditions, as e.g., encountered by underwater plants or plants at the bottom of the rainforest (sun flecks). As rapid light curves are measured in artificial light, the measurement as such is not affected by the ambient light fluctuations. On the other hand, the integrated light history of the sample is reflected in the particular characteristics of the rapid light curve.

VI. Intrinsic Heterogeneity of Variable Chlorophyll Fluorescence

Determination of maximal fluorescence yield, F_m or F_m' , is of central importance for the quenching analysis using the saturation pulse method. As outlined in section V.A.1, this method is based on the assumption that during a saturation pulse the quantum yield of photochemical energy conversion transiently is suppressed to zero and that the quantum yield of nonradiative energy dissipation does not change. Whether or not the latter assumption is justified depends on the interpretation of the detailed kinetics of the fluorescence rise during a pulse of saturating light, the properties of which reveal a fundamental

intrinsic heterogeneity of variable fluorescence. As this heterogeneity is based on the peculiarities of PS II photochemistry as such, it is distinct from heterogeneities involving different types of cells or populations of PS II centers (Section IV.D.). While existence of the latter does not affect the saturation pulse method as such, the intrinsic heterogeneity of variable fluorescence bears on the very principle of this method. A deeper understanding of the underlying mechanisms may either question or support its general applicability. Therefore, this aspect is given particular attention here (see discussions of the same topic in Chapters 6, Vredenberg; 19, Bruce and Vasil'ev; 30, Falkowski et al.).

A. Polyphasic Rise of Fluorescence Yield upon Onset of Saturating Light

In standard applications of PAM fluorometry, the effect of a saturation pulse is just recorded as a spike reflecting the increase of fluorescence yield to a maximal level of F_m or F_m' . In order to resolve kinetic details of this fluorescence change, the light-on characteristics as well as time resolution of the detector system have to be fast. As was first shown by Deslosme (1967), the fluorescence rise upon onset of saturating light is polyphasic, consisting of two fundamentally different parts, so-called 'photochemical' and 'thermal' phases, with the latter comprising 30–50% of the total rise. A detailed study of the polyphasic fluorescence rise has revealed that the thermal part actually consists of several subphases, which are affected differently by preillumination, electron acceptors and inhibition of the PS II donor and acceptor sides (Schreiber, 1986; Neubauer and Schreiber, 1987; Schreiber and Neubauer, 1987; Chapter 12, Strasser et al.). As shown in Fig. 11, fluorescence rises in the photochemical phase from F_0 to a first intermediate level, I_1 (also called 'J'), and then in two thermal phases via a second intermediate level, I_2 (also called 'I'), to a peak level, P , which in dark adapted samples is equivalent to F_m . Whereas the rate of the photochemical phase is limited by light intensity, the rate of the thermal phases saturates at high light intensities (ca. $1,500 \mu\text{mol photons m}^{-2}\text{s}^{-1}$, Neubauer and Schreiber, 1987), with the consequence that maximal fluorescence cannot be reached within less than about 200 ms. The I_1 level ('J') increases with light intensity, saturating at distinctly higher intensities than the thermal phases (ca. $10,000 \mu\text{mol photons m}^{-2}\text{s}^{-1}$, Neubauer and Schreiber, 1987). More recently, Reto Strasser

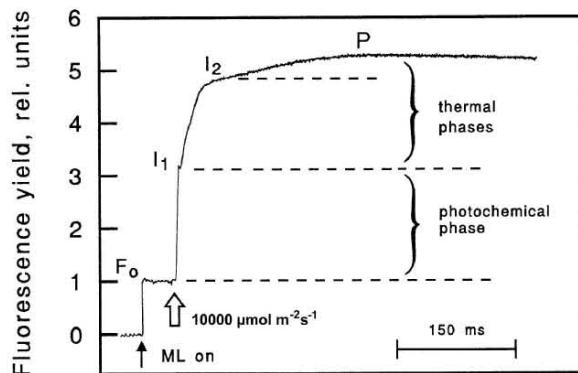


Fig. 11. Polyphasic Chl fluorescence rise in spinach leaf upon onset of saturating light as measured with Pulse Amplitude Modulation (PAM) fluorometer. Notations: F_0 , dark-level fluorescence yield, monitored with weak measuring light (ML); I_1 , first intermediate level (also called 'J'); I_2 , second intermediate level (also called 'I'); P , peak level reached in saturating light, equivalent to maximal fluorescence yield, F_m . Modified from Schreiber et al. (1994).

and co-workers have studied the polyphasic rise using a nonmodulated measuring system and have demonstrated its analytical value in numerous applications (Strasser and Govindjee, 1992; Srivastava et al., 1995; Strasser et al., 1995; B. Strasser, 1997; Stirbet et al., 1998; Chapter 12, Strasser et al.). Strasser and Govindjee (1992) chose a different nomenclature, with O-J-I-P corresponding to O- I_1 - I_2 - F_m . The reasons for their choice are given in Strasser et al. (1995). One of the reasons was that these authors first did not realize the equivalence of the various levels determined by modulated and nonmodulated measuring systems, and the other was the simplicity of the new format. It should be noted, however, that I_1 (measured by PAM fluorometry) is defined for saturating light intensity (see above). Hence, equivalence of I_1 to J (measured by nonmodulated system) can be expected only, if the latter is also assessed at saturating intensity.

The polyphasic rise of fluorescence yield upon onset of saturating light is very similar in a large variety of photosynthetic organisms and, hence, seems to reflect fundamental properties of PSII. While the rise from F_0 to I_1 (or 'J') can be understood in a conventional way as representing the kinetics of Q_A^- accumulation, an interpretation of the thermal phases is more difficult. In this context, the following observations are relevant:

- DCMU eliminates the thermal phases, with the amplitude of the remaining photochemical phase being raised close to the F_m -level (Neubauer and

Schreiber, 1987; Schreiber and Krieger, 1996; Schreiber et al., 1998) (see also Fig. 12 above).

- Hill reagents, like ferricyanide and methylviologen, specifically eliminate the I_2 -Fm (or I to P) phase (Neubauer and Schreiber, 1987; Hormann et al., 1994).
- Preillumination by saturating single turnover flashes causes lowering of I_1 (or J), which follows a period-4 oscillation (S-state dependent quenching) (Delosme, 1971; Joliot and Joliot, 1973; Schreiber and Neubauer, 1987; Srivastava et al., 1999).
- All treatments known to slow down electron donation to $P680^+$ preferentially suppress the thermal phases (I_1 - I_2 -Fm or J-I-P), thus resulting in a corresponding lowering of Fv/Fm (Schreiber and Neubauer, 1987; B. Strasser, 1997).
- Only highly active samples with intact thylakoid membranes display high Fv/Fm values, with a large contribution of the I_1 - I_2 (or J to I) phase to variable fluorescence (Schreiber and Krieger, 1996; see also Fig. 12).
- The I_1 - I_2 (or J to I) phase is paralleled by a decrease of the electric field-indicating electrochromic bandshift around 515 nm (Schreiber and Neubauer, 1990).
- The fluorescence yield observed briefly after a saturating single turnover flash is close to the I_1 (or J) level (Schreiber, 1986; Schreiber et al., 1995a; Schreiber and Krieger, 1996; Samson and Bruce, 1996).
- The half-rise time of the O- I_1 (or O to J) phase is almost identical in the absence and presence of DCMU.

In view of the last two observations it may be concluded that Q_A is fully reduced at I_1 (or J) and that the fluorescence yield at that point is lowered with respect to Fm by a special type of nonphotochemical quenching. As this quenching disappears during the thermal phases (I_1 - I_2 -Fm or J-I-P), it does not affect Fm-determination by PAM-fluorometry and the standard saturation pulse method. However, the possibility has to be considered that at least part of this quenching is already present before application of a

saturation pulse. In this case, assessment of maximal fluorescence yield at I_1 or I_2 (J or I) possibly would be more appropriate than at Fm (or P). In order to solve this question, the nature of the quenching at I_1 and I_2 (J and I) has to be clarified.

Based on the effects of DCMU and Hill reagents (see above) it was suggested that the quenching at I_2 (or I) is caused by oxidized plastoquinone (Schreiber, 1986; Neubauer and Schreiber, 1987). Nonphotochemical quenching by oxidized plastoquinone (PQ-quenching) was first reported by Vernotte et al. (1979). Whereas this type of quenching is rather weak in intact organisms (5–15% lowering of Fm), it is considerably enhanced in fractionated membrane preparations (Van Gorkom et al., 1974; Hsu and Lee, 1995; Kurreck et al., 2000; Pospisil and Dau, 2000). In Fig. 12 polyphasic rise curves of spinach chloroplasts and PS II enriched membrane fragments (BBY; after Berthold et al., 1981) are compared in the absence and presence of DCMU. If it is assumed that DCMU stabilizes PQ-quenching by preventing PQ-reduction, 7% and 36% of variable Chl fluorescence are controlled by PQ-quenching in chloroplasts and in BBY membrane fragments, respectively. It may be noted that variable fluorescence and, hence, Fv/Fm are much smaller in BBY fragments than in chloroplasts (by 37% in the presence and by 60% in the absence

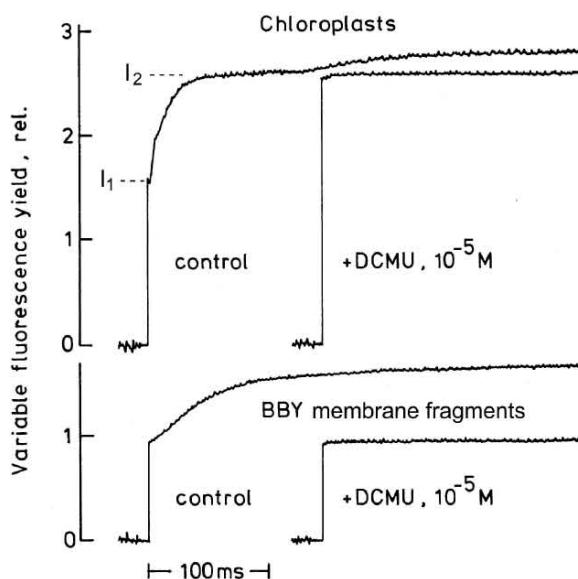


Fig. 12. Comparison of polyphasic Chl fluorescence rise curves in spinach chloroplasts and Berthold-Babcock Yocom (BBY) PS II fragments of spinach thylakoids. Suppression of plastoquinone (PQ)-quenching was made by the addition of 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU).

of DCMU). In BBY fragments the fluorescence rise completely lacks the I_1 - I_2 (or J to I) phase (Pospisil and Dau, 2000). This observation may be considered particularly important for the interpretation of the nonphotochemical quenching at the I_1 (or J) level (Sections VI.B and VI.C).

If the I_2 -Fm (or I-P) phase is due to suppression of nonphotochemical quenching by oxidized PQ, it is not related to energy conversion at PS II reaction centers and, therefore, should not be included in the assessment of maximal fluorescence yield by the saturation pulse method. This argues in favor of the use of short (ca. 50 ms) saturation pulses for assessment of Fv/Fm on the basis of I_2 (or I). In practice, however, the difference between I_2 and Fm (or between I and P) disappears upon illumination at relatively low light intensities (Schreiber et al., 1995a). Therefore, if no suitable device for assessment of Fv/Fm on the basis of I_2 (or I) is available, this problem can be avoided by assessment of Fv/Fm using weak background light ($10\text{--}30 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$). Actually, in most practical situations, the latter approach is advantageous, as the accuracy of Fm-determination is much higher, when a plateau is reached and the fluorescence signal can be averaged over several hundred milliseconds. Furthermore, in some organisms, e.g., most cyanobacteria and diatoms, maximal Fv/Fm values are not observed after dark adaptation but after adaptation to moderate light intensities (Section VII.A.).

B. I_1 (or J)-level and $F_{50\mu\text{s}}$ Measured by the Pump-and-Probe Method

As to the quenching at I_1 (or J), the distinct influences of the S-states and of various chemical treatments (see above) suggest that it is closely linked to properties of the PS II donor side (Schreiber and Neubauer, 1987; B. Strasser, 1997). On the other hand, its elimination by DCMU or by the prereduction of the PQ-pool also point to a decisive role of the PS II acceptor side. It is clear that during the first turnovers of the PS II reaction center, following onset of saturating illumination, the state of the PS II donor side is closely linked with that of the acceptor side. If DCMU is present, or if the PQ-pool (as well as Q_B) is prerduced, not more than one turnover is possible and consequently not more than one positive charge can accumulate at the donor-side. In the absence of DCMU, the turnover rate at PS II reaction centers is limited by the rate of electron transfer from

Q_A to Q_B and the rate of replacement of Q_BH_2 by Q_B , which will be high as long as the PQ-pool is not yet filled up. Hence, during the first tens of milliseconds (i.e., during the course of the I_1 - I_2 phase) there is the unusual situation that Q_A is practically fully reduced and nevertheless PS II turnover rate is close to maximal. As long as this happens, there is a high density of up to four positive charges at the donor and up to two negative charges at the acceptor side. It has been suggested that the resulting electrical field not only causes a large electrochromic bandshift around 515 nm, but is also involved in the quenching at I_1 (or J), which disappears in parallel with the electrochromic bandshift (Schreiber and Neubauer, 1990).

The situation is somewhat different when maximal fluorescence yield is assessed by the pump-and-probe method (Mauzerall, 1972; Falkowski et al., 1986a,b). This method applies μs probe flashes shortly before and ca. $50\mu\text{s}$ after firing of a saturating single turnover pump flash for assessment of fluorescence yields F and Fm (corresponding to $F_{50\mu\text{s}}$), respectively. As Q_A reduction is induced by just one single turnover flash, accumulation of more than one positive charge at the donor side may not be possible, just like in the presence of DCMU. Nevertheless it was found that the maximal fluorescence yield assessed $30\text{--}100\mu\text{s}$ after a single turnover saturating flash (for simplicity called $F_{50\mu\text{s}}$) does correspond to the I_1 (or J-level) (Schreiber, 1986; Schreiber et al., 1994; Schreiber and Krieger, 1996; Samson and Bruce, 1996), which suggests that $F_{50\mu\text{s}}$ is affected by the same quenching mechanism as I_1 (or J).

It was proposed that, similarly as suggested above for quenching at the I_2 (or I)-level, antenna quenching by oxidized PQ (Vernotte et al., 1979) is also responsible for the low fluorescence yields of I_1 (or J) and $F_{50\mu\text{s}}$ (Kramer et al., 1995; Prasil et al., 1996; Samson and Bruce, 1996; Vasil'ev and Bruce, 1998; Koblizek et al., 2001). Such PQ-quenching would not only affect $F_{50\mu\text{s}}$, but in general all fluorescence levels measured in the presence of oxidized PQ, including F_o , F'_o , F and I_1 measured by PAM fluorometry. It would also introduce considerable uncertainties in the determination of nonphotochemical quenching (Samson et al., 1999). At most physiological conditions PQ is largely oxidized and, hence, such nonradiative energy dissipation by PQ would cause a basic inefficiency of photosynthesis. Furthermore, it would mean that quantum yield is consistently overestimated by the saturation pulse method, as this inefficiency would be transiently removed during a saturation

pulse when PQ becomes fully reduced. Actually, if the PQ-quenching hypothesis were correct, only about half of variable fluorescence would be correlated with Q_A reduction, whereas the other half would reflect reduction of the PQ-pool. (For an entirely different point of view, see Chapter 6, Vredenberg).

An argument put forward in favor of a PQ-quenching mechanism is the fact that both I_1 and $F_{50\mu s}$ are increased when PQ is prereduced by actinic illumination (Kramer et al., 1995; Prasil et al., 1996; Samson and Bruce, 1996). It was also claimed that PQ-reduction leads to a significant increase of the effective absorption cross-section of PS II, as measured by the pump-and-probe method (Samson and Bruce, 1996; Prasil et al., 1996). On the other hand, a strong argument against PQ-quenching is the finding that DCMU not only removes most of the quenching at I_1 (Neubauer and Schreiber, 1987; see also Fig. 12), but also raises the maximal fluorescence yield induced by a single turnover flash to the I_2 -level measured with a multiple turnover saturation pulse (Schreiber and Krieger, 1996). DCMU should stabilize PQ-quenching and not remove it (see Fig. 12).

Figure 13 shows parallel measurements by PAM

fluorometry and by the pump-and-probe method using a Xe-PAM fluorometer (the Xe-PAM uses single turnover xenon discharge flashes instead of LED pulses for fluorescence excitation; see Schreiber et al., 1993). It may be noticed that even in the presence of DCMU briefly after the pump flash $F_{50\mu s}$ is non-photochemically quenched. It takes ca. 1 ms until this quenching is relaxed (Schreiber and Krieger, 1996). While it may be assumed that a similar quenching with consequent relaxation is also induced in the absence of DCMU, this cannot be distinguished as clearly as when DCMU is present. Any relaxation of nonphotochemical quenching (fluorescence rise) within the first 1 ms following a flash is overlapped by the larger *increase* of photochemical quenching (fluorescence decrease) associated with the rapid oxidation of Q_A^- by Q_B . In saturating continuous light, such nonphotochemical quenching may be expected to be induced with every PS II turnover, as long as the PQ-pool is not yet fully reduced.

Obviously, the observed DCMU-effect questions the assumption of Falkowski and Kolber (1995) that Fm-determination by the pump-and-probe method is not changed by DCMU. While it is true that DCMU

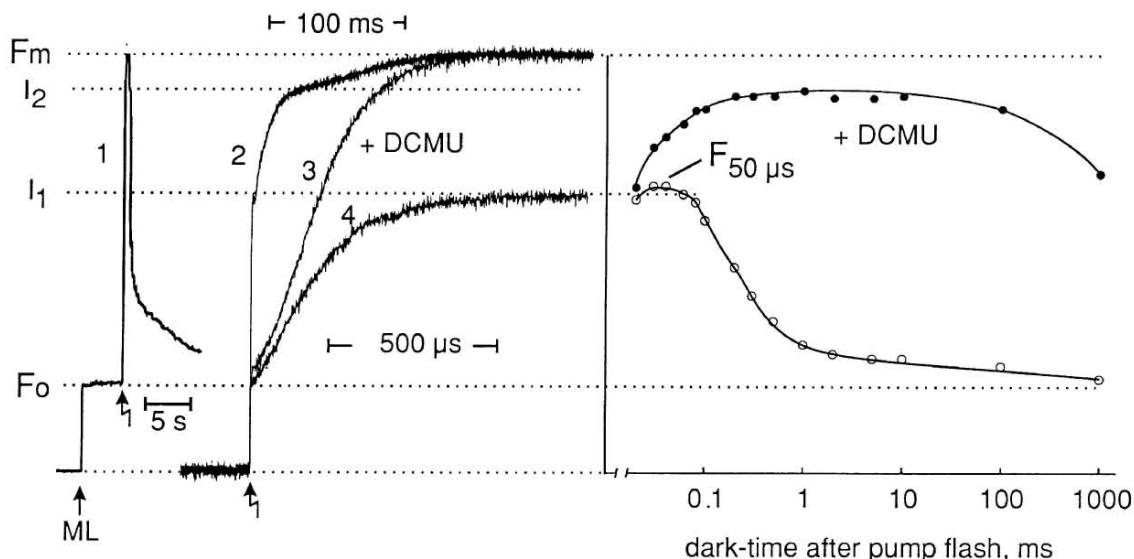


Fig. 13. Assessment of maximal Chl fluorescence yield by different experimental approaches. From left: Pulse-amplitude-modulation and saturation pulse method (1); polyphasic rise upon onset of continuous saturating light (2–4); pump-and-probe method (right panel). The same high intensity LED array with an intensity of $12,000 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ served as saturation pulse source in the recordings 1–4 and also as measuring light in the recordings 2–4. Please note the different time scales: the 5s bar applies to curve 1, the 100 ms bar to curve 2, and the 500 μs bar to curves 3 and 4. All measurements with dark adapted spinach chloroplasts at $0.5 \text{ mg Chl l}^{-1}$ in the same cuvette and optical geometry. Where 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) was added, its final concentration was $5 \mu\text{M}$. Notations: F_o , dark-level fluorescence yield, monitored with weak measuring light (ML); I_1 , first intermediate level (also called 'J'); I_2 , second intermediate level (also called 'I'); F_m , maximal fluorescence yield reached in saturating light (also called P); $F_{50\mu s}$, fluorescence yield measured 50 μs after a pump flash with the help of a probe flash. Data from Schreiber and Krieger (1996).

hardly increases $F_{50\mu s}$, this clearly is due to a transient type of nonphotochemical quenching, which is induced by the pump flash and disappears within 1 ms. Different findings also may be related to the fact that DCMU-binding depends on the redox state of Q_B^- (Lavergne, 1982; Laasch et al., 1983; Urbach et al., 1984). Very high DCMU concentrations are required to displace Q_B^- , which is known to be present in intact chloroplasts and algae (Wollman, 1978; Rutherford et al., 1984).

While Samson and Bruce (1996) confirmed that DCMU raises the Fm determined by the pump-and-probe method, these authors still assumed that oxidized PQ causes the quenching at I_1 . More recently, however, Yaakoubd et al. (2002) reported experiments suggesting that this type of quenching must be related to Q_B -bound PQ and not to free PQ. Vasil'ev and Bruce (1998) and Vasil'ev et al. (1998) presented evidence from picosecond time-resolved fluorescence measurements, which appears to support a PQ-quenching mechanism at the antenna level. The Fm in the presence of background light was characterized by a large 3.1 ns decay component, which disappeared upon addition of 5-hydroxy-naphthoquinone, supposedly acting as an antenna quencher. It was concluded that oxidized plastoquinone acts similarly. Modeling of the decay kinetics supported this conclusion. However, there remains some uncertainty concerning the significance of the naphthoquinone effect. Substituted naphthoquinones are known to compete with plastoquinone for the Q_B binding site and in addition to antenna quenching also act as DCMU-analogs (Vermaas, 1984). Furthermore, the fitting of fluorescence decay kinetics strongly depends on assumptions of PS II heterogeneity (Roelofs et al., 1992) and on the assumed model of excitation dynamics within PS II (Section VI.C. and Fig. 14). Vasil'ev and Bruce (1998) invoke a 30–35% contribution of ‘inactive centers’ (Chylla and Whitmarsh, 1987) to explain the 3.1 ns decay component that is observed at Fm, but not at $F_{50\mu s}$. Alternatively, this components could also result from recombination fluorescence (Schreiber and Krieger, 1996; Section VI.C.).

In view of the DCMU-effect, any PQ-quenching at the I_1 (or J)-level (or of $F_{50\mu s}$) somehow must involve Q_B (Schreiber, 1986; Schreiber and Neubauer, 1987; Vasil'ev and Bruce, 1998; Kolber et al., 1998; Yaakoubd et al., 2002), i.e., one molecule out of the PQ-pool, temporarily bound to the PS II reaction center complex. Therefore, the term ‘ Q_B -quenching’ appears more appropriate to this author, although

the mechanism of this quenching remains to be clarified (see discussion in the following section). Elucidation of the Q_B -quenching mechanism is not only of utmost importance for the interpretation of data obtained with the saturation pulse and pump-and-probe methods, but also for an understanding of PS II primary reactions (for a review, see Samson et al., 1999). Obviously, for such understanding the scheme presented in Fig. 2 is too simple and a more profound approach is required. The following ‘excursion’ in section VI.C. into details of primary steps of PS II is not written for the general user of PAM fluorometry but rather for readers interested in basic aspects of PS II photochemistry and Chl fluorescence quenching mechanisms.

C. Quenching Related to PS II Charge Recombination and Q_B -quenching

As suggested by Schreiber and Neubauer (1987) as well as by Samson and Bruce (1996) it is tempting to consider a link between photochemical ($O-I_1$ or O to J) and thermal (I_1-I_2 or J to I) phases of the fluorescence rise in saturating light and the Q_1/Q_2 quenchers defined by Joliot and Joliot (1977, 1979). The latter authors described a large intrinsic heterogeneity of variable Chl fluorescence, which was clearly distinct from the small α, β -heterogeneity observed by Anastasios Melis and coworkers (Melis and Homann, 1976; Melis and Duysens, 1979; Melis and Schreiber, 1979). A first single turnover saturating flash was found to reduce a fraction of quenchers, called Q_1 (equivalent to Q_A), with high quantum efficiency, whereas several flashes were required to reduce the remaining fraction of quenchers, called Q_2 (hypothetical alternative acceptor with unknown molecular identity).

While the principal findings of the Joliot's experiment recently were confirmed (Schreiber, 2002), a serious argument against Q_1/Q_2 heterogeneity is the fact that during the past 30 years all efforts have failed to identify a molecule, which might correspond to Q_2 , despite considerable progress in the elucidation of PS II molecular structure. A different interpretation is suggested by Lavergne and Rappaport (1998). These authors showed that there is no need to invoke two types of PS II acceptors, if rapid recombination of $P680^+Q_A^-$ is assumed. Recombination is favored by the long lifetime of $P680^+$, which in presence of hydroxylamine extends into the 100 μs time range.

The recombination hypothesis is supported by measurements of delayed Chl fluorescence. Upon

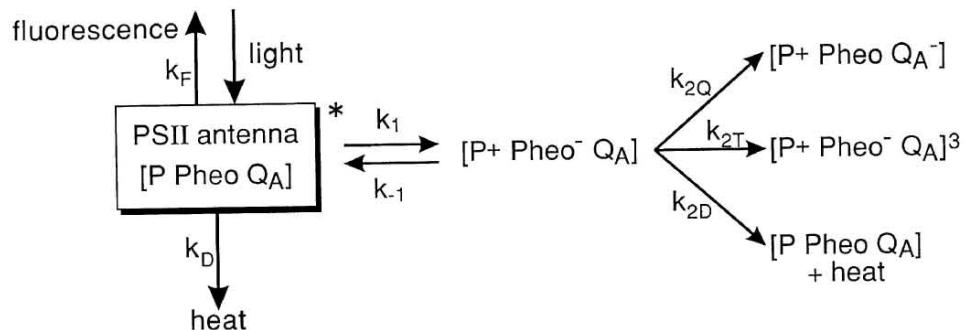


Fig. 14. Equilibration of excitation energy between PS II antenna and reaction center according to the reversible radical pair model (Schatz et al., 1988; scheme reproduced from Schreiber and Krieger, 1996). Two distinct ways of formation of singlet excited Chl in PS II antenna (symbolized by *) are distinguished: Direct excitation by light or ‘delayed excitation’ via recombination of primary radical pair P^+Pheo^- , so that overall fluorescence consists of prompt and recombination fluorescence. It is assumed that in the presence of oxidized Q_A^- the rate constant of charge stabilization, k_{2Q} , is much larger than the rate constants of radiative recombination, k_{-1} , nonradiative recombination, k_{2D} , and triplet formation, k_{2T} . Hence, F_o is dominated by prompt fluorescence. Radical pair formation also occurs, although with decreased k_1 , when Q_A^- is reduced (not shown in the scheme). In the state $P^+Pheo^-Q_A^-$ k_{-1} is stimulated, resulting in a high yield of radiative recombination (recombination fluorescence). Hence, F_m consists of prompt and recombination fluorescence. Recombination fluorescence is selectively ‘quenched’ by the nonradiative loss processes at the reaction centers, characterized by k_{2T} and k_{2D} . On the other hand, prompt and recombination fluorescence are equally ‘quenched’ by nonradiative antenna deexcitation, characterized by k_D .

charge recombination, energy is released in the form of heat and delayed fluorescence (for reviews see Lavorel, 1975; Jursinic, 1986; Bilger and Schreiber, 1990; Chapter 13, Tyystjarvi and Vass). Delayed fluorescence (more briefly also called luminescence) can be measured by repetitive pulse excitation in a similar way as pulse-modulated fluorescence (Schreiber and Schliwa, 1987b). By measuring the $50\ \mu s$ luminescence component, recombination yield at PS II can be followed quasi-continuously in parallel with the quenching parameters of prompt fluorescence. It was shown that $50\ \mu s$ luminescence is strongly stimulated upon addition of hydroxylamine in parallel with increases of photochemical and nonphotochemical quenching (Schreiber and Neubauer, 1989; Schreiber and Neubauer, 1990).

While the Joliot's experiment and its interpretation by Lavergne and Rappaport (1998) illustrate the potential impact of PS II charge recombination on the fluorescence yield measured after a pump flash, it has to be considered that in this experiment DCMU was present, which prevents any participation of Q_B^- in the quenching mechanism, as Q_B^- is known to be displaced by DCMU from its binding site (Velthuys, 1981). Hence, the mechanism of the apparent Q_B^- -quenching discussed in section VI.B. must differ from that of the ‘recombination quenching’ suggested by Lavergne and Rappaport (1998).

In the absence of DCMU, in principle Q_B^- could participate in PS II photochemistry via the ‘inactive

branch’ of the reaction center complex (Schreiber, 2002). However, for explanation of apparent Q_B^- -quenching a *direct* participation of Q_B^- in the quenching mechanism may not be required. In this context it is important to recall that Q_B^- not only acts as an acceptor of electrons from reduced Q_A^- , but by doing so also helps maintain a high density of positive charges at the PS II donor-side and, hence, a strong transmembrane electrical field, as long as it is reoxidized by the PQ-pool (Section VI.B.). While this field will drive $Q_A^-/P680^+$ charge recombination, it is highly unlikely that this will lead to substantial net Q_A^- oxidation in control samples, differently from the situation in the presence of hydroxylamine, which inhibits water splitting (Lavergne and Rappaport, 1998).

Besides $Q_A^-/P680^+$ charge recombination, the recombination of the primary radical pair $Pheo^-/P680^+$ has to be also considered (Schreiber and Krieger, 1996). According to the reversible radical pair model of PS II (Schatz et al., 1988), primary charge separation is reversible (‘shallow trap’). Even in the presence of Q_A^- the quantum yield of radical pair formation amounts to 50–70% (Van Mieghem et al., 1995; Hillmann et al., 1995; Renger et al., 1995). The fluorescence increase upon reduction of Q_A^- is not only due to a decrease of the rate of radical pair formation (k_1), but also to an increase of the rate of radical pair recombination (k_{-1}) (see scheme in Fig. 14). Hence, there is a dynamic reversible movement of excitons between antenna and reaction center, which is con-

trolled by the various rate constants. As suggested by Schreiber and Krieger (1996), these features can be expected to give rise to a fundamental heterogeneity of variable fluorescence. When Q_A^- becomes reduced, one part of the consequent fluorescence increase results from the prevention of radical pair formation and, hence, corresponds to 'prompt fluorescence'. Another part results from the radiative recombination of the radical pair to the ground state and, hence, corresponds to 'recombination fluorescence.' It is important to realize that in the presence of Q_A^- any increase of the rate constants of nonradiative charge recombination (k_{2D}) and triplet formation (k_{2T}) will cause nonphotochemical quenching, which will affect recombination fluorescence more than prompt fluorescence. It has been proposed that the transient inefficiency caused by a saturating flash, as identified during the first 1 ms following a pump flash in the presence of DCMU (see Fig. 13), is due to a transient stimulation of k_{2T} and/or k_{2D} (Schreiber and Krieger, 1996). A stimulation of k_{2T} by a local electrical field was reported (Van Mieghem et al., 1995). Fluorescence lifetime measurements by Goss et al. (1995) also showed that zeaxanthin-independent nonphotochemical quenching correlates with a significant increase in the rate constants k_{2T} and/or k_{2D} . Hence, for the time being, the recombination hypothesis put forward by Schreiber and Krieger (1996) appears to give the most plausible explanation for apparent Q_B^- -quenching (for a different point of view, see Chapter 19, Bruce and Vasil'ev).

Vredenberg (2000) has proposed a different model for energy trapping at PS II reaction centers and for quenching of Chl fluorescence that also assumes reversible trapping by centers with reduced Q_A^- (see also Chapter 6, Vredenberg). In contrast to Schreiber and Krieger (1996), however, Wim Vredenberg assumes that reduced pheophytin (Pheo^-) will accumulate when electrons are donated to the state $\text{P}^+ \text{Pheo}^-$ and that maximal fluorescence yield requires quantitative formation of the state $\text{P} \text{Pheo}^- Q_A^-$. This assumption, however, has to be reconciled with the fact that maximal fluorescence yield (with $\text{Fm}/\text{Fo} > 5$) is stable for hours in the dark even in the presence of molecular oxygen, when PS II is blocked by a combination of DCMU and hydroxylamine (Bennoun, 1970; U. Schreiber, unpublished). When Pheo^- accumulates upon illumination under anaerobic conditions, strong fluorescence *quenching* is observed (Klimov et al., 1977; Heber et al., 1985), which disappears upon addition of oxygen. Pheo^- causes a similar absorbance

change around 820 nm as $\text{P}700^+$ and $\text{P}680^+$, which can be measured routinely in the absence of oxygen in parallel to light-driven development of fluorescence quenching. In the presence of oxygen a corresponding absorbance change is not observed in parallel to a light-driven fluorescence rise to Fm (U. Schreiber and C. Neubauer, unpublished).

According to Schreiber and Krieger (1996), Q_B^- -quenching is equivalent to preferential quenching of recombination fluorescence via increases in the competing nonradiative loss processes at the reaction center, which may be induced by a local electrical field as well as by changes in membrane conformation or membrane integrity. It may be emphasized that with physiologically healthy samples such quenching is 'artificial,' in the sense that it occurs only under extreme light conditions in short time windows during a multiple turnover saturation pulse (up to 50 ms) or following a single turnover saturating flash (up to 1 ms). With damaged or fractionated samples, k_{2T} and/or k_{2D} may reach values that prevent recombination fluorescence even under moderate light conditions. In this sense, recombination fluorescence (as well as the I_1 - I_2 or J-I phase) may be considered an indicator of PS II integrity. This statement is in line with the following observations:

- Maximal variable fluorescence (as well as maximal values of Fv/Fm and PS II quantum yield) of dark-adapted samples varies largely depending on the physiological state and the integrity of the samples. Hence, Fm/Fo may decrease from about 6 to 1, when BBY-fragments are isolated, starting from intact leaves, via intact chloroplasts, envelope free chloroplasts, washed thylakoids to detergent treated membranes. While with proper precautions PS II functionally remains intact, the properties of variable fluorescence change dramatically. It is generally observed, that the decrease of Fv goes along with a preferential suppression of the I_1 - I_2 (or J to I) phase. In BBY-particles the I_1 - I_2 (or J to I) phase is completely lacking (see Fig. 12).
- When intact chloroplasts are irradiated at low temperatures (6°C) with strong light ($4,000 \mu\text{mol photons m}^{-2}\text{s}^{-1}$), the observed decrease in PS II quantum yield (photoinhibition) is paralleled by preferential suppression of the I_1 - I_2 (or J to I) phase (Christian Neubauer and Ulrich Schreiber, unpublished). On the other hand, when zeaxanthin dependent nonphotochemical quenching is

created by moderate illumination, O-I₁ (or O to J) and I₁-I₂ (or J to I) phases are quenched to the same extent (Genty et al. 1990; Schreiber et al. 1995a). In the latter case, nonradiative deexcitation in the antenna is stimulated (rate constant k_D in the scheme of Fig. 14), affecting both yields of prompt and recombination fluorescence.

In view of the above arguments, it appears unlikely that the apparent Q_B-quenching, which causes the lowering of I₁(or J) and of F_{50μs} with respect to Fm, is already effective in lowering the PS II quantum yield before application of a saturation pulse or a pump flash. After dark-adaptation or during moderate continuous illumination, Q_A is fully or mostly oxidized, with the consequence that recombination fluorescence is negligibly small, as k_Q>k₋₁ (see scheme in Fig. 14). Significant quenching of recombination fluorescence should occur only after Q_A-reduction, with the additional prerequisites of Q_B being oxidized and k_{2T} (and/or k_{2D}) being stimulated. In practice, this means that Fm should be determined after complete reduction of the PQ-pool, i.e., at the I₂-level.

As to Fm determination by the pump-and-probe method, F_{50μs} is subject to Q_B-quenching (here considered to be caused by selective quenching of recombination fluorescence), the extent of which is determined by the reduction state of Q_B (and PQ-pool). As most of this quenching is induced by the pump flash (via Q_A reduction and stimulation of a local field), the Fm corresponding to the situation before application of the pump flash is underestimated, which leads to underestimation of the effective PS II quantum yield in most physiologically relevant situations. The same considerations apply when a maximal fluorescence level is induced within 100 μs by a rapid sequence of flashes (Kolber et al., 1998) or by saturating 20–100 μs LED pulses (Koblizek et al., 2001).

If proven to be true, Q_B-quenching (i.e., selective quenching of recombination fluorescence) can be expected to affect the determination of the functional PS II absorption cross-section, σ_{PSII}, by the pump-and-probe method. σ_{PSII} is the product of the light-harvesting capability of the antenna pigments and the efficiency of excitation transfer to the reaction center (Ley and Mauzerall, 1982). It can be determined by gradually increasing the intensity of the pump flash and following the flash-intensity saturation curve of the fluorescence increase (Ley and Mauzerall, 1982; Falkowski et al., 1986b). Any stimulation of nonradiative deexcitation, irrespective of whether it

affects excitons directly originating from excitation or from recombination, should cause a decrease of σ_{PSII}. Hence, also stimulation of quenching at I₁(or J) and of F_{50μs} should be paralleled by a decrease in σ_{PSII}. As shown by Koblizek et al. (2001), σ_{PSII} indeed shows a period-4 oscillation in parallel with corresponding changes of F_{50μs}, which are also observed at the I₁-level (Schreiber and Neubauer, 1987). As the redox state of the PQ-pool affects the redox state of Q_B, it will also affect the apparent σ_{PSII}, provided the above stated preconditions (Q_A reduced, large local field) are fulfilled. At moderate light intensities, the Q_B-quenching mechanism will not be effective and σ_{PSII}-determination should not be significantly affected by the PQ-redox state. This aspect is particularly problematic when using the pump-and-probe method and dealing with unicellular algae, which show pronounced changes of the PQ-reduction state in the dark (Schreiber et al., 1971; Schreiber and Vidaver, 1974, 1975) associated with chlororespiratory electron flow (Bennoun, 1982; 1998; 2002 Gans and Rebeillé, 1990; Wilhelm and Duval, 1990) and NADPH-dehydrogenase activity (Mi et al., 1992; Mano et al., 1995; Schreiber et al., 1995a). Prasil et al. (1996) reported a decrease of σ_{PSII} measured by the pump-and-probe method in the order of 30% associated with the reduction of the PQ-pool. If the above considerations are correct, the real decrease of cross-section would be much smaller, as most of the apparent Q_B-quenching would be artificially induced by the pump flashes.

While the detailed mechanism of Q_B-quenching remains to be elucidated, for the time being the arguments presented above allow me to offer the following conclusions:

- Besides the normal photochemical and nonphotochemical quenching, which can be assessed by the saturation pulse method during steady state illumination or in the course of slow induction kinetics (Section IV), there is an additional type of ‘artificial’ nonphotochemical quenching observed in the sub-ms time range following a saturating flash or during the first 10–50 ms after onset of saturating continuous light.
- This type of quenching on the one hand complicates the interpretation of rapid fluorescence transients and pump-and-probe data, but on the other hand offers valuable information on the state of PS II donor and acceptor sides as well as on the

integrity of PS II reaction centers.

- The term Q_B -quenching was coined because this type of quenching is observed only when the Q_B site is not occupied by DCMU and Q_B is oxidized. However, it should be emphasized that a *direct* participation of Q_B in the quenching reaction may *not* be required.
- A plausible working hypothesis, which is supported by a number of observations, assumes that Q_B -quenching is caused by preferential suppression of recombination fluorescence, when nonradiative radical pair recombination is stimulated at PS II centers.
- According to this hypothesis, Q_B -quenching is effective only when Q_A is reduced *and* at the same time nonradiative loss processes at the reaction center are stimulated.
- Q_B -quenching does not affect quantum yield determination by the saturation pulse method, as long as the PQ-pool is fully reduced in the course of the saturation pulse.
- On the other hand, Q_B -quenching systematically affects Fm and quantum yield determination by the pump-and-probe method.

These conclusions, if proven to be true, are of considerable practical relevance for the interpretation of data obtained with different experimental approaches.

VII. Pulse Amplitude Modulation (PAM) Fluorometry for Special Applications

As already pointed out in the Introduction, progress in Chl fluorescence has been stimulated by dynamic interaction between basic research, applied research and instrument development. Due to recent technical progress in optoelectronics and photonics, it has become possible to develop instruments with exceptional sensitivity and selectivity, which can be applied in practically all situations where photosynthesis takes place *in vivo*. In some cases, new devices have opened the way for the study of organisms, which previously were hardly accessible by other methods. This is particularly evident with respect to photosynthesis in

aquatic environments, like oceans, rivers and lakes (Chapter 30, Falkowski et al.; Chapter 31, Raven and Maberly). For example, with the help of a submersible PAM fluorometer (DIVING-PAM), for the first time detailed information on the photosynthetic properties of photosymbiont-containing invertebrates *in situ* were obtained. This includes measurements on ascidians containing Prochloron as photosymbiont (Schreiber et al., 1997), corals (Beer et al., 1998; Jones et al., 1998; Ralph et al., 1999) and sponges (Beer and Ilan, 1998). The same instrument has also been applied for the study of seagrass communities *in situ* (Ralph et al., 1998). While a detailed description of all different types of PAM fluorometers and their numerous applications would be out of scope of this chapter, a few instruments with exceptional measuring principles are briefly described.

A. Phytoplankton Analysis by 4-Wavelength Excitation Technique

Since the early work of Lorenzen (1966), Chl fluorescence has become increasingly important for assessment of phytoplankton primary productivity (Falkowski and Kiefer, 1985; Kiefer and Reynolds, 1992; Kolber and Falkowski, 1993; Falkowski and Kolber, 1995; Chapter 30, Falkowski et al.). Special instruments were developed to measure Chl content as well as the most relevant photosynthetic parameters of phytoplankton in water samples down to $0.1 \mu\text{g} \text{Chl l}^{-1}$ (Kolber and Falkowski, 1993; Schreiber et al., 1993; Kolber et al., 1998). Until recently, the available instrumentation had been limited by the fact that it could not distinguish between the fluorescence emitted from different types of phytoplankton, like green algae, diatoms/dinoflagellates and cyanobacteria. In principle, such distinction is possible on the basis of the specific fluorescence excitation properties of differently pigmented phytoplankton groups (Yentsch and Yentsch, 1979; Schreiber et al., 1993). Excitation energy is transferred with high efficiency from various accessory pigments (Chl *b*, Chl *c*, fucoxanthin, carotenoids, peridinin, phycocyanin, phycoerythrin) to Chl *a*, from which most of the overall fluorescence is emitted. Kolbowski and Schreiber (1995) developed a microprocessor-controlled PAM fluorometer that employs an array of light-emitting diodes (LED) to excite Chl fluorescence alternating by $10 \mu\text{s}$ measuring light pulses at four different wavelengths and to illuminate samples with actinic light and saturation pulses. In the meantime, a compact version of this

PHYTO-PAM Chl fluorometer has become available (described in Schreiber, 1998), which has profited from recent progress in strong LEDs emitting in the green and blue wavelength regions. Current instruments feature 470, 520, 645 and 665 nm measuring light. At these wavelengths large differences in the excitation spectra of green algae, diatoms/dinoflagellates and cyanobacteria are observed, which constitute the three major phytoplankton groups. In practice, ‘reference excitation spectra,’ which are measured with each individual instrument and stored in computer-memory, are the basis for deconvolution of the overall signal into the contributions of these three groups. Chl contents are determined on the basis of the deconvoluted Fo-values, which are measured with weak measuring light. The saturation pulse method is applied for assessment of photosynthetic activity (Section IV).

Figure 15 gives an example of light response curves of effective quantum yield, $\Delta F/F_m'$, and relative electron transport rate, ETR, as measured with a water sample from Sydney Harbor (from 10 m depth). This is an example of Rapid Light Curves, with 30 s intervals at each intensity step (Section V.B.). In the given water sample, the content of cyanobacteria was below the detection limit. The diatoms/dinoflagellates showed a rise of quantum yield in the range of low light intensities, thus being clearly distinguishable from the green algae. This unusual light response, which was also observed in a pure culture of the diatom *Phaeodactylum tricornutum* (U. Schreiber, unpublished), may reflect partial reduction of the PS II acceptor pool in the dark, possibly via the NADPH-dehydrogenase, as previously observed in cyanobacteria (Mi et al., 1992).

Beutler et al. (1998) introduced a submersible fluorometer, which applies five different excitation wavelengths for differentiating between up to four different spectral groups. These researchers demonstrated the possibility of rapid depth profiling of the distribution of various groups of microalgae in lakes, rivers and the sea (Beutler et al., 2002).

B. Systems for Assessment of Photosynthesis at Single Cell Level

With the help of a photomultiplier, the sensitivity of fluorescence detection can be strongly enhanced. Although photomultipliers do not tolerate strong background light, nevertheless the saturation pulse

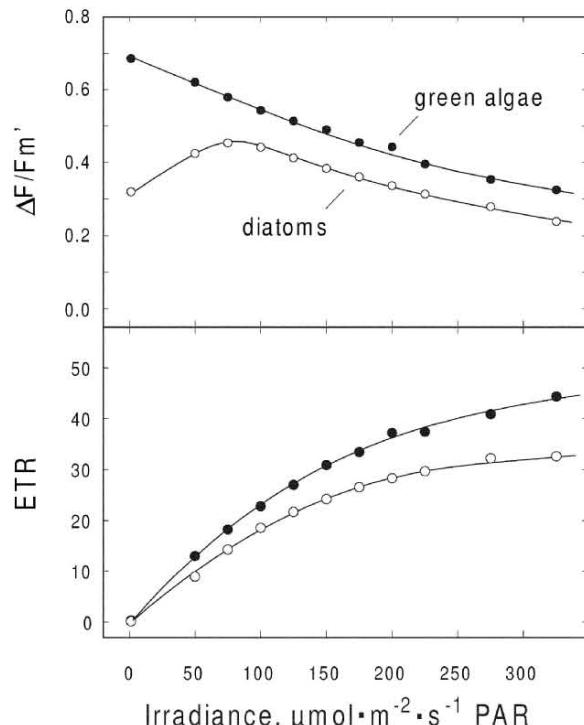


Fig. 15. Light-response curves of green algae and diatoms in a water sample from Sydney Harbor (10 m depth). Measurement with PHYTO-PAM fluorometer using 30 s illumination intervals at each intensity step. $\Delta F/F_m'$, effective PS II quantum yield ETR, relative electron transport rate calculated from fluorescence parameters. The irradiance-scale ($\mu\text{mol} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$ PAR) refers to the quantum flux density of photosynthetically active radiation. Data from Schreiber (1998).

method can be applied, when not only the measuring light, but also the actinic light is appropriately pulse modulated (Section II.). For this purpose, all light qualities have to be under microprocessor control (PAM-CONTROL family of fluorometers). The signal/noise level in such fluorometers is determined by the amplitude of the overall signal, which means in practice that Chl fluorescence detection is limited by the amplitude of the unavoidable background signal. At high photomultiplier gain, even in the absence of Chl a small pulse modulated signal is generated, due to fluorescence of various system components, like optical filters, lenses, fiber optics etc. PAM fluorometers with extremely low background signal levels were developed, which allow assessment of photosynthesis at the single cell or even single chloroplast level, using essentially the same measuring protocols as with whole leaves (Schreiber, 1998).

1. Epifluorescence Microscope System

Epifluorescence microscopes can be readily adapted for PAM fluorometry in conjunction with a PAM-CONTROL unit (MICROSCOPY-PAM). A blue LED is installed in place of the usual Xe-arc lamp in the excitation pathway of the microscope. As the LED light is effectively focused on the object, a single LED is sufficiently strong to serve not only for measuring light (i.e., fluorescence excitation), but for actinic light and saturation pulses as well. By visual inspection, with the help of an x-y stage an object can be centered in the field of view, which can be narrowed down with the help of an iris diaphragm, such that the fluorescence characteristics of a particular cell or chloroplast can be selectively assessed.

An important practical application of the MICROSCOPY-PAM relates to the study of guard-cell photosynthesis (Schreiber, 1998; Goh et al., 1999). Previous pioneering work of Eduardo Zeiger and coworkers (Zeiger et al., 1980; Melis and Zeiger, 1982) had shown that basic information on photosynthesis in single guard cells could be obtained by Chl fluorescence measurements. While this early work revealed an apparently normal Kautsky effect in guard cells, it could not distinguish between photochemical and nonphotochemical quenching. Figure 16 shows dark-light induction curves of single guard cell pairs of *Vicia faba* in the presence and absence of molecular O₂, recorded with repetitive application of saturation pulses. Removal of O₂ causes a dramatic loss of photosynthetic activity, which is reflected by negative values of ΔF/F_{m'} (negative spikes induced by saturation pulses) during actinic illumination. O₂-dependent electron flow apparently plays a key role in guard-cell photosynthesis (Goh et al., 1999), and in the absence of O₂ the PS II acceptor side becomes reduced to a point that reduced pheophytin can accumulate during a saturation pulse, which acts as a quencher of variable fluorescence (Klimov et al., 1977; Heber et al., 1985; Schreiber and Neubauer, 1990; for a different point of view, see a discussion in Chapter 6, Vredenberg). This example illustrates the analytical value of the saturation pulse method. The Kautsky effect as such is very similar +/−O₂, so that without quenching analysis the observed P-S decline could be mistaken to reflect activation of photosynthetic electron flow and membrane energization. In reality, quenching analysis reveals that electron transport is completely blocked and illumination

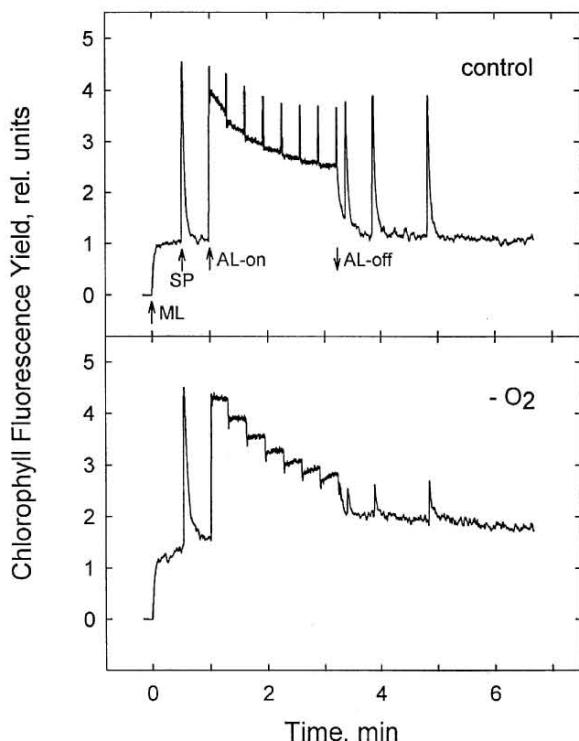


Fig. 16. Dark-light induction curves with repetitive saturation pulses for quenching analysis measured on a single guard cell pair of *Vicia faba* in the presence and absence of O₂. Measurement with MICROSCOPY-PAM fluorometer. Notations: ML, measuring light; SP, saturation pulse; AL, actinic light. Data from Schreiber (1998).

causes a largely irreversible loss of PS II quantum yield (photodamage).

2. Microfiber System

In conjunction with a fiberoptic microprobe the PAM-CONTROL unit can also be used for ultrasensitive fluorescence measurements in different layers of photosynthetically active material (MICROFIBER-PAM). A similar, although somewhat less sensitive measuring system based on a standard PAM fluorometer had been previously described for assessment of photosynthesis within leaves (Schreiber et al., 1996). The fiberoptic microprobe is based on a multimode fibercoupler that functions like a beam-splitter, to the split side of which an LED light source and a photomultiplier are connected, whereas the other side is in contact with the investigated object. While the single fibers have a diameter of 100 μm, the fiber at

the object side can be pulled out to a tapered endpiece of 10–30 μm diameter, which is suited to penetrate into deeper layers of photosynthesizing tissue. The fiber position and depth of penetration normally is controlled by a micromanipulator. With the help of miniature collimating optics, the light of a single blue LED is effectively coupled into the fiber, with the intensity at the tapered end being sufficiently high that the same light may serve not only for measuring light, but for actinic light and saturation pulses as well (in analogy to MICROSCOPY-PAM). Hence, the PS II quantum yield, $\Delta F/F_m'$, can be determined without need of external light sources.

The microfiber system allows insights below the surface layer of an object, which are not possible with microscopy or imaging techniques. In this way heterogeneity of photosynthetic parameters across a leaf can be assessed. Such heterogeneity can e.g., be expected after illumination of a leaf with excessive light. As the intensity of the incident light drops rapidly within the leaf (Vogelmann and Björn, 1984; Bornman et al., 1991), light which is excessive at the surface will cease being so at deeper cell layers. Figure 17 shows an experiment with a *Syringa vulgaris* leaf, with F_v/F_m as a measure of maximal quantum yield. As expected, the loss of F_v/F_m caused by photoinhibition decreased with the depth of the assessed tissue layer (Schreiber et al., 1996). It may be concluded that overall photoinhibition normally is overestimated if, as has been common practice, it is assessed by F_v/F_m measurements from the leaf surface.

C. Imaging of Photosynthetic Activity

Chl fluorescence imaging was pioneered by Omasa et al. (1987) and Daley et al. (1989). This early work required empirical calibration of the fluorescence images against other methods, such as gas exchange or measurements of relative electron transport rate by PAM fluorometry, in order to obtain images of photosynthetic activity. Genty and Meyer (1995) as well as Siebke and Weis (1995) introduced imaging systems capable of assessing fluorescence at two levels of light intensity (actinic illumination and saturation pulse), thus allowing derivation of the fluorescence parameter $\Delta F/F_m'$ and, hence, assessment of relative electron transport rate. Oxborough and Baker (1997) developed an ultrasensitive system based on a Peltier-cooled CCD-camera and a fluorescence microscope, which is capable of generating fluorescence images

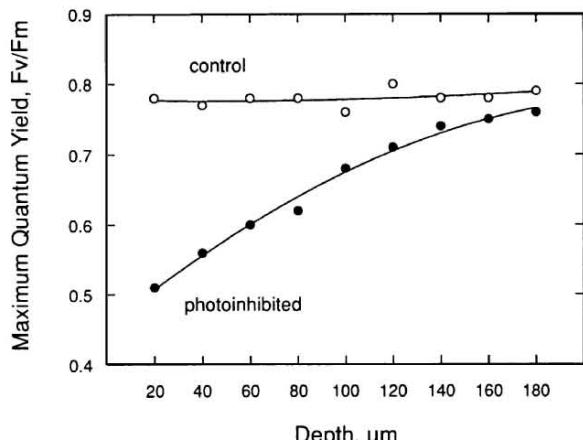


Fig. 17. Assessment of F_v/F_m as a measure of PS II quantum yield at different depths within a leaf of *Syringa vulgaris*. Comparison of control leaf with leaf illuminated for 5 min at 5,000 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ at the upper surface (photo inhibited). Measurement with MICROFIBER-PAM fluorometer. Data from Schreiber et al. (1996).

at irradiance levels from less than 0.1 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ to more than 8,500 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$. This system for the first time allowed assessment of F_v/F_m images with spatial resolution down to single cells and chloroplasts. Nedbal et al. (2000) reported on a kinetic fluorescence imaging system, which applies modulated measuring light, similarly to a PAM fluorometer, derived from an array of 700 LEDs. This system, which employs two separate tungsten-halogen light sources for actinic light and saturation pulses, not only is capable of saturation pulse quenching analysis, but even can tolerate ambient daylight. Holub et al. (2000) have developed a Fluorescence Lifetime Imaging Microscope that has been used to measure Chl fluorescence lifetime images of leaf surfaces as well as of single algal cells.

Recently, a compact PAM fluorometer for measuring images of photosynthetic activity was developed, which incorporates most recent advances in CCD-camera technology and data exchange between camera and PC (IMAGING-PAM Chlorophyll Fluorometer). This system will be briefly described here. Image capture is synchronized with the pulse modulated measuring light. Digitization occurs within the camera, such that no framegrabber is required, and the digitized data are transferred at 400 megabit/s (firewire, IEEE1394 port) to a PC. With only LEDs serving as light sources, the system is portable and can be battery powered. Its standard measuring head employs a circular 3-wavelength

LED-array for measuring not only images of fluorescence (F_0 , F and F_m), but of absorbed PAR as well. Blue LEDs at the same time provide measuring light, actinic light and saturation pulses. Fluorescence is measured at wavelengths >640 nm. With the help of red (650 nm) and near-infrared (780 nm) LEDs also images of absorbed PAR are derived, with 650 nm being representative for photosynthetically active radiation and 780 nm serving as a non-photosynthetically active reference. The intensities of the 650 and 780 nm measuring light are adjusted such that with a white flower petal the two images (i.e., remitted light) display identical intensities. It is assumed that the reflectance and scattering properties of a flower petal are similar to those of a leaf and that 780 nm light is not absorbed by photosynthetically active pigments. With increasing Chl content the red signal decreases, while the near-infrared signal remains constant. The PAR-absorptivity coefficient, a , is calculated pixel by pixel according to the equation: $a = 1 - (\text{red signal} / \text{near-infrared signal})$. For example, if the near-infrared signal is 5 times more intense than the red signal, then $a = 0.8$, i.e., 80% of the incident red light is absorbed. Maximal values of PAR-absorption determined in this way are around 0.89 and mean values around 0.85, i.e., close to the value reported by Björkman and Demmig (1987). Necrotic spots absorb red and near infrared light to a similar extent, leading to low values of PAR-absorptivity.

Knowledge of absorbed PAR is essential for quantifying photosynthetic activity. The saturation pulse method as such can only provide information on PS II quantum yield. The actual photosynthetic activity depends on Chl content and the absorbed PAR. Normally, it is assumed that 84% of the incident photons are absorbed (Section V.B.). While this approach is feasible in the study of normal leaves, when fluorescence is averaged over a larger area, it may become problematic when spatial heterogeneities are analyzed, as e.g., induced by virus infection, which may involve not only differences in PS II quantum yield but in Chl content as well. Generally, leaf senescence is characterized by decreases of Chl content and of photosynthetic capacity on a leaf area basis. At the same time, however, the capacity on a Chl basis may even increase (Adams et al., 1990b). With plant pathology being a major field of application for fluorescence imaging (Balachandran et al., 1994) and most pathogens inducing early senescence, images of PAR-absorptivity provide important complementary information to fluorescence images.

In principle, the same measuring routines can be

applied with the IMAGING-PAM fluorometer as with standard PAM fluorometers, as e.g., recording of dark-light induction curves with repetitive application of saturation pulses for quenching analysis (Figs. 5–7) and of light-response curves (Figs. 10 and 15). At low measuring pulse frequency the measuring light intensity is sufficiently weak for assessment of the quasi-dark fluorescence level, F_0 , and maximal quantum yield, F_v/F_m . During continuous illumination, measuring light frequency is increased, leading to increased time resolution and signal/noise.

In the experiment of Fig. 18, a defined heterogeneity of fluorescence parameters was artificially induced by dipping the tip of a *Nerium oleander* leaf for 5 min into 45 °C water, a treatment that does not lead to any visible damage. Images of PAR-absorptivity, effective quantum yield, $\Delta F/F_m'$, relative rate of photosynthesis and non-photochemical quenching, NPQ, are presented. While PAR-absorptivity is relatively homogenous over the imaged leaf area (average value 0.861), the fluorescence based parameters reveal pronounced heterogeneity between the heated and non-heated parts of the leaf. The most complex information is provided by the nonphotochemical quenching parameter (NPQ), which allows us to differentiate within the tip region several zones of heat-induced damage, likely to reflect small differences in the effective temperature during heat-treatment. Obviously, the heat effect ranges from nearly complete suppression of NPQ at the very tip, to strong stimulation of NPQ close to the border to the non-heated part of the leaf. Stimulation of NPQ by heat treatment is well known (Bilger and Schreiber, 1987; Schreiber and Bilger, 1987) (see also Fig. 7). It is likely to reflect stimulation of O_2 -dependent electron flow upon suppression of Calvin-Benson cycle activity. While Calvin-Benson cycle consumes ATP and, hence, lowers the pH-gradient, the Mehler-Ascorbate-Peroxidase cycle does not require ATP, and thus gives rise to strong membrane energization. The NPQ-image in Fig. 18 suggests that 45 °C is not only close to the critical temperature for suppression of Calvin-Benson cycle activity, but also of the Mehler-Ascorbate-Peroxidase cycle. Furthermore, these data remind us that the relative rate of photosynthesis measured on the basis of fluorescence parameters does not only reflect CO_2 assimilation, but O_2 -dependent electron flow as well (Section III. B.). The latter appears to be stimulated along with NPQ, when the former becomes suppressed, e.g., by stress induced damage.

With the IMAGING-PAM fluorescence images can

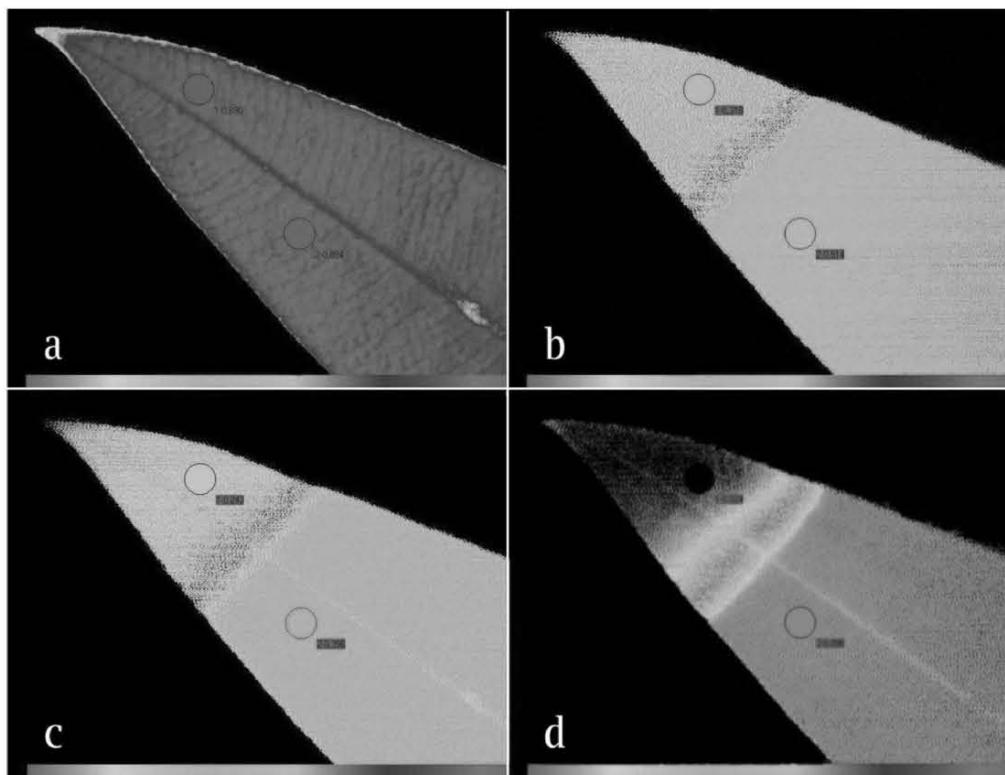


Fig. 18. Assessment of photosynthesis in leaf of *Nerium oleander* by fluorescence imaging. Heterogeneity was artificially induced by dipping the leaf tip for 5 min in 45 °C water. Continuous illumination at 400 μmol photons $\text{m}^{-2} \text{s}^{-1}$. The imaged parameters are: a) Absorptivity of photosynthetically active radiation, b) effective PS II quantum yield, $\Delta F/F_{\text{m}'}$, c) relative rate of photosynthesis, d) non-photochemical quenching, NPQ. Relative values ranging from 0 to 1 are displayed using the indicated false color scale. Measurements with the IMAGING-PAM fluorometer. For viewing the original color images, see Color Plate 2, Fig. 1.

be saved at a maximal rate of 1 image/5s. Hence, it is possible to follow spatiotemporal changes of fluorescence parameters. Using this method, very recently the propagation of a heat-stress signal via the leaf vein system was demonstrated (Schreiber et al., 2003). A small spot of a dandelion leaf was intensely heated using a near-infrared laser pulse. After the laser pulse, there was an immediate stimulation of photochemical quenching, qP , and of effective quantum yield, $\Delta F/F_{\text{m}'}$, along the vein system of the leaf (not shown here). With a delay of about 20 s, nonphotochemical quenching, assessed via the quenching coefficient qN , propagated over the leaf vein system, accompanied by declines in the fluorescence parameters qP and $\Delta F/F_{\text{m}'}$. Images showing the qN -responses are presented in Fig. 19. While 20 s after the heating event the qN response was restricted to the close vicinity of the hole burnt by the laser beam, within the following 50 s a spectacular propagation of the qN response took place, which first was limited to the right hand

side of the mid rib and then in the course of another 90 s also propagated to the left hand side of the mid rib. Obviously, the primary localized heating effect has triggered a signal that propagated via the leaf vein system. This signal could involve depolarization of cellular membrane potentials, moving from cell to cell via the plasmodesmata (Schreiber et al., 2003). Phloem cells in the leaf vein system may be particularly ‘excitable’ for the transmission of this response. Action potentials or variation potentials are considered plant-generated stress signals in response to environmental stress stimuli (Vian et al. 1999; Volkov et al., 2000). Chl fluorescence imaging can be expected to play an important role in the characterization of such signals and the elucidation of the underlying mechanisms.

The examples of PAM fluorometry presented in this section demonstrate the wide range of practical applications of Chl fluorescence measurements, which have been largely extending beyond the original field

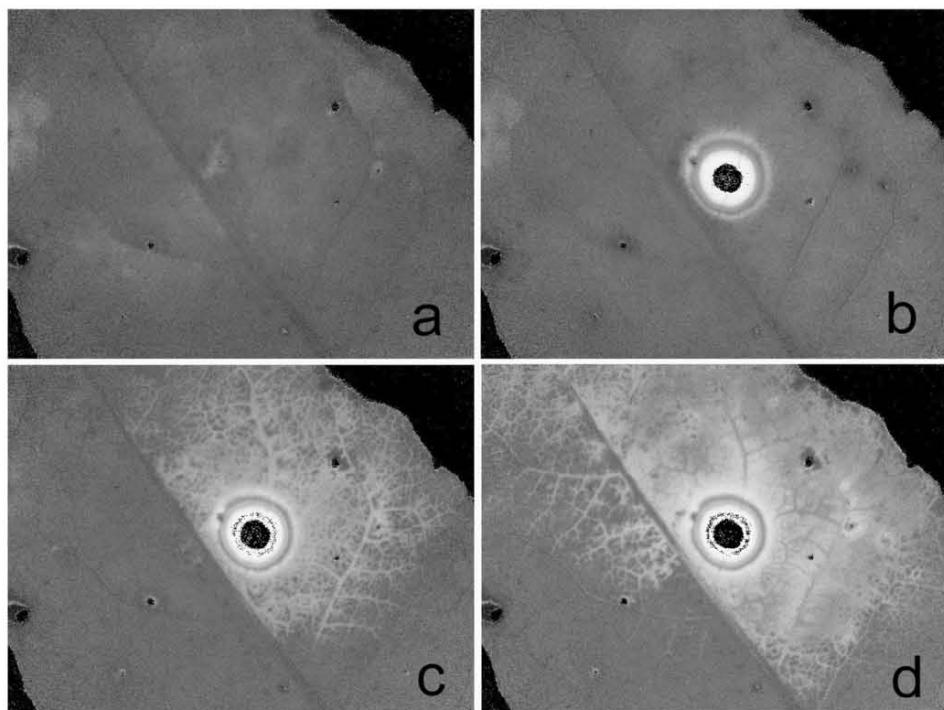


Fig. 19. Coefficient of nonphotochemical quenching, qN , imaged at the upper surface of a dandelion leaf (*Taraxacum officinale*) showing spatiotemporal variations after spot heating by a pulse of near-infrared laser light. The leaf was illuminated by blue light (470 nm) at $80 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$. Saturation pulses for assessment of quenching parameters were applied every 10 s. The displayed images were recorded: a) 10 s before laser pulse heating, b) 20 s after laser pulse heating, c) after additional 60 s, d) after additional 60 s. qN values between 0 and 1 are coded by a scale of gray tones ranging from black to white. The gray tone dominating before the laser pulse corresponds to $qN \approx 0.4$ whereas the bright tone spreading via the vein system after the laser pulse corresponds to $qN \approx 0.7$. Measurements with the IMAGING-PAM fluorometer. See Color Plate 2, Fig. 2 for color images of the same measurements.

of photosynthesis research. This development may be expected to continue in the near future with further progress in instrumentation following the general technical progress.

Acknowledgments

I thank Jérôme Lavergne and Anja Krieger for stimulating discussions and good advice. Rolf Gademann is thanked for help with the preparation of the figures. Support by the Deutsche Forschungsgemeinschaft over many years (from 1971 to 1998) is gratefully acknowledged.

References

- Adams III WW, Demmig-Adams B, Winter K and Schreiber U (1990a) The ratio of variable to maximum chlorophyll fluorescence from Photosystem II, measured in leaves at ambient temperature and at 77K, as an indicator of the photon yield of photosynthesis. *Planta* 180: 166–174
- Adams III WW, Winter K, Schreiber U and Schramel P (1990b) Photosynthesis and chlorophyll fluorescence characteristics in relationship to changes in pigment and element composition of leaves of *Platanus occidentalis* L. during autumnal leaf senescence. *Plant Physiol* 93: 1184–1190
- Allen JF (1992) Protein phosphorylation in regulation of photosynthesis. *Biochim Biophys Acta* 1098: 275–335
- Apostol S, Briantais JM, Moise N, Cerovics ZG and Moya I (2001) Photoinactivation of photosynthetic electron transport chain by accumulation of over-saturating light pulses given to dark adapted pea leaves. *Photosynth Res* 67: 215–227
- Asada K (1999) The water-water cycle in chloroplasts: Scavenging of active oxygen and dissipation of excess photons. *Ann Rev Plant Physiol Plant Mol Biol* 50: 601–639
- Asada K and Badger M (1984) Photoreduction of $^{18}\text{O}_2$ and $^{18}\text{H}_2\text{O}_2$ with a concomitant evolution of $^{16}\text{O}_2$ in intact spinach chloroplasts. Evidence for scavenging of hydrogen peroxide by peroxidase. *Plant Cell Physiol* 25: 1169–1179
- Balachandran S, Osmond CB and Daley PF (1994) Diagnosis of earliest strain-specific interactions between tobacco mosaic virus and chloroplasts of tobacco leaves *in vivo* by means of chlorophyll fluorescence imaging. *Plant Physiol* 104: 1059–1065
- Beer S and Ilan M (1998) In situ measurements of photosynthetic irradiance responses of two Red Sea sponges growing under

- dim light conditions. *Mar Biol* 131: 613–617
- Beer S, Ilan M, Eshel A, Weil A and Brickner I (1998) Use of pulse amplitude modulated (PAM) fluorometry for in situ measurements of photosynthesis in two Red Sea faviid corals. *Mar Biol* 131: 607–612
- Bennoun P (1970) Réoxydation du quencher de fluorescence ‘Q’ en présence de 3-(3,4-dichlorophényl)-1,1-diméthylurée. *Biochim Biophys Acta* 216: 357–363
- Bennoun P (1982) Evidence for a respiratory chain in the chloroplast. *Proc Natl Acad Sci USA* 79: 4352–4356
- Bennoun P (1998) Chlororespiration, 16 years later. In: Rochaix JD, Goldschmidt-Clermont M and Merchant S (eds) *The Molecular Biology of Chloroplasts and Mitochondria in Chlamydomonas*, pp 675–683. Kluwer Academic Publishers, Dordrecht
- Bennoun P (2002) The present model for chlororespiration. *Photosynth Res* 73: 273–277
- Berthold DA, Babcock GT and Yocom CF (1981) A highly resolved, oxygen-evolving Photosystem II preparation from spinach thylakoid membranes. *FEBS Lett* 134: 231–234
- Beutler M, Wiltshire KH, Meyer B, Moldaenke C and Dau H (1998). Rapid depth-profiling of the distribution of ‘spectral groups’ of microalgae in lakes, rivers and in the sea. In: Garab G (ed) *Photosynthesis: Mechanisms and Effects*. Vol V, pp 4301–4304. Kluwer Academic Publishers, Dordrecht
- Beutler M, Wiltshire KH, Meyer B, Moldaenke C, Lüring C, Meyerhöfer M, Hansen U-P and Dau H (2002) A fluorometric method for differentiation of algal populations *in vivo* and *in situ*. *Photosynth Res* 72: 39–53
- Bilger W and Björkman O (1990) Role of the xanthophyll cycle in photoprotection elucidated by measurements of light-induced absorbance changes, fluorescence and photosynthesis in leaves of *Hedera canariensis*. *Photosynth Res* 25: 173–186
- Bilger W and Schreiber U (1986) Energy-dependent quenching of dark-level chlorophyll fluorescence in intact leaves. *Photosynth Res* 10: 303–308
- Bilger W and Schreiber U (1990) Chlorophyll luminescence as an indicator of stress-induced damage to the photosynthetic apparatus. Effect of heat-stress in isolated chloroplasts. *Photosynth Res* 25: 161–171
- Bilger W, Schreiber U and Lange OL (1987) Chlorophyll fluorescence as an indicator of heat induced limitation of photosynthesis in *Arbutus unedo*. In: Tenhunen JD, Catarino FM, Lange OL and Oechel WC (eds) *Plant Response to Stress*, pp 391–399. Springer Verlag Berlin, Heidelberg
- Bilger W, Schreiber U and Bock M (1995) Determination of the quenching coefficients of Photosystem II and of non-photochemical quenching of chlorophyll fluorescence in the field. *Oecologia* 102: 425–432
- Björkman O (1987) Low-temperature chlorophyll fluorescence in leaves and its relationship to photon yield of photosynthesis in photoinhibition. In: Kyle DJ, Osmond CB and Arntzen CJ (eds) *Photoinhibition*, pp. 123–144. Elsevier, Amsterdam
- Björkman O and Demmig B (1987) Photon yield of O_2 -evolution and chloroplast fluorescence characteristics at 77 K among vascular plants of diverse origins. *Planta* 170: 489–504
- Björkman O and Demmig-Adams B (1994) Regulation of photosynthetic light energy capture, conversion, and dissipation in leaves of higher plants. In: Schulze E-D and Caldwell MM (eds) *Ecophysiology of Photosynthesis. Series Ecological Studies*, Vol 100, pp 17–47. Springer-Verlag, Berlin
- Black MT, Brearley TH and Horton P (1986) Heterogeneity in chloroplast Photosystem II. *Photosynth Res* 8: 193–207
- Bolhar-Nordenkampf HR, Long SP, Baker NR, Öquist G, Schreiber U and Lechner EG (1989) Chlorophyll fluorescence as a probe of the photosynthetic competence of leaves in the field: A review of current instrumentation. *Functional Ecol* 3: 497–514
- Bonaventura C and Myers J (1969) Fluorescence and oxygen evolution from *Chlorella pyrenoidosa*. *Biochim Biophys Acta* 189: 366–383
- Bornman JF, Vogelman TC and Martin G (1991) Measurement of chlorophyll fluorescence within leaves with a fiber optic microprobe. *Plant Cell Environ* 14: 719–725
- Briantais JM, Vernotte C, Picaud M and Krause GH (1979) A quantitative study of the slow decline of chlorophyll a fluorescence in isolated chloroplasts. *Biochim Biophys Acta* 548: 128–138
- Briantais JM, Vernotte C, Krause GH and Weis E (1986) Chlorophyll a fluorescence of higher plants: Chloroplasts and leaves. In: Govindjee, Amesz J and Fork CD (eds) *Light Emission by Plants and Bacteria*, pp 539–583. Academic Press, New York
- Bradbury M and Baker NR (1981) Analysis of the slow phases of the *in vivo* chlorophyll fluorescence induction curve. Changes in the redox state of Photosystem II electron acceptors and fluorescence emission from Photosystem I and II. *Biochim Biophys Acta* 635: 542–551
- Bukhov NG, Wiese C, Neimanis S and Heber U (1996) Control of Photosystem II in spinach leaves by continuous light and by light pulses given in the dark. *Photosynth Res* 50: 181–191
- Butler WL (1978) Energy distribution in the photochemical apparatus of photosynthesis. *Annu Rev Plant Physiol* 29: 345–378
- Cao J and Govindjee (1990) Chlorophyll a fluorescence transient as an indicator of active and inactive Photosystem II in thylakoid membranes. *Biochim Biophys Acta* 1015: 180–188
- Chylla RA and Whitmarsh J (1990) Light saturation response of inactive Photosystem II reaction centers in spinach. *Photosynth Res* 25: 39–48
- Chylla RA, Garab G and Whitmarsh J (1987) Evidence for slow turnover in a fraction of Photosystem II complexes in thylakoid membranes. *Biochim Biophys Acta* 894: 562–571
- Crofts J and Horton P (1991) Dissipation of excitation energy by Photosystem II particles at low pH. *Biochim Biophys Acta* 1058: 187–193
- Daley PF, Raschke K, Ball JT and Berry JA (1989) Topography of photosynthetic activity of leaves obtained from video images of chlorophyll fluorescence. *Plant Physiol* 90: 1233–1238
- Dau H (1994) Molecular mechanisms and quantitative models of variable Photosystem II fluorescence. *Photochem Photobiol* 60: 1–23
- Demmig B and Winter K (1988) Light response of CO_2 assimilation, reduction state of Q and radiationless energy dissipation in intact leaves. *Aust J Plant Physiol* 15: 151–162
- Demmig-Adams B (1990) Carotenoids and photoprotection in plants: A role for the xanthophyll zeaxanthin. *Biochim Biophys Acta* 1020: 1–24
- Demmig-Adams B and Adams WW III (1992) Photoprotection and other responses of plants to high light stress. *Annu Rev Plant Physiol Plant Mol Biol* 43: 599–626
- Delosme R (1967) Étude de l’induction de fluorescence des algues vertes et des chloroplastes au début d’une illumination intense.

- Biochim Biophys Acta 143: 108–128
- Delosme R (1971) Variations du rendement de fluorescence de la chlorophylle *in vivo* sous l'action d'éclairs de forte intensité. C R Acad Sci Paris 272: 2828–2831
- Dietz KJ, Schreiber U and Heber U (1985) The relationship between the redox state of Q_A and photosynthesis in leaves at various carbon-dioxide, oxygen and light regimes. Planta 166: 219–226
- Duysens LNM and Sweers HE (1963) Mechanism of the two photochemical reactions in algae as studied by means of fluorescence. In: Japanese Society of Plant Physiologists (ed) Studies on Microalgae and Photosynthetic Bacteria, pp 353–372. University of Tokyo Press, Tokyo
- Earl HJ and Tollenhaar M (1998) Relationship between thylakoid electron transport and photosynthetic CO_2 uptake in leaves of three maize (*Zea mays* L) hybrids. Photosynth Res 58: 245–257
- Falkowski PG and Kiefer DA (1985) Chlorophyll *a* fluorescence in phytoplankton: Relationship to photosynthesis and biomass. J Plankton Res 7: 715–731
- Falkowski PG and Kolber Z (1995) Variations in chlorophyll fluorescence yields in phytoplankton in the world oceans. Aust J Plant Physiol 22: 341–355
- Falkowski PG, Fujita Y, Ley AC and Mauzerall D (1986a) Evidence for cyclic electron flow around Photosystem II in *Chlorella pyrenoidosa*. Plant Physiol 81: 310–312
- Falkowski PG, Wyman K, Ley AC and Mauzerall D (1986b) Relationship of steady state photosynthesis to fluorescence in eucaryotic algae. Biochim Biophys Acta 849: 183–192
- Franck UF, Hoffmann N, Arenz H und Schreiber U (1969) Chlorophyllfluoreszenz als Indikator der photochemischen Primärprozesse der Photosynthese. Ber Bunsenges Phys Chem 73: 871–879
- Gans P and Rebeillé F (1990) Control in the dark of the plastoquinone redox state by mitochondrial activity in *Chlamydomonas reinhardtii*. Biochim Biophys Acta 1015: 150–155
- Genty B and Meyer S (1994) Quantitative mapping of leaf photosynthesis using chlorophyll fluorescence imaging. Aust J Plant Physiol 22: 277–284
- Genty B, Briantais JM and Baker NR (1989) The relationship between the quantum yield of photosynthetic electron transport and quenching of chlorophyll fluorescence. Biochim Biophys Acta 990: 87–92
- Genty B, Wonders J and Baker NR (1990) Non-photochemical quenching of F_0 in leaves is emission wavelength dependent: Consequences for quenching analysis and its interpretation. Photosynth Res 26: 133–139
- Gilbert M, Wilhelm C and Richter M (2000) Bio-optical modelling of oxygen evolution using *in vivo* fluorescence: Comparison of measured and calculated photosynthesis/irradiance (P-I) curves in four representative phytoplankton species. J Plant Physiol 157: 307–314
- Gilmore AM, Itoh S and Govindjee (2000) Global spectral-kinetic analysis of room temperature chlorophyll *a* fluorescence from light-harvesting mutants of barley. Phil Trans Roy Soc Lond B 355: 1371–1384
- Goh CH, Schreiber U and Hedrich R (1999) New approach of monitoring changes in chlorophyll-*a* fluorescence of single guard-cells and protoplasts in response to physiological stimuli. Plant Cell Environ 22: 1057–1070
- Goss R, Richter M, Wagner B and Holzwarth AR (1995) In: Mathis P (ed) Photosynthesis: From Light to Biosphere, Vol IV, pp 87–90. Kluwer Academic Publishers, Dordrecht
- Govindjee (1990) Photosystem II heterogeneity: The acceptor side. Photosynth Res 25: 151–160
- Govindjee (1995) Sixty-three years since Kautsky: Chlorophyll *a* fluorescence. Aust J Plant Physiol 22: 131–160
- Govindjee and Jursinic P (1979) Photosynthesis and fast changes in light emission by plants. Photochem Photobiol Rev 4: 125–205
- Govindjee and Papageorgiou G (1971) Chlorophyll fluorescence and photosynthesis: Fluorescence transients. In: Giese AC (ed) Photophysiology, Vol 6, pp 1–46. Academic Press, New York
- Govindjee, Ichimura S, Cederstrand C and Rabinowitch E (1960) Effect of combining far-red light with shorter wave light on the excitation of fluorescence in *Chlorella*. Arch Biochem Biophys 89: 322–323
- Govindjee, Amesz J and Fork DC (eds) (1986) Light Emission by Plants and Bacteria. Academic Press, Orlando
- Graan T and Ort DR (1986) Detection of oxygen-evolving Photosystem II centers inactive in plastoquinone reduction. Biochim Biophys Acta 852: 320–330
- Heber U, Kirk MR and Boardman NK (1979) Photoreactions of cytochrome b_{590} and cyclic electron flow in Photosystem II of intact chloroplasts. Biochim Biophys Acta 546: 292–306
- Heber U, Kobayashi Y, Leegood RC and Walker DA (1985) Low fluorescence yield in anaerobic chloroplasts and stimulation of chlorophyll *a* fluorescence by oxygen and inhibitors that block electron flow between Photosystem II and I. Proc Royal Soc B London 225: 41–53
- Heimann S (1998) Charakterisierung der Chloroplasten-cytochrome mit dem LED-Array-Spektralphotometer unter besonderer Berücksichtigung des Cytochrom b-559. PhD Thesis. Universität Würzburg, Germany
- Hillmann B, Bretel K, Van Mieghem F, Kamowski A, Rutherford AW and Schlodder E (1995) Charge recombination reactions in Photosystem II. 2. Transient absorbance difference spectra and their temperature dependence. Biochemistry 34: 4814–4827
- Holub O, Seufferheld MJ, Gohlke C, Govindjee and Clegg RM (2000) Fluorescence lifetime imaging (FLI) in real time—a new technique in photosynthesis research. Photosynthetica 38: 581–599
- Hormann H, Neubauer C and Schreiber U (1994) On the relationship between chlorophyll fluorescence quenching and the quantum yield of electron transport in isolated thylakoids. Photosynth Res 40: 93–106
- Horton P (1983) Relations between electron transport and carbon assimilation; simultaneous measurements of chlorophyll fluorescence, transthylakoid pH gradient and O_2 evolution in isolated chloroplasts. Proc R Soc Lond B 217: 405–416
- Horton P and Bowyer JR (1990) Chlorophyll fluorescence transients. In: Harwood JL, Bowyer JR (eds) Methods in Plant Biochemistry, Vol 4, pp 259–296. Academic Press, New York
- Horton P and Hague A (1988) Studies on the induction of chlorophyll fluorescence in barley protoplasts. IV. Resolution of non-photochemical quenching. Biochim Biophys Acta 932: 107–115
- Hsu B-D and Lee J-Y (1995) Fluorescence quenching by plastoquinone in an oxygen-evolving Photosystem-II-enriched preparation. J Photochem Photobiol 30: 57–61
- Joliot A and Joliot P (1964) Étude cinétique de la réaction photochimique libérant l'oxygène au cours de la photosynthèse.

- CR Acad Sci Paris 258: 4622–4625
- Joliot P and Joliot A (1973) Different types of quenching involved in Photosystem II centers. *Biochim Biophys Acta* 305: 302–316
- Joliot P and Joliot A (1977) Evidence for a double hit process in Photosystem II based on fluorescence studies. *Biochim Biophys Acta* 462: 559–574
- Joliot P and Joliot A (1979) Comparative study of the fluorescence yield and of the C550 absorption change at room temperature. *Biochim Biophys Acta* 546: 93–105
- Jones RJ, Hoegh-Guldberg O, Larkum AWD and Schreiber U (1998) Temperature-induced bleaching of corals begins with impairment of the CO₂ fixation mechanism in zooxanthellae. *Plant Cell Environ* 21: 1219–1230
- Joshi MK and Mohanty P (1995) Probing photosynthetic performance by chlorophyll *a* fluorescence: Analysis and interpretation of fluorescence parameters. *J Sci Industr Res* 54: 155–174
- Jursinic PA (1986) Delayed fluorescence: Current concepts and status. In: Govindjee, Amesz J and Fork DC (eds) *Light Emission by Plants and Bacteria*, pp 291–328. Academic Press, New York
- Kautsky H and Franck U (1943) Chlorophyllfluoreszenz und Kohlensäureassimilation. *Biochem Z* 315: 139–232
- Kautsky H and Hirsch A (1931) Neue Versuche zur Kohlensäureassimilation. *Naturwissenschaften* 19: 964
- Kautsky H, Appel W and Amann H (1960) Die Fluoreszenzkurve und die Photochemie der Pflanze. *Biochem Z* 332: 277–292
- Ke B (2001) *Photosynthesis: Photobiochemistry and Photobiophysics*. Kluwer Academic Publishers, Dordrecht
- Kiefer DA and Reynolds RA (1992) Advances in understanding phytoplankton fluorescence and photosynthesis. In: Falkowski PG (ed) *Primary Productivity and Biogeochemical Cycles in the Sea*, pp 155–174. Plenum press, New York
- Klimov VV, Klevanik AV, Shuvalov VA and Krasnovsky AA (1977) Reduction of pheophytin in the primary light reaction of Photosystem II. *FEBS Lett* 82: 183–186
- Klughammer C and Schreiber U (1994) An improved method, using saturating light pulses, for the determination of Photosystem I quantum yield via P700⁺-absorbance changes at 830 nm. *Planta* 192: 261–268
- Klughammer C and Schreiber U (1998) Measuring P700 absorbance changes in the near infrared spectral region with a dual wavelength pulse modulation system. In: Garab G (ed) *Photosynthesis: Mechanisms and Effects*. Vol V, pp 4357–4360. Kluwer Academic Publishers, Dordrecht
- Klughammer C, Kolbowski J and Schreiber U (1990) LED Array Spectrophotometer for measurement of time resolved difference spectra. *Photosynth Res* 25: 317–327
- Klughammer C, Heimann S and Schreiber U (1998) Inhibition of cytochrome b₅₆₃ oxidation by triorganotins in spinach chloroplasts. *Photosynth Res* 56: 117–130
- Koblizek M, Kaftan D and Nedbal L (2001) On the relationship between the non-photochemical quenching of the chlorophyll fluorescence and the Photosystem II light harvesting efficiency. A repetitive flash fluorescence study. *Photosynth Res* 68: 141–152
- Kolber Z and Falkowski PG (1993) Use of active fluorescence to estimate phytoplankton photosynthesis in situ. *Limnol Oceanogr* 38: 1646–1665
- Kolber Z, Prasil O and Falkowski P (1998) Measurements of variable chlorophyll fluorescence using fast repetition rate technique. I. Defining methodology and experimental protocols. *Biochim Biophys Acta* 1367: 88–106
- Kolbowski J and Schreiber U (1995) Computer-controlled phytoplankton analyzer based on 4-wavelengths PAM chlorophyll fluorometer. In: Mathis P (ed) *Photosynthesis: from Light to Biosphere*, Vol V, pp 825–828. Kluwer Academic Publishers, Dordrecht
- Kolbowski J, Reising H and Schreiber U (1990) Computer controlled pulse modulation system for analysis of photoacoustic signals in the time domain. *Photosynth Res* 25: 309–316
- Krall JP and Edwards GE (1990) Quantum yields of Photosystem II electron transport and carbon dioxide fixation in C4 plants. *Aus J Plant Physiol* 17: 579–588
- Krall JP and Edwards GE (1991) Environmental effects on the relationship between the quantum yields of carbon assimilation and in vivo PS II electron transport in maize. *Aus J Plant Physiol* 18: 267–278
- Kramer DM, DiMarco G and Loreto F (1995) Contribution of plastoquinone quenching to saturation pulse-induced rise of chlorophyll fluorescence in leaves. In: Mathis P (ed) *Photosynthesis: From Light to Biosphere*. Vol. I, pp 147–150. Kluwer Academic Publishers, Dordrecht
- Krause G and Weis E (1991) Chlorophyll fluorescence and photosynthesis: The basics. *Ann Rev Plant Physiol Plant Mol Biol* 42: 313–49
- Krause GH, Briantais JM and Vernotte C (1982) Photoinduced quenching of chlorophyll fluorescence in intact chloroplasts and algae. Resolution into two components. *Biochim Biophys Acta* 679: 116–24
- Krieger A and Weis E (1993) The role of calcium in the pH dependent control of Photosystem II. *Photosynth Res* 37: 117–130
- Kurrek J, Schödel R and Renger G (2000) Investigation of the plastoquinone pool size and fluorescence quenching in thylakoid membranes and Photosystem II (PS II) membrane fragments. *Photosynth Res* 63: 171–182
- Laasch H, Urbach W and Schreiber U (1983) Binary flash-induced oscillations of (¹⁴C)DCMU binding to the Photosystem II acceptor complex. *FEBS Lett* 159: 275–279
- Lam E, Baltimore B, Ortiz W, Choller S, Melis A and Malkin R (1983) Characterization of a resolved oxygen-evolving Photosystem II preparation from spinach thylakoids. *Biochim Biophys Acta* 724: 201–211
- Lavergne J (1982) Mode of action of dichlorophenyldimethylurea. Evidence that the inhibitor competes with plastoquinone for binding to a common site on the acceptor side of Photosystem II. *Biochim Biophys Acta* 682: 345–353
- Lavergne J and Briantais J-M (1996) Photosystem II heterogeneity. In: Ort DR and Yocum CF (eds) *Oxygenic Photosynthesis: The Light Reactions*, pp 265–287. Kluwer Academic Publishers, Dordrecht
- Lavergne J and Leci E (1993) Properties of inactive Photosystem II centers. *Photosynth Res* 35: 323–343
- Lavergne J and Rappaport F (1998) Stabilization of charge separation and photochemical misses in Photosystem II. *Biochemistry* 37: 7899–7906
- Lavergne J and Trissl HW (1995) Theory of fluorescence induction in Photosystem II: Derivation of analytical expressions in a model including exciton-radical-pair equilibrium and restricted energy transfer between photosynthetic units. *Biophys J* 68: 2474–2492
- Lavorel J (1975) Luminescence. In: Govindjee (ed) *Bioenergetics of Photosynthesis*, pp 223–317. Academic Press, New York

- Lavorel J and Etienne AL (1977) In vivo chlorophyll fluorescence. In: Barber J (ed) Primary Processes of Photosynthesis, pp 203–268. Elsevier, Amsterdam
- Lazar D (1999) Chlorophyll a fluorescence induction. *Biochim Biophys Acta* 1412: 1–28
- Ley AC and Mauzerall D (1982) Absolute absorption cross sections for Photosystem II and the minimum quantum requirement for photosynthesis in *Chlorella vulgaris*. *Biochim Biophys Acta* 680: 95–106
- Long SP, Postl WF and Bolhar-Nordenkampf HR (1993) Quantum yields for uptake of carbon dioxide in C₃ vascular plants of contrasting habitats and taxonomic groupings. *Planta* 189: 226–234
- Lorenzen CJ (1966) A method for the continuous measurement of in vivo chlorophyll concentration. *Deep-Sea Res* 13: 223–227
- Malkin S and Kok B (1966) Fluorescence induction studies in isolated chloroplasts. I. Number of components involved in the reaction and quantum yields. *Biochim Biophys Acta* 126: 413–432
- Mano J, Miyake C, Schreiber U and Asada K (1995) Photoactivation of electron flow from NADPH to plastoquinone in spinach chloroplasts. *Plant Cell Physiol* 36: 1589–1598
- Mauzerall D (1972) Light induced fluorescence changes in *Chlorella*, and the primary photoreactions for the production of oxygen. *Proc Natl Acad Sci USA* 69: 1358–1362
- Melis A (1991) Dynamics of photosynthetic membrane composition and function. *Biochim Biophys Acta* 1058: 87–106
- Melis A and Duyssens LNM (1979) Biphasic energy conversion kinetics and absorbance difference spectra of Photosystem II of chloroplasts. Evidence for two different PS II reaction centers. *Photochem Photobiol* 29: 373–382
- Melis A and Homann PH (1976) Heterogeneity of photochemical centres in system II of chloroplasts. *Photochem Photobiol* 23: 343–350
- Melis A and Schreiber U (1979) The kinetic relationship between the C-550 absorbance change, the reduction of Q (A₃₂₀) and the variable fluorescence yield change in chloroplasts at room temperature. *Biochim Biophys Acta* 547: 47–57
- Melis A and Zeiger E (1982) Chlorophyll fluorescence transients in mesophyll and guard cells. Modulation of guard cell photophosphorylation by CO₂. *Plant Physiol* 69: 642–647
- Mi H, Endo T, Schreiber U, Ogawa T and Asada K (1992) Electron donation from cyclic and respiratory flows to photosynthetic intersystem chain is mediated by pyridine nucleotide dehydrogenase in the cyanobacterium *Synechocystis* PCC 6803. *Plant Cell Physiol* 33: 1233–1237
- Mi H, Klughammer C and Schreiber U (2000) Light-induced dynamic changes of NADPH fluorescence in *Synechocystis* PCC 6803 and its ndhB-defective mutant M55. *Plant Cell Physiol* 41: 1129–1135
- Mohammed GH, Binder WD and Gillies SL (1995) Chlorophyll fluorescence: A review of its practical forestry applications and instrumentation. *Scand J Forest Res* 10: 383–410
- Munday JCM Jr. and Govindjee (1969) Light induced changes of the fluorescence yield of chlorophyll a in vivo. III. The dip and the peak in the fluorescence transient of *Chlorella pyrenoidosa*. *Biophys J* 9: 1–21
- Murata N, Nishimura M and Takamiya A (1966) Fluorescence of chlorophyll in photosynthetic systems. II. Induction of fluorescence in isolated spinach chloroplasts. *Biochim Biophys Acta* 120: 23–33
- Nedbal L, Soukupova J, Kaftan D, Whitmarsh J and Trtilek M (2000) Kinetic imaging of chlorophyll fluorescence using modulated light. *Photosynth Res* 66: 3–12
- Neubauer C and Schreiber U (1987) The polyphasic rise of chlorophyll fluorescence upon onset of strong continuous illumination: I. Saturation characteristics and partial control by the Photosystem II acceptor side. *Z Naturforsch* 42c: 1246–1254
- Oberhuber W, Dai Z-Y and Edwards GE (1993) Light dependence of quantum yields of Photosystem II and CO₂ fixation in C₃ and C₄ plants. *Photosynth Res* 35: 265–274
- Ögren E and Baker NR (1985) Evaluation of a technique for the measurement of chlorophyll fluorescence from leaves exposed to continuous white light. *Plant Cell Environ* 8: 539–547
- Omasa K, Shimazaki KI, Aiga I, Larcher W and Onoe M (1987) Image analysis of chlorophyll fluorescence transients for diagnosing the photosynthetic system of attached leaves. *Plant Physiol* 84: 748–752
- Oxborough K and Baker NR (1997) An instrument capable of imaging chlorophyll-a fluorescence from intact leaves at very low irradiance and at cellular and subcellular levels of organization. *Plant Cell Environ* 20: 1473–1483
- Papageorgiou G (1975) Chlorophyll fluorescence: An intrinsic probe of photosynthesis. In: Govindjee (ed) Bioenergetics of Photosynthesis, pp 319–371. Academic Press, New York
- Papageorgiou G and Alygizaki-Zorba A (1997) A sensitive method for the estimation of the cytoplasmic osmolality of cyanobacterial cells using chlorophyll fluorescence. *Biochim Biophys Acta* 1335: 1–4
- Papageorgiou G, Govindjee, Govindjee R, Mimuro M, Stamatakis K, Alygizaki-Zorba A and Murata N (1999) Light-induced and osmotically-induced changes in chlorophyll a fluorescence in two *Synechocystis* sp. PCC6803 strains that differ in membrane lipid unsaturation. *Photosynth Res* 59: 125–136
- Pfündel E (1998) Estimating the contribution of Photosystem I to total leaf fluorescence. *Photosynth Res* 56: 185–195
- Pfündel E and Bilger W (1994) Regulation and possible function of the violaxanthin cycle. *Photosynth Res* 42: 89–109
- Pospisil P and Dau H (2000) Chlorophyll fluorescence transients of Photosystem II membrane particles as a tool for studying photosynthetic oxygen evolution. *Photosynth Res* 65: 41–52
- Prasol O, Kolber Z, Berry J and Falkowski PG (1996) Cyclic electron flow around Photosystem II in vivo. *Photosynth Res* 48: 395–410
- Quick WP and Horton P (1984) Studies on the induction of chlorophyll fluorescence quenching by redox state and transthalakoid pH gradient. *Proc R Soc Lond B* 220: 371–382
- Quick WP and Stitt M (1989) An examination of factors contributing to non-photochemical quenching of chlorophyll fluorescence in barley leaves. *Biochim Biophys Acta* 977: 287–296
- Radmer RJ and Kok B (1976) Photoreduction of O₂ primes and replaces CO₂ assimilation. *Plant Physiol* 58: 336–40
- Ralph PJ, Gademann R and Dennison WC (1998). In situ seagrass photosynthesis measured using a submersible, pulse-amplitude modulated fluorometer. *Mar Biol* 132: 367–373
- Ralph PJ, Gademann R, Larkum AWD, and Schreiber U (1999) In-situ underwater measurements of photosynthetic activity of coral zooxanthellae and other reef-dwelling dinoflagellate endosymbionts. *Marine Ecology-Progr Ser* 180: 139–147
- Rascher U, Liebig M and Lüttge U (2000) Evaluation of instant light-response curves of chlorophyll fluorescence parameters

- obtained with a portable chlorophyll fluorometer on site in the field. *Plant Cell Environ* 23: 1397–1405
- Reising H and Schreiber U (1992) Pulse-modulated photoacoustic measurements reveal strong gas-uptake component at high CO₂-concentrations. *Photosynth. Res.* 31: 227–238
- Renger G (1973) Studies of the mechanism of destabilization of the positive charges trapped in the photosynthetic watersplitting enzyme Y by a deactivation accelerating agent. *Biochim Biophys Acta* 314: 390–402
- Renger G and Schreiber U (1986) Practical applications of fluorometric methods to algae and higher plant research. In: Govindjee, Ames J and Fork DC (eds) *Light Emission by Plants and Bacteria*, pp 587–619. Academic Press, Orlando
- Renger G, Eckert HJ, Bergmann A, Bernarding J, Liu B, Nipowitzki A, Reifarth F and Eichler HJ (1995) Fluorescence and spectroscopic studies of exciton trapping and electron transfer in Photosystem II of higher plants. *Aust J Plant Physiol* 22: 167–181
- Roelofs TA, Lee CH and Holzwarth AR (1992) Global target analysis of picosecond chlorophyll fluorescence kinetics from pea chloroplasts. *Biophys J* 61: 1147–1163
- Rutherford W, Govindjee and Inoue Y (1984) Charge accumulation and photochemistry in leaves studied by thermoluminescence and delayed light emission. *Proc Natl Acad Sci USA* 81: 1107–1111
- Samson G and Bruce D (1996) Origins of the low yield of chlorophyll fluorescence induced by single turnover flash in spinach thylakoids. *Biochim Biophys Acta* 1276: 147–153
- Samson G, Prasol O and Yaakoubi B (1999) Photochemical and thermal phases of chlorophyll *a* fluorescence. *Photosynthetica* 37: 163–182
- Schatz GH, Brock H and Holzwarth AR (1988) Kinetic and energetic model for the primary processes in Photosystem II. *Biophys J* 54: 397–405
- Schreiber U (1979) Cold-induced uncoupling of energy transfer between phycobilisomes and chlorophyll in *Anacystis nidulans*: Antagonistic effects of monovalent and divalent cations, and of high and low pH. *FEBS Lett* 107: 4–9
- Schreiber U (1980) Reversible uncoupling of energy transfer between phycobilins and chlorophyll in *Anacystis nidulans*: Light stimulation of cold-induced phycobilisome detachment. *Biochim Biophys Acta* 591: 361–371
- Schreiber U (1983) Chlorophyll fluorescence yield changes as a tool in plant physiology. The measuring system. *Photosynth Res* 4: 361–373
- Schreiber U (1986) Detection of rapid induction kinetics with a new type of high-frequency modulated chlorophyll fluorometer. *Photosynth Res* 9: 261–272
- Schreiber U (1997) Chlorophyll Fluorescence and Photosynthetic Energy Conversion. Simple Introductory Experiments with the TEACHING-PAM Chlorophyll Fluorometer. Heinz Walz GmbH, Effeltrich, Germany <<http://www.walz.com>>
- Schreiber U (1998) Chlorophyll fluorescence: New instruments for special applications. In: Garab G (ed) *Photosynthesis: Mechanisms and Effects*, Vol V, pp 4253–4258. Kluwer Academic Publishers, Dordrecht
- Schreiber U (2002) Assessment of maximal fluorescence yield: Donor-side dependent quenching and Q_B-quenching. In: Van Kooten O and Snel JFH (eds) *Plant Spectrofluorometry: Applications and Basic Research*, pp 23–47. Rozenberg Publishers, Amsterdam
- Schreiber U and Berry JA (1977) Heat-induced chlorophyll fluorescence changes in intact leaves correlated with damage of the photosynthetic apparatus. *Planta* 136: 233–238
- Schreiber U and Bilger W (1987) Rapid assessment of stress effects on plant leaves by chlorophyll fluorescence measurements. In: Tenhunen JD, Catarino FM, Lange OL and Oechel WC (eds) *Plant Response to Stress*, pp 27–53. Springer Verlag-Berlin-Heidelberg
- Schreiber U and Bilger W (1993) Progress in chlorophyll fluorescence research: Major developments during the last years in retrospect. *Progress in Botany* 54: 151–173
- Schreiber U and Krieger A (1996) Hypothesis: Two fundamentally different types of variable chlorophyll fluorescence in vivo. *FEBS Lett* 397: 131–135
- Schreiber U and Neubauer C (1987) The polyphasic rise of chlorophyll fluorescence upon onset of strong continuous illumination: II. Partial control by the Photosystem II donor side and possible ways of interpretation. *Z Naturforsch* 42c: 1255–1264
- Schreiber U and Neubauer C (1989) Correlation between dissipative fluorescence quenching at Photosystem II and 50 μs recombination luminescence. *FEBS Lett* 258: 339–342
- Schreiber U and Neubauer C (1990) O₂-dependent electron flow, membrane energization and the mechanism of non-photochemical quenching of chlorophyll fluorescence. *Photosynth Res* 25: 279–293
- Schreiber U and Schliwa U (1987a) Fluorometer zur Messung lichtinduzierter Veränderungen der Fluoreszenzausbeute einer zu untersuchenden Probe. Patentschrift DE 3518527C2
- Schreiber U and Schliwa U (1987b) A solid-state instrument for measurement of chlorophyll fluorescence induction in plants. *Photosynth Res* 11: 173–182
- Schreiber U and Vidaver W (1974) Chlorophyll fluorescence induction in anaerobic *Scenedesmus obliquus*. *Biochim Biophys Acta* 386: 97–112
- Schreiber U and Vidaver W (1975) Analysis of anaerobic fluorescence decay in *Scenedesmus obliquus*. *Biochim Biophys Acta* 386: 37–51
- Schreiber U, Bauer R and Franck U (1971) Chlorophyll fluorescence induction in green plants at oxygen deficiency. In: Forti G, Avron M and Melandri A (eds) *Proceedings of the 2nd International Congress on Photosynthesis*, pp 169–179. Junk, The Hague
- Schreiber U, Groberman L and Vidaver W (1975) A portable solid state fluorometer for the measurement of chlorophyll fluorescence induction in plants. *Rev Sc Instr* 46: 538–542
- Schreiber U, Bilger W and Schliwa U (1986) Continuous recording of photochemical and non-photochemical chlorophyll fluorescence quenching with a new type of modulation fluorometer. *Photosynth Res* 10: 51–62
- Schreiber U, Klughammer C and Neubauer C (1988) Measuring P700 absorbance changes around 830 nm with a new type of pulse modulation system. *Z Naturforsch* 43c: 686–698
- Schreiber U, Neubauer C and Klughammer C (1989) Devices and methods for room-temperature fluorescence analysis. *Phil Trans R Soc Lond B* 323: 241–251
- Schreiber U, Neubauer C and Schliwa U (1993) PAM fluorometer based on medium-frequency pulsed Xe-flash measuring light: A highly sensitive new tool in basic and applied photosynthesis research. *Photosynth Res* 36: 65–72
- Schreiber U, Bilger W and Neubauer C (1994) Chlorophyll fluorescence as a non-intrusive indicator for rapid assessment of in

- vivo photosynthesis. In: Schulze E-D and Caldwell MM (eds) *Ecophysiology of Photosynthesis*. Series Ecological Studies, Vol 100, pp 49–70. Springer-Verlag, Berlin
- Schreiber U, Hormann H, Neubauer C and Klughammer C (1995a) Assessment of Photosystem II photochemical quantum yield by chlorophyll fluorescence quenching analysis. *Aust J Plant Physiol* 22: 209–220
- Schreiber U, Endo T, Mi H and Asada K (1995b) Quenching analysis of chlorophyll fluorescence by the saturation pulse method: Particular aspects relating to the study of eucaryotic algae and cyanobacteria. *Plant Cell Physiol* 36: 873–882
- Schreiber U, Hormann H, Asada K and Neubauer C (1995c) O₂-dependent electron flow in spinach chloroplasts: Properties and possible regulation of the Mehler-Ascorbate Peroxidase Cycle. In: Mathis P (ed) *Photosynthesis: from Light to Biosphere*. Vol II, pp 813–818. Kluwer Academic Publishers, Dordrecht
- Schreiber U, Kühl M, Klimant I and Reising H (1996) Measurement of chlorophyll fluorescence within leaves using a modified PAM fluorometer with a fiber-optic microprobe. *Photosynth Res* 47:103–109
- Schreiber U, Gademann R, Ralph PJ and Larkum AWD (1997) Assessment of photosynthetic performance of prochloron in *Lissoclinum patella* in hospite by chlorophyll fluorescence measurements. *Plant Cell Physiol* 38: 945–951
- Schreiber U, Bilger W, Hormann H and Neubauer C (1998) Chlorophyll fluorescence as a diagnostic tool: Basics and some aspects of practical relevance. In: Raghavendra AS (ed) *Photosynthesis. A Comprehensive Treatise*, pp. 320–336. Cambridge University Press, Cambridge
- Schreiber U, Walz H and Kolbowski J (2003) Propagation of spatial variations of chlorophyll fluorescence parameters in dandelion leaves induced by laser spot heating. <http://www.pam-news.de/ar/03-01/PAMNews03-01.html> (June 22, 2003)
- Seaton GGR and Walker DA (1990) Chlorophyll fluorescence as a measure of photosynthetic carbon assimilation. *Proc Roy Soc London Ser B* 242: 29–35
- Shen YK, Chow WS, Park YI and Anderson JM (1996) Photoinactivation of Photosystem II by cumulative exposure to short light pulses during the induction period of photosynthesis. *Photosynth Res* 47: 51–59
- Siebke K and Weis E (1995) Assimilation images of leaves of *Glechoma hederacea*: Analysis of non-synchronous stomata related oscillations. *Planta* 196: 155–165
- Srivastava A, Strasser RJ and Govindjee (1995) Polyphasic rise of chlorophyll a fluorescence in herbicide resistant D1 mutants of *Chlamydomonas reinhardtii*. *Photosynth Res* 43: 131–141
- Srivastava A, Strasser RT and Govindjee (1999) Greening of peas: Parallel measurements of 77K emission spectra, OJIP chlorophyll fluorescence transient, period four oscillation of initial fluorescence level, delayed light emission, and P700⁺. *Photosynthetica* 3: 365–392
- Stamatakis K and Papageorgiou G (2001) The osmolality of the cell suspension regulates phycobilisome-to-Photosystem I excitation transfers in cyanobacteria. *Biochim Biophys Acta* 1506: 172–181
- Stirbet A, Govindjee, Strasser B and Strasser RT (1998) Chlorophyll a fluorescence induction in higher plants: Modelling and numerical simulation. *J Theor Biol* 193: 131–151
- Strasser B (1997) Donor side capacity of Photosystem II probed by chlorophyll fluorescence transients. *Photosynth Res* 52: 147–155
- Strasser RJ and Govindjee (1992) On the O-J-I-P fluorescence transients in leaves and D1 mutants of *Chlamydomonas reinhardtii*. In: Murata N (ed) *Research in Photosynthesis*. Vol II, pp 20–32. Kluwer Academic Publishers, Dordrecht
- Strasser RJ, Srivastava A and Govindjee (1995) Polyphasic chlorophyll a fluorescence transient in plants and cyanobacteria. *Photochem Photobiol* 61: 32–42
- Urbach W, Laasch H and Schreiber U (1984) Redox-state dependent changes of inhibitor-binding to the Photosystem II acceptor complex. *Z Naturforsch* 39c: 392–397
- van Gorkom HJ, Tamminga JJ, Havemann J and van der Linden IK (1974) Primary reactions, plastoquinone and fluorescence yield in subchloroplast fragments prepared with deoxycholate. *Biochim Biophys Acta* 347:417–438
- van Kooten O and Snel JFH (1990) The use of chlorophyll fluorescence nomenclature in plant stress physiology. *Photosynth Res* 25: 147–150
- van Mieghem F, Brettel K, Hillmann B, Kamrowski A, Rutherford AW and Schlodder E (1995) Charge recombination reactions in Photosystem II. 1. Yields, recombination pathways, and kinetics of the primary pair. *Biochemistry* 34: 4798–4813
- Vasil'ev S and Bruce D (1998) Nonphotochemical quenching of excitation energy in Photosystem II. A picosecond time-resolved study of the low yield of chlorophyll a fluorescence induced by single-turnover flash in isolated spinach thylakoids. *Biochemistry* 37: 11046–11054
- Vasil'ev S, Wiebe S and Bruce D (1998) Non-photochemical quenching of chlorophyll fluorescence in photosynthesis. 5-hydroxy-1,4-naphthoquinone in spinach thylakoids as a model for antenna quenching mechanisms. *Biochim Biophys Acta* 1363: 147–156
- Velthuys BR (1981) Electron dependent competition between plastoquinone and inhibitors for the binding to PS II. *FEBS Lett* 126: 277–281
- Velthuys BR and Amesz J (1974) Charge accumulation at the reducing site of system 2 of photosynthesis. *Biochim Biophys Acta* 325: 138–148
- Vermaas WFJ (1984) The interaction of quinones, herbicides and bicarbonate with their binding environment at the acceptor side of Photosystem II in photosynthesis. PhD Thesis, Landbouwhogeschool Wageningen, The Netherlands
- Vernotte C, Etienne AL and Briantais JM (1979) Quenching of the system II chlorophyll fluorescence by the plastoquinone pool. *Biochim Biophys Acta* 545: 519–527
- Vian A, Henty-Vian C and Davies E (1999) Rapid and systemic accumulation of chloroplast mRNA binding protein transcripts after flame stimulus in tomato. *Plant Physiol* 121: 517–524
- Volkov AG, Collins DJ and Mwesigwa J (2000) Plant electrophysiology: Pentachlorophenol induces fast action potentials in soybean. *Plant Science* 153: 185–190
- Vogelmann TC (1993) Plant tissue optics. *Ann Rev Plant Physiol Plant Mol Biol* 44: 231–251
- Vogelmann TC and Björn LO (1984) Measurement of light gradients and spectral regime in plant tissue with a fibre optic probe. *Physiol Plant* 60: 361–368
- Vredenberg WJ (2000) A three-state model for energy trapping and chlorophyll fluorescence in Photosystem II incorporating radical pair recombination. *Biophys J* 79: 25–38
- Walker DA, Sivak MN, Prinsley RT and Cheeseborough JK (1983) Simultaneous measurement of oscillations in oxygen evolution and chlorophyll a fluorescence in leaf pieces. *Plant*

- Physiol 73:542–549
- White AJ and Critchley C (1999) Rapid light curves: A new fluorescence method to assess the state of the photosynthetic apparatus. *Photosynth Res* 59: 63–72
- Wilhelm C and Duval JC (1990) Fluorescence induction kinetics as a tool to detect photorespiratory activity in the prasinophycean alga *Mantoniella squamata*. *Biochim Biophys Acta* 1016: 197–202
- Wollman (1978) Determination and modification of the redox state of the secondary acceptor of Photosystem II in the dark. *Biochim Biophys Acta* 503: 263–273
- Yaakoubd B, Andersen R, Desjardins Y and Samson G (2002) Contributions of the free oxidized and Q_B-bound plastoquinone molecules to the thermal phase of chlorophyll-*a* fluorescence. *Photosynth Res* 74: 251–257
- Yentsch CS and Yentsch CM (1979) Fluorescence spectral signatures: The characterization of phytoplankton populations by the use of excitation and emission spectra. *J Mar Res* 37: 471–483
- Zeiger E, Armond P and Melis A (1980) Fluorescence properties of guard cell chloroplasts: evidence for linear electron transport and light harvesting pigment of Photosystem I and II. *Plant Physiol* 67: 17–20

