New Emitter-Detector-Cuvette Assembly for Measuring Modulated Chlorophyll Fluorescence of Highly Diluted Suspensions in Conjunction with the Standard PAM Fluorometer

Ulrich Schreiber

Julius-von-Sachs-Institut für Biowissenschaften der Universität Würzburg, Lehrstuhl Botanik I, Mittlerer Dallenbergweg 64, D-97082 Würzburg, Bundesrepublik Deutschland

Dedicated to Professor Aloysius Wild on the occasion of his 65th birthday

Z. Naturforsch. **49 c,** 646–656 (1994); received May 18/July 7, 1994

Chlorophyll Fluorescence, Photosynthesis, PAM Fluorometer, Quenching Analysis, Unicellular Algae, Cyanobacteria, Phytoplankton

A new emitter-detector-cuvette assembly for the standard PAM fluorometer is described which leads to substantial improvement of signal/noise ratio and increased flexibility with respect to the choice of excitation and emission wavelengths. These features are particularly useful for work with very dilute suspensions of unicellular algae and isolated chloroplasts. Instead of fiber optics perspex rods are applied for guiding excitation light to a mirrored 10×10×45 mm cuvette and from there at 90° angle to the photodetector, similarly as recently reported for a PAM fluorometer based on Xe-flash measuring light (Schreiber *et al.* (1993), Photosynth. Res. **36,** 65–72). While the detection limit of the new system does not reach that of the Xe-PAM fluorometer, it is approximately two orders of magnitude higher than that of the standard system. Rapid induction kinetics can be measured at low chlorophyll concentrations down to 0.1 $\mu g \cdot ml^{-1}$. Satisfactory quenching analysis for detection of active chlorophyll concentration is still possible at 5 μg chlorophyll $\cdot l^{-1}$. The various optical factors contributing to the improved sensitivity are analyzed. An accessory device is described by which the frequency of the measuring light pulses generated by the PAM fluorometer is lowered in order to reduce the actinic effect of the measuring light. The performance of the new system using different excitation and emission wavelengths is demonstrated in measurements with green algae, cyanobacteria and leaves. Applying a newly available blue light-emitting diode with 450 nm peak emission, short wavelength fluorescence enriched in PS II emission can be measured, which is characterized by high values of variable fluorescence relative to maximal fluorescence. Using measuring light covering five different wavelength ranges the fluorescence contributions from cyanobacteria and green algae can be distinguished on the basis of distinct differences in their excitation spectra. This approach should become useful for an estimation of content and activity of different types of phytoplankton in natural surface waters.

Introduction

Chlorophyll fluorescence provides a complex signal of photosynthesizing organisms, giving information on various aspects of the photosynthetic process (for review see Briantais *et al.*, 1986; Renger and Schreiber, 1986; Krause and Weis, 1991; Schreiber *et al.*, 1994). In the past, fluorescence measurements have been mostly applied to the study of higher plant photosynthesis, using intact leaves or isolated chloroplasts, in which case signal amplitudes are rather large. In order to apply the "light-doubling method" for quenching analysis (Bradbury and Baker, 1981) it had been of primary concern to develop selective modulation fluorometers, which allow the measurement of fluorescence *yield* against a large background of non-modulated signals (Schreiber, 1984; Quick and Horton, 1984; Dietz *et al.*, 1985; Ögren and Baker, 1985; Schreiber *et al.*, 1986). The so-called pulse-amplitude-modulation (PAM) measuring

Abbreviations: Chl, chlorophyll; LED, light-emitting diode; Fo, minimal fluorescence yield of dark-adapted sample; Fm, maximal fluorescence yield of dark-adapted sample; Fm′, maximal fluorescence yield of illuminated sample; Fv, variable fluorescence yield Fm–Fo; ΔF, increase of fluorescence yield upon rapid induction of Fm′ by pulse of saturating light.

Reprint requests to Dr. U. Schreiber.
Telefax: (0931) 71446.

0939–5075/94/0900–0646 $ 06.00

principle (Schreiber, 1986) has proven particularly useful, as it combines high sensitivity, selectivity and time resolution. The presently available commercial instruments (see *e.g.* Bolhar-Nordenkampf *et al.*, 1989) were developed with emphasis on the study of photosynthesis in intact leaves and of isolated chloroplasts at relatively high chlorophyll concentrations. Hence, it is mostly research on higher plant photosynthesis where so far remarkable progress has been made on the basis of modulation fluorometry (see *e.g.* Demmig-Adams, 1990; Krause and Weis, 1991; Walker, 1992; Schreiber and Bilger, 1993).

Recently, we have reported on a new PAM fluorometer, which uses Xe-discharge flashes for pulsed measuring light (Schreiber *et al.*, 1993) and displays exceptional sensitivity in measurements with extremely dilute suspensions, as *e.g.* phytoplankton in natural surface waters at chlorophyll concentrations in the order of $0.1-10\ \mu g \cdot l^{-1}$. The substantially increased sensitivity of this instrument with respect to the standard PAM fluorometer (Walz, Effeltrich, F.R.G.) was in part reached by optimization of optical parameters and cuvette geometry for measurements with dilute suspensions.

The present communication describes a new emitter-detector cuvette assembly for the standard PAM fluorometer which displays similar optical features and cuvette geometry as the Xe-PAM fluorometer (Schreiber *et al.*, 1993). While the detection limit of this new system does not reach that of the Xe-PAM fluorometer, it is approximately two orders of magnitude higher than that of the presently available standard system. A major advantage of the new system is that it combines the high time resolution and signal stability of the standard PAM fluorometer (100 kHz modulation frequency and LED output stability around 10^{-4}) with improved sensitivity, selectivity and flexibility in the choice of excitation and emission wavelengths. These features are particularly important for reliable photosynthesis investigations of unicellular algae with different antenna organization, for the study of which the PAM fluorometer so far had not been optimized, as recently pointed out by Ting and Owens (1992) and Büchel and Wilhelm (1993).

Features of the New Emitter-Detector-Cuvette Assembly

The main components of the new emitter-detector-cuvette assembly are displayed in the block diagram of Fig. 1. Optics and cuvette geometry are very similar to those previously described for the Xe-PAM fluorometer (Schreiber *et al.*, 1993). The

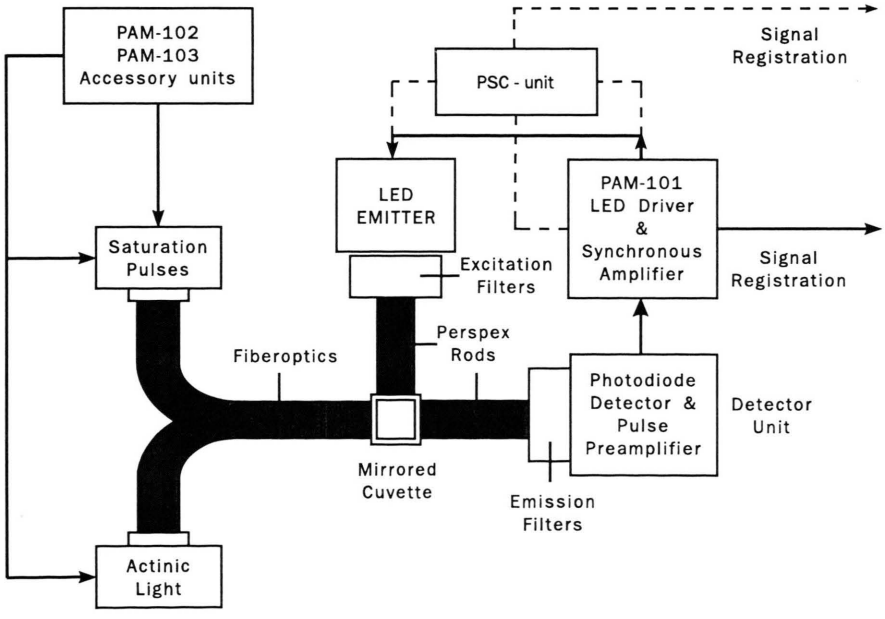

Fig. 1. Block diagram of the new emitter-detector-cuvette assembly for the standard PAM fluorometer providing flexibility in the choice of excitation and emission wavelengths. The PSC unit (pulse sequence converter) can serve for lowering the integrated measuring light intensity to 1/8 (see section below). Further details, see text.

major difference is that, as with the normal PAM fluorometer, an LED instead of a Xe-discharge lamp is used for measuring light. Therefore, the new emitter-detector unit can be connected to the standard PAM fluorometer just by exchanging the connectors for emitter and detector at the PAM-101 Main Control Unit. This has the practical advantage that the new unit is compatible with the various accessory light sources, associated with the PAM-102 and PAM-103 modules, including single- and multiple-turnover flashlamps. For maximal resolution the so-called "Auto 100 kHz" function is essential: Before actinic illumination *quasi*-dark fluorescence yield, Fo, is monitored at 1.6 kHz modulation frequency and simultaneously with the onset of actinic illumination there is an increase to 100 kHz, thus substantially improving signal/noise ratio and time resolution (for an example see Fig. 2 below).

The main differences with respect to the standard emitter-detector unit of the PAM fluorometer consist in the following features:

(1) In the emitter part various types of LED-measuring light sources and different optical filters can be readily exchanged.

(2) The optical pathways connecting emitter, detector and sample do not involve fiber optics. Instead 10×10×100 mm quartz, glass or perspex rods are used, with a 90° angle between excitation and emission pathways.

(3) A standard 10×10×45 mm quartz or glass cuvette is applied, with the option that one or two (at 90° angle) sides are mirrored. When two sides are mirrored, the fiber optics are connected to the bottom side of the cuvette.

(4) The optical filters in front of the detector are exchangeable.

(5) The multibranched fiber optics, which are *not* used for guiding measuring light to the cuvette and fluorescence to the detector, are connected either to the transparent bottom of the cuvette or to an unmirrored side window at 90° angle to the emission pathway, in order to apply various types of actinic illumination.

(6) For applications which require extremely low measuring light intensities, a special "pulse sequence converter (PSC unit) can be applied which suppresses 7/8 of the LED pulses with a corresponding decrease in integrated intensity (see section below).

Performance of the New Emitter-Detector Unit

The performance of the new emitter-detector unit can be best judged by comparison with the standard system under the special conditions it has been developed for, *i.e.* in experiments with rather dilute suspensions.

In Fig. 2 rapid induction kinetics of green unicellular algae *Dunaliella parva* at chlorophyll concentrations of 0.1 and 1 μg·ml^{-1} are shown and compared with a recording at 50 μg·ml^{-1} using the standard system with the KS-101 suspension cuvette. It is apparent that in terms of signal/noise the recording at 1 μg Chl·ml^{-1} with the new system is somewhat superior to that at 50 μg Chl·ml^{-1} with the standard system. At 1 μg Chl·ml^{-1} with the standard system the signal is

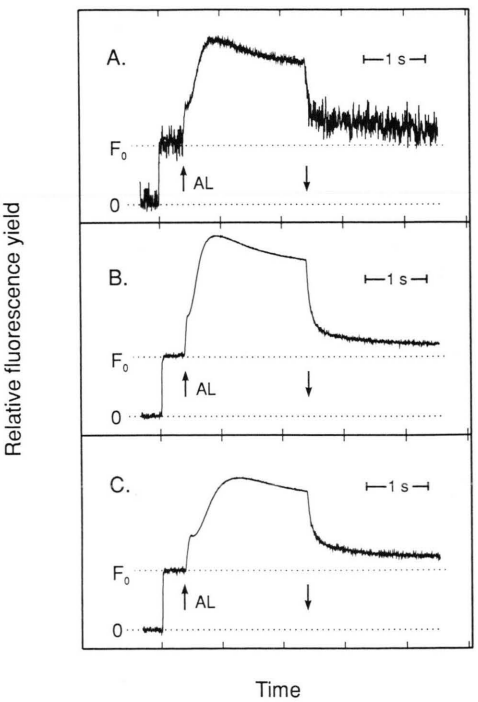

Fig. 2. Rapid induction kinetics of the green algae *Dunaliella parva* as measured with the new emitter-detector-cuvette assembly and the standard system of the PAM fluorometer. A. Dilute suspension of 0.1 μg Chl/ml; measurement with new emitter-detector unit using double-mirrored cuvette. B. Suspension of 1 μg Chl/ml; new emitter-detector-cuvette assembly. C. Dense suspension of 50 μg Chl/ml; standard emitter-detector unit (ED-101, Walz) in conjunction with suspension cuvette KS-101. Actinic light (AL), 650 nm from a light-emitting diode source (H-3000 array) providing 360 μmol quanta/m^2 s in the case of experiments A–B and 155 μmol quanta/m^2 s in experiment C (due to intensity loss caused by fiber optics).

dominated by noise, such that an evaluation of fluorescence information is not possible (not shown). On the other hand, even at 0.1 µg Chl · ml^{-1} the induction curves measured with the new system are quite satisfactory. In principle, further signal/noise improvement could be achieved by signal averaging.

When a suspension of *Dunaliella parva* at 200 µg Chl · l^{-1} was stepwise diluted with growth medium and at each chlorophyll concentration the maximal fluorescence yield was measured by a 1 s saturating light pulse, a linear plot of the signal amplitude *vs.* chlorophyll concentration resulted (data not shown). For zero chlorophyll concentration this plot extrapolated to a background signal which corresponds to the fluorescence displayed at 8 µg Chl · l^{-1}. Under identical conditions the signal amplitude observed with the cuvette containing distilled water was only half of that found with the growth medium, *i.e.* equivalent to maximal fluorescence at 4 µg Chl · l^{-1}. For comparison, the standard emitter-detector unit in conjunction with the fiber optics and the KS-101 suspension cuvette displays a background signal corresponding to Fm at 2000 µg Chl · l^{-1} when the cuvette is filled with water. This signal is due to detector filter fluorescence excited by measuring light reflected from the mirrored bottom of the cuvette and also to reflected measuring light passing the detector filter. Both components become strongly reduced at elevated chlorophyll concentrations when the measuring light is effectively absorbed.

At a given signal/noise ratio the noise will decrease with the signal. Therefore, the relatively low background signal observed with the new emitter-detector-cuvette assembly favours a low limit for determination of *active* chlorophyll, which may be defined as the chlorophyll concentration at which starting at a *quasi*-dark level, Fo, the signal change by saturation pulse-induced reaction centre closure amounts to twice the noise level, optimized by appropriate electronic damping. The thus-defined limit depends to some extent on the maximal measuring light intensity which can be applied without substantial center closure and, hence, also varies with the quantum capture efficiency and physiological state of the investigated sample. Using dark-adapted spinach chloroplasts, with the new system accurate measurements of Fv/Fm were possible down to approximately 1 nM chlorophyll, which compares with 20 pM chlorophyll when the Xe-PAM fluorometer is used (Schreiber *et al.*, 1993). It should be noted that the detection limits cited in the literature for other fluorometers normally apply for overall chlorophyll, irrespective of whether this is active or inactive. Furthermore, only with determination of active chlorophyll a clear-cut distinction from other fluorescing compounds, *e.g.* in natural surface waters, is possible.

In recent years, *semi*-quantitative methods have been developed for estimation of photosynthetic activity from fluorescence measurements on the basis of fluorescence quenching analysis (Schreiber *et al.*, 1986; Weis and Berry, 1987; Genty *et al.*, 1989). The empirical parameter ΔF/Fm′, which is a measure of effective PS II quantum yield, has proven particularly useful in applied research, as its determination does not require knowledge of Fo (Genty *et al.*, 1989). Hence, an increase of fluorescence beyond Fo by maximal measuring light intensity is of no concern for ΔF/Fm′ determination when assessment is during steady-state illumination and 100 kHz measuring pulse frequency can be applied, with the advantage of a high signal/noise ratio. Fig. 3 shows the responses of a very dilute suspension of *Ankistrodesmus braunii*

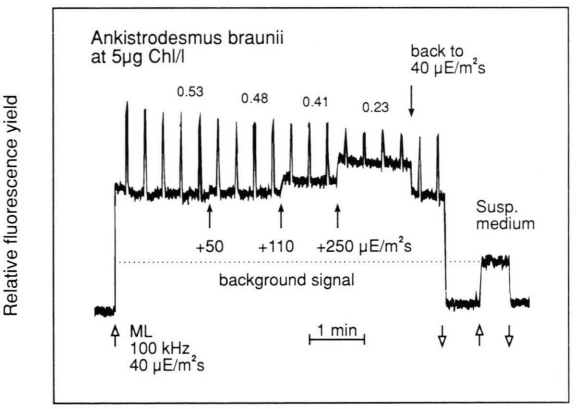

Fig. 3. Fluorescence response of a very dilute suspension of *Ankistrodesmus braunii* at 5 µg Chl/l using maximal measuring light (ML) intensity and 100 kHz pulse frequency with the new emitter-detector-cuvette assembly. Additional actinic light (650 nm) was applied at the indicated intensities during the course of the recording. Saturating light pulses (2 s duration) were applied for assessment of ΔF/Fm′. The signal obtained with pure suspension medium is also shown. This has to be subtracted for evaluation of Fm′.

($5\ \mu g \cdot l^{-1}$) at maximal measuring light intensity (integrated intensity of 40 μmol quanta/m^2 s of incident light). The recording is first without additional actinic illumination and then additional actinic light is added stepwise, yielding overall intensities of 90, 200 and 450 μmol quanta/m^2 s. Even at the given low chlorophyll content, it is possible to assess the fluorescence increase induced by a saturation pulse with relatively high accuracy. For correct calculation of ΔF/Fm′ the background signal obtained with pure medium has to be subtracted from Fm′. As expected, the ΔF/Fm′ value decreases with increasing quantum flux density. The product of ΔF/Fm′×PAR constitutes a relative measure of photosynthetic electron transport rate (Genty *et al.*, 1989; Seaton and Walker, 1990; Oberhuber *et al.*, 1993; Hormann *et al.*, 1994). Hence, plots of ΔF/Fm′×PAR *versus* PAR are equivalent to light-saturation curves of photosynthesis, which provide valuable information on the capacity and adaptational state of the organisms (for an example with unicellular algae and phytoplankton, see Schreiber *et al.*, 1993).

Various Factors Contributing to Improved Performance

In the preceding section it was shown that the new emitter-detector-cuvette assembly is substantially improved with respect to the standard system, in terms of a higher signal/noise ratio, a lower background signal and a lower detection limit. Notably, the improvements dealt with so far have been achieved exclusively by changes in optical features and cuvette geometry. The question arises what are the relative contributions of the various factors which led to such improved performance. To answer this question the fluorescence intensity of an ethanolic chloroplast extract ($5\ \mu g\ Chl \cdot ml^{-1}$) was measured with the same PAM fluorometer using either the standard emitter-detector unit (ED-101) with the four-armed fiber optics (101-F) and the suspension cuvette (KS-101) or the new emitter-detector-cuvette assembly with a cuvette mirrored at two sides (90° angle). In order to facilitate direct comparison of signal levels, two LEDs were selected for the two emitter units to give in combination with the short-pass filters identical measuring light intensities. It was also assured that the sensitivities of the detector-preamplifier units were identical. It was found that the amplitude of the signal measured in the new assembly is 63 times larger than the signal in the standard system, which in the past has been mostly used for studies using isolated chloroplasts at chlorophyll concentrations around $30-70\ \mu g \cdot ml^{-1}$ (see *e.g.* Neubauer and Schreiber, 1989; Hormann *et al.*, 1994).

The overall signal improvement can be explained by three different factors, namely increased guidance of measuring light from emitter to cuvette, enhanced conductance of fluorescence from cuvette to detector, and favorable cuvette properties, involving features like volume, internal reflection and absorption. These three factors

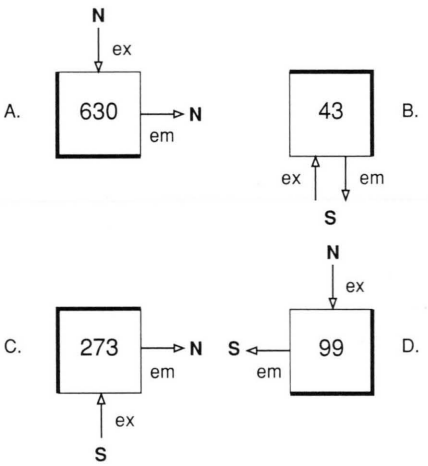

Fig. 4. Comparison of fluorescence signals obtained with an ethanolic chlorophyll extract using the standard PAM-101 Main Control Unit with different arrangements of optical components with respect to the sample contained in a 10×10×45 mm cuvette mirrored at two sides. N, component of the *new* assembly, involving a perspex rod guiding excitation light (ex) from the LED-measuring light source to the cuvette or emission (em) from the cuvette to the detector. S, component of the *standard* assembly, involving fiber optics. The broad-lined walls indicate position of the mirrored walls of the cuvette. The numbers indicate the measured fluorescence signal in a given configuration. A. New emitter-detector-cuvette assembly. B. Standard fiber optics interfacing double-mirrored cuvette; assessment of cuvette factor. C. Excitation *via* fiber optics and perspex rod in detection path; assessment of excitation pathway factor. D. Quartz rod in excitation pathway and fiber optics for guiding fluorescence to detector; assessment of emission pathway factor. For comparison, the standard emitter-detector unit ED-101 (Walz) in combination with the standard KS-101 suspension cuvette (Walz) showed a signal of 10 units. See text for further details.

were quantified as schematically depicted in Fig. 4. The same PAM fluorometer was used, varying the connected emitters and/or detectors of the standard (S) and the new (N) assemblies. The standard assembly involves guidance of measuring light or fluorescence by the fiber optics (101-F4, Walz), while perspex rods are used in the new assembly. The ethanolic chlorophyll solution was contained in a quartz cuvette with two sides mirrored at right angle. Quantification of the three "stimulation factors" involves fluorescence measurements in four different positions and emitter-detector combinations. The numbers refer to the measured signal amplitudes. A relative amplitude of 10 corresponds to the signal measured with the PAM fluorometer using the standard suspension cuvette (KS-101, Walz). On the basis of these measurements the following "stimulation factors" are obtained: 1) overall stimulation, f = 63; 2) excitation pathway, f_1 = 2.3, by comparison of positions A and C; 3) emission pathway, f_2 = 6.3, by comparison of positions A and D; 4) cuvette properties, f_3 = 4.3, by comparison of signal in position B with that obtained using the standard suspension cuvette.

From a practical point of view, these three "stimulation factors" carry different weights. Factor f_2 (improved fluorescence collection) is not only the largest but also the most valuable, as it applies under all conditions using the new emitter-detector-cuvette assembly. Factor f_1 (increased measuring light intensity) will be less useful in cases where low measuring light intensity is important (*e.g.* determinations of Fo or maximal Fv/Fm of dark-adapted samples) unless the integrated measuring light intensity is lowered by additional system modifications, which cause a lowering in the frequency of the LED pulses (see section on pulse sequence conversion below). Factor f_3 (cuvette properties) depends on the type of 10×10 mm cuvette used in the new assembly. It drops to a value of 2.5 with an unmirrored cuvette and a factor of 3.4 with only one mirrored side (opposite to detection pathway).

It has been pointed out by Ting and Owens (1992) and Büchel and Wilhelm (1993) that fluorescence measurements with the standard PAM fluorometer can be problematic when photosynthesizing organisms with highly effective antenna systems are investigated, such that measuring light intensity must be very low in order to avoid an actinic effect. As shown above, in principle part of this problem can be overcome by rather simple and straightforward modifications in the arrangement of the optical components. Even with the standard emitter-detector unit, already by connecting the fiber optics to a double-mirrored cuvette the signal can be increased by a factor of 4.3. And if a perspex rod is used instead of the fiber optics to carry the fluorescence from the cuvette to the detector, the additional stimulation factor f_2 = 6.3 applies, giving an overal signal increase of 27. This can be taken advantage of to measure at an increased signal/noise ratio or to lower the measuring light intensity, in order to avoid an actinic effect.

Pulse & Signal Converter for Low Measuring Light Applications

The highest signal/noise ratio and, hence, the lowest detection limit is obtained at maximal measuring light intensities. Actually, the noise amplitude at the output of the PAM fluorometer is not changed when measuring light is turned on/off and is independent of signal amplitude. Hence, as fluorescence intensity is a linear function of measuring light intensity, the signal/noise ratio is proportional to measuring light intensity. Unfortunately, for the physiological reasons outlined above, in practice use of maximal measuring light intensity is not always possible, in particular when detection of the dark fluorescence level, Fo, is essential. In these cases also the stimulation factor f_1 = 2.3 obtained with the new emitter-detector-cuvette assembly (see Fig. 4) cannot be taken advantage of. However, there is the possibility to overcome this limitation by lowering the frequency with which measuring light pulses are applied. Obviously, the intensity of an individual pulse can remain high, while at the same time the quantum flux density integrated over a longer time period can be lowered by decreasing pulse frequency.

The Main Control Unit of the PAM fluorometer provides only two fixed pulse frequencies (1.6 and 100 kHz). Therefore a special "pulse & signal converter" (PSC) was developed, interfacing the new emitter-detector unit and the PAM fluorometer. The continuous current pulses originating at the

emitter output at the PAM-101 unit are converted in the PSC unit into 5 ms "packages" of pulses (8 pulses at 1.6 kHz or 500 pulses at 100 kHz) separated by 40 ms dark time. This leads to a decrease of integrated measuring light intensity by a factor of 8. As a consequence of the periodic measuring light application, at the output of the PAM-101 a pulse-signal originates which displays a frequency of 25 Hz which is transformed into a continuous signal in the PSC unit.

In Fig. 5 two recordings of the "*quasi*-dark" fluorescence level, Fo, and of slow induction kinetics of *Dunaliella parva* at a chlorophyll concentration of 100 $\mu g \cdot l^{-1}$ are displayed. Curve A was recorded at maximal measuring light intensity (setting 12 at PAM-101) without application of the PSC unit and curve B was measured under identical conditions, except that the integrated measuring light intensity was decreased by a factor of 8 by application of the PSC unit. On first sight, the two recordings appear identical, which is indeed almost true for the variable part of fluorescence. There is, however, a distinct difference in the fluorescence yield before application of a saturation pulse. Obviously, at the given measuring light intensity (0.7 μmol quanta/m^2 s) a special fraction of PS II reaction centers closes, while the remainder is not affected. Very recently it was shown in detailed experiments with isolated chloroplasts that the closure of these centers is accompanied by a considerable decrease in the quantum yield of PS II driven electron transport (Hormann *et al.*, 1994). During actinic illumination at physiologically relevant quantum flux densities these centers will be generally closed and the observed values of $\Delta F/Fm'$ (empirical index of effective PS II quantum yield) will be independent of measuring light intensities applied with the PAM fluorometer, *i.e.* also unaltered by the use of the PSC unit. In the example of Fig. 5, $\Delta F/Fm' = 0.32$ during steady-state illumination, with and without PSC unit.

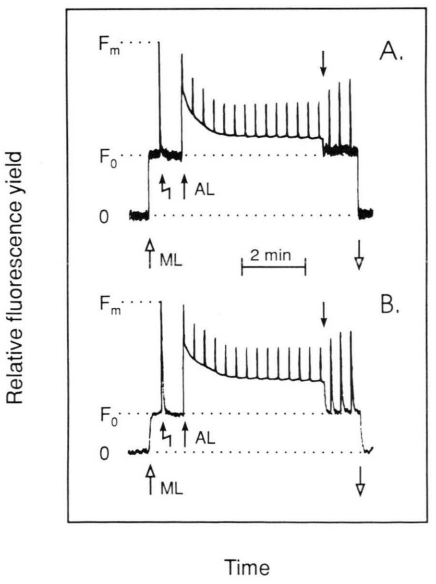

Fig. 5. Dark-light induction curves of *Dunaliella parva* at 0.1 μg Chl/ml using a relatively high measuring light intensity without and with application of the pulse-sequence-converter (PSC) unit. A. Without PSC unit at an integrated measuring light intensity of 0.7 μmol quanta/m^2 s. B. With PSC unit at an integrated measuring light intensity of 0.08 μmol quanta/m^2 s. In both cases 650 nm excitation and the double-mirrored cuvette were used. Actinic intensity, 85 μmol quanta/m^2 s of 650 nm.

It may be concluded that the PSC unit can be useful in experiments which involve organisms with high quantum capture efficiency, for which the dark fluorescence, Fo, and maximal PS II quantum efficiency (estimated from the empirical index Fv/Fm) shall be determined. This improvement is achieved at the cost of time resolution which is substantially decreased due to the low pulse frequency and strong damping in the sample & hold circuit. Hence, using the PSC unit only slow fluorescence changes can be recorded, with a response time of 0.3 s (70% of total signal change) which is just sufficient to determine Fm with a 1 s saturation pulse.

Different Excitation and Emission Wavelengths

In the standard emitter-detector unit of the PAM fluorometer the components determining excitation/emission characteristics are fixed for optimal performance in measurements using leaves and isolated chloroplasts. For this purpose fluorescence is excited by a red LED (peak 650 nm, Type H-3000, Stanley, Japan) the longer wavelength emission of which is eliminated by a short-pass filter ($\lambda < 680$ nm, Type DT-Cyan special, Balzers, Liechtenstein). The photodetector, which is protected by a long-pass filter ($\lambda >$ 710 nm, Type RG 9, Schott, Germany), consists of a PIN photodiode (Type S 1723, Hamamatsu,

Japan) with maximal sensitivity in the near-infrared region (700–1000 nm). The same components have also proven best suited to achieve maximal sensitivity with the new emitter-detector-cuvette assembly, not only using chloroplasts and green algae, but as well algae with different pigment antenna and cyanobacteria. This is true despite of the fact that other wavelengths may be more strongly absorbed than 650 nm, as *e.g.* 620–580 nm light in cyanobacteria, because the 650–670 nm LEDs have a considerably higher light output than LEDs with shorter emission peaks. This is apparent from Table I (upper part) where the relative fluorescence intensities of the green alga *Ankistrodesmus braunii* and the cyanobacterium *Synechococcus leopoliensis* (strain B 1402-1) are shown for fluorescence excitation by 5 separate LEDs displaying different emission wavelengths. The applied LEDs belong to the strongest presently available types at the given wavelengths. In this comparison fluorescence emission at wavelengths above 710 nm was measured. The measured fluorescence intensities reflect the intensity of LED emission as well as the efficiency of light absorption by the fluorescent PS II. It is apparent, that with the present LED technology there is an intensity gap in the green region, where only very weak fluorescence signals can be excited. On the other hand, in the blue region very recently a rather strong LED has become available, which can induce almost half of the fluorescence found with 650 nm excitation using green algae. With cyanobacteria blue light induces only weak fluorescence, as only few of this light is absorbed by the fluorescent PS II (see Fig. 6 below).

When shorter wavelength excitation is used, it is possible to measure not only long wavelength fluorescence but the entire emission range starting from 650 nm. This results in an increased signal amplitude. In Table I (lower part) the signal amplitudes of F > 650 nm, F > 710 nm and 710 > F > 650 nm with blue excitation are compared using dilute samples of *Synechococcus* and *Ankistrodesmus*. The gain by including short-wavelength fluorescence (approx. factor of 3) makes up for the loss in available measuring light intensity when a 450 nm LED is used instead of a 650 nm LED. In addition, there is the important advantage that short-wavelength fluorescence can be measured which is enriched in PS II fluorescence (Kyle *et al.*, 1983). It has been reported by Genty *et al.* (1990) that the ratio Fv/Fm, which is an empirical index of PS II quantum yield (Kitajima and Butler, 1975) is higher when measured below 700 nm.

Table I. Comparison of fluorescence signals from *Ankistrodesmus braunii* and *Synechococcus leopoliensis* with different wavelengths of excitation and emission. Dilute suspensions containing approximately 0.5 μg Chl/ml were used and the *quasi*-dark level fluorescence yield, Fo, was evaluated. All light-emitting diodes were operated at the same pulse current. Maximal signals were measured with 650 nm excitation (Stanley H-3000 LED), where integrated measuring light intensity amounted to 0.02 μmol quanta/m^2 s, and normalized to 100. See legend of Fig. 7 for details on light-emitting diodes and excitation filters. The different emission ranges were selected by the following filters: F > 650 nm, Schott RG645 2 mm; F > 710 nm, Schott RG9 1 mm; 710 nm > F > 650 nm, Balzers Calflex-X special and Schott RG645 2 mm.

Species	Relative fluorescence intensity (F > 710 nm) using light-emitting diodes with different peak wavelengths for excitation				
	650 nm	620 nm	590 nm	560 nm	450 nm
Ankistrodesmus	100	12.2	9.8	0.46	42.7
Synechococcus	100	30.0	25.9	0.86	3.2

Species	Relative fluorescence intensity with blue excitation (peak 450 nm) in dependence of different ranges of emission wavelengths selected by filters		
	F > 650 nm	F > 710 nm	710 nm > F > 650 nm
Ankistrodesmus	320	100	73
Synechococcus	286	100	73

Table II shows Fv/Fm ratios of F > 650 nm, F > 710 nm and 710 > F > 650 nm for *Ankistrodesmus braunii*, a spinach leaf and a maize leaf. In the latter cases, 10×10 mm leaf segments were placed diagonally into the cuvette holder, thus forming 90° angles with respect to excitation and emission pathways. Blue measuring light was used. For F > 710 nm also the ratios measured with 650 nm excitation (as with standard PAM detector unit) are given. The observed differences in Fv/Fm are somewhat smaller than reported by Genty *et al.* (1990), although the maximal values of Fv/Fm are even higher. It may be mentioned, that due to the special cuvette geometry and filter selection (see above) in the given examples there is practically no stray light signal when the cuvette is filled with suspension medium alone or empty. This feature favours particularly high Fv/Fm values.

Table II. Comparison of Fv/Fm values obtained with algae and leaf samples monitoring different ranges of fluorescence emission wavelengths with blue or red excitation. The *Ankistrodesmus* suspension contained 2 μg Chl/ml. See legend of Table I for details on filters used for selection of different emission ranges. For further details, see text.

Specimen	F > 650 exc. 450	F > 710 exc. 450	F > 710 exc. 650	710 > F > 650 exc. 450
Ankistrodesmus	0.80	0.79	0.80	0.81
Spinach leaf	0.85	0.84	0.84	0.88
Maize leaf	0.82	0.82	0.84	0.88

Differential Excitation of Cyanobacteria and Green Algae Fluorescence

The new emitter-detector unit may be envisaged to find application in the study of phytoplankton in natural waters like lakes and rivers, where chlorophyll concentrations of $5-100\ \mu g \cdot l^{-1}$ are not uncommon. As shown above, at these low concentrations valuable information on chlorophyll content and photochemical activity can be gained by quenching analysis with the saturation pulse method. A straightforward quantification, however, is complicated by the fact, that the natural phytoplankton consists of a mixture of different algal groups, including chlorophyceae, cyanophyceae, xanthophyceae, cryptophyceae and phaeophyceae. One possibility to distinguish the fluorescence contributions from these various components is based on differences in antenna composition and organization which cause differences in fluorescence excitation spectra (Fork and Mohanty, 1986; Govindjee and Satoh, 1986). These differences are particularly pronounced when cyanobacteria and green algae are compared. Fig. 6 shows dark-light induction curves of *Synechococcus* and *Ankistrodesmus* measured with excitation by a 620 nm LED and a 450 nm LED. Identical photon flux densities (0.1 μmol quanta/m^2 s) were used, as measured with a Licor Microquantum Sensor. It is apparent that with 620 nm excitation the cyanobacteria display a relatively high fluorescence yield, whereas the green algae show a low signal. The proportions are turned around when 450 nm excitation is applied. Then fluorescence yield of *Synechococcus* is very low, while *Ankistrodesmus* shows a high yield. This can be readily explained by the fact that fluorescence emission, and variable fluorescence in particular, at room temperature primarily originates from PS II and that in cyanobacteria contrary to green algae, the phycobiliproteins and not Chl *a/b* proteins constitute the major light-harvesting antenna of PS II. At 620 nm there is a peak in phycocyanin absorbance, which is low in the blue region. On the other hand, chlorophyll absorbance is quite low at 620 nm and high in the blue region.

In Fig. 7 coarse excitation spectra are presented for *Synechococcus* and *Ankistrodesmus braunii* with the differently coloured LEDs serving as broad band "monochromatic" excitation sources. The photon flux densities of photosynthetic active

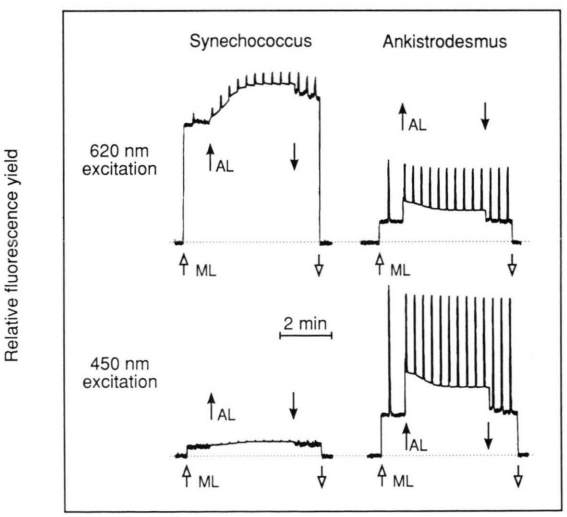

Fig. 6. Dark-light induction curves of *Synechococcus leopoliensis* and *Ankistrodesmus braunii* measured with excitation by light-emitting diodes with peak emission at 620 nm and 450 nm. Identical photon flux densities (0.1 μmol quanta/m^2 s) were used for excitation with the two wavelength ranges. For details on the types of light-emitting diodes and filters, see legend to Fig. 7. Measuring light, ML. Actinic light, AL, was 150 μmol quanta/m^2 s at 650 nm. The cells were harvested during the logarithmic phase of their growth, and diluted to 0.5 μg Chl/ml by growth medium.

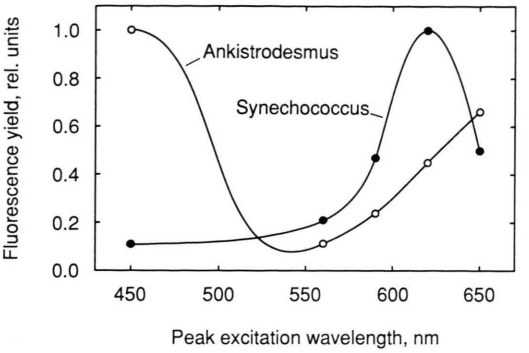

Fig. 7. Relative fluorescence yield of the green algae *Ankistrodesmus braunii* and the cyanobacteria *Synechococcus leopoliensis* with broad band excitation using differently colored light-emitting diodes. The data points correspond to the following light-emitting diodes + filters with the corresponding peak wavelengths: 650 nm, Stanley H-3000 + Balzers DT Cyan special short-pass filter $\lambda < 680$ nm; 620 nm, Hewlett-Packard DG00 + Balzers DT Cyan special short-pass filter $\lambda < 650$ nm + Balzers Calflex-X special short-pass filter $\lambda < 770$ nm; 590 nm, Hewlett-Packard BL00 + Balzers Calflex-X special short-pass filter $\lambda < 770$ nm; 560 nm, Hewlett-Packard HLMP 3950 + Balzers DT Green dichroic filter; 450 nm, Ledtronics L 200 CWB1K + Balzers DT Cyan dichroic filter. Measuring light intensity was in all cases adjusted to the same quantum flux density of 0.1 μE/m^2 s. Fluorescence was measured at $\lambda > 710$ nm using a 1 mm RG9 filter (Schott) in front of the detector. The *quasi*-dark level, Fo, was assessed.

radiation were adjusted to the same values. The indicated wavelengths correspond to the peaks of LED emission. On the basis of such information the relative contribution of *Synechococcus* and *Ankistrodesmus* in mixtures of the two can be calculated. Quantification becomes more complicated when mixtures including the other algal groups are given. In principle, however, as long as the relevant components and their excitation spectra are known, it should be possible to deconvolute their relative contributions by measurements of fluorescence yield with excitation at a number of different wavelengths. For this purpose computer assisted deconvolution methods, as *e.g.* developed for quantification of cytochrome absorbance changes *in vivo* (Klughammer *et al.*, 1990) should be useful. Such work presently is in progress in our laboratory.

Conclusions

With the new emitter-detector-cuvette assembly described in this communication, the range of applications of the standard PAM fluorometer can be considerably extended. In particular, the following features may be emphasized:

1) Signal amplitude can be increased by more than a factor of 60 due to very efficient optical coupling of LED-measuring light source, suspension cuvette with mirrored walls and photodetector.

2) LED-measuring light sources with different emission peaks can be used in combination with appropriate filters for selective excitation of different antenna systems.

3) Using short-wavelength LEDs the short-wavelength fluorescence can be measured, which is primarily originating in PS II and displaying high Fv/Fm values.

4) As the new unit is operated in conjunction with the standard PAM fluorometer, the great stability and time resolution of this system can be taken advantage of. This is particularly useful for rapid kinetic measurements.

5) Due to the substantially increased sensitivity, measurements can be routinely carried out at low chlorophyll concentrations, where light gradient formation and fluorescence reabsorption in the cuvette become negligibly small. This is *e.g.* important for the study of PS II heterogeneity.

6) In principle, with the same system a number of other possible applications besides chlorophyll fluorescence measurements can be envisaged. These include recording of 9-aminoacridine fluorescence (or of other fluorescent amines) to assess transthylakoidal proton gradients and measurements of light scattering. In particular, by a small modification (moving the detector pathway to be in line with the emitter pathway) it is possible to measure also absorbance changes by intrinsic components (like P 515 or zeaxanthin) and of added substances (like Cyt *c* or methyl purple) with high time resolution and high signal/noise ratio. This will be reported on in a separate publication (Schreiber, in preparation).

Acknowledgements

I wish to thank Ulrich Schliwa, Horst Kobold, Heinz Reising and Henning Hormann for help and technical assistance. This work was supported by the Deutsche Forschungsgemeinschaft (SFB 176 and SFB 251).

Bolhar-Nordenkampf H. R., Long S. P., Baker N. R., Öquist G., Schreiber U. and Lechner E. (1989), Chlorophyll fluorescence as a probe of the photosynthetic competence of leaves in the field: A review of current instrumentation. Functional Ecology **3,** 497–514.

Bradbury M. and Baker N. R. (1981), Analysis of the slow phase 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 **63,** 542–551.

Briantais J. M., Vernotte C., Krause G. H. and Weis E. (1986), Chlorophyll *a* fluorescence of higher plants, chloroplasts and leaves, in: Light Emission by Plants and Bacteria (Govindjee, J. Amesz and D. C. Fork, eds.). Academic Press, New York, pp. 539–583.

Büchel C. and Wilhelm C. (1993), *In vivo* analysis of slow chlorophyll fluorescence induction kinetics in algae: Progress, problems and perspectives. Photochem. Photobiol. **58,** 137–148.

Demmig-Adams B. (1990), Carotenoids and photoprotection in plants: A possible role for the xanthophyll zeaxanthin. Biochim. Biophys. Acta **1020,** 1–24.

Dietz K. J., 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.

Fork D. C. and Mohanty P. (1986), Fluorescence and other characteristics of blue-green algae (cyanobacteria), red algae and cryptomonads, in: Light Emission by Plants and Bacteria (Govindjee, J. Amesz and D. C. Fork, eds.). Academic Press, Orlando, pp. 451–496.

Genty B., Briantais J. M. and Baker N. R. (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 N. R. (1990), Non-photochemical quenching of Fo in leaves is emission wavelength-dependent: consequences for quenching analysis and its interpretation. Photosynth. Res. **26,** 133–139.

Govindjee and Satoh K. (1986), Fluorescence properties of chlorophyll *b*- and chlorophyll *c*-containing algae, in: Light Emission by Plants and Bacteria (Govindjee, J. Amesz and D. C. Fork, eds.). Academic Press, Orlando, pp. 497–537.

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.

Kitajima M. and Butler W. L. (1975), Quenching of chlorophyll fluorescence and primary photochemistry in chloroplasts by dibromothymoquinone. Biochim. Biophys. Acta **376,** 105–115.

Klughammer C., Kolbowski J. and Schreiber U. (1990), LED Array Spectrophotometer for measurement of time-resolved difference spectra. Photosynth. Res. **25,** 317–327.

Krause G. H. and Weis E. (1991), Chlorophyll fluorescence and photosynthesis: the basics. Annu. Rev. Plant Physiol. Plant Mol. Biol. **42,** 313–319.

Kyle D. J., Baker N. R. and Arntzen C. J. (1983), Spectral characterization of photosystem I fluorescence at room temperature using thylakoid protein phosphorylation. Photochem. Photophys. **5,** 79–85.

Neubauer C. and Schreiber U. (1989), Photochemical and non-photochemical quenching of chlorophyll fluorescence induced by hydrogen peroxide. Z. Naturforsch. **44c,** 262–270.

Oberhuber W., Dai Z.-Y. and Edwards G. E. (1993), Light dependence of quantum yields of photosystem II and CO_2 fixation in C_3 and C_4 plants. Photosynth. Res. **35,** 265–274.

Ögren E. and Baker N. R. (1985), Evaluation of a technique for the measurement of chlorophyll fluorescence from leaves exposed to continuous white light. Plant Cell Environ. **8,** 539–547.

Quick W. P. and Horton P. (1984), Studies on the induction of chlorophyll fluorescence in barley protoplasts. II. Resolution of fluorescence quenching by redox state and the transthylakoid pH gradient. Proc. R. Soc. Lond. B. **220,** 371–382.

Renger G. and Schreiber U. (1986), Practical applications of fluorometric methods to algae and higher plant research, in: Light Emission by Plants and Bacteria (Govindjee, J. Amesz and D. C. Fork, eds.). Academic Press, New York, pp. 587–619.

Schreiber U. (1984), Chlorophyll fluorescence yield changes as a tool in plant physiology. The measuring system. Photosynth. Res. **4,** 361–373.

Schreiber U., Bilger W. and Neubauer C. (1994), Chlorophyll fluorescence as a non-intrusive indicator for rapid assessment of *in vivo* photosynthesis, in: Ecophysiology of Photosynthesis. Ecological Studies, **Vol. 100** (E. D. Schulze and M. M. Caldwell, eds.). Springer Verlag, Berlin, pp. 49–70.

Schreiber U. and Bilger W. (1993), Progress in chlorophyll fluorescence research: Major developments during the past years in retrospect, in: Progress in Botany, **Vol. 54** (U. Lüttge and H. Ziegler, eds.). Springer Verlag, Berlin, pp. 151–153.

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., Schliwa U. and Bilger W. (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. (1986), Detection of rapid induction kinetics with a new type of high-frequency modulated chlorophyll fluorometer. Photosynth. Res. **9,** 261–272.

Seaton G. G. R. and Walker D. A. (1990), Chlorophyll fluorescence as a measure of photosynthetic carbon assimilation. Proc. R. Soc. Lond. B. **242,** 29–35.

Ting C. S. and Owens T. G. (1992), Limitation of the pulse-modulated technique for measuring the fluorescence characteristics of algae. Plant Physiol. **100,** 367–373.

Walker D. A. (1992), Excited leaves. New Phytol. **121,** 325-345.

Weis E. and Berry J. (1987), Quantum efficiency of photosystem II in relation to energy-dependent quenching of chlorophyll fluorescence. Biochim. Biophys. Acta **894,** 198–208.