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Technical communication

PAM fluorometer based on medium-frequency pulsed Xe-flash measuring light: A highly sensitive new tool in basic and applied photosynthesis research

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Abstract

A newly developed modulation fluorometer is described which employs repetitive 1 μs Xe-flashes for excitation light. Similar to the standard PAM Chlorophyll Fluorometer, which uses 1 μs LED pulses for measuring light, the integrated measuring light intensity is sufficiently low to monitor the dark-fluorescence level, F_o. The maximal fluorescence yield, F_m, can be determined with high selectivity upon application of a saturating light pulse. The Xe-PAM displays exceptionally high sensitivity, enabling quenching analysis at chlorophyll concentrations as low as 1 μg/l, thus allowing to assess photosynthesis of phytoplankton in natural waters like lakes, rivers and oceans. Due to high flexibility in the choice of excitation and emission wavelengths, this system also provides the experimental basis for a thorough study of fluorescence and photosynthesis properties of various algae classes with differing antenna organisation. By appropriate modifications, the instrument may as well be used to measure with great sensitivity and selectivity other types of fluorescence (e.g. NADPH-fluorescence), as well as light-scattering and absorbance changes.

Introduction

In recent years there has been considerable progress in instrumentation and methodology for the study of photosynthesis by chlorophyll fluorescence measurements (for reviews see Horton and Bowyer 1990, Demmig-Adams 1990, Krause and Weis 1991, Walker 1992, Schreiber and Bilger 1993, Schreiber et al. 1993). On the basis of the original 'light-doubling' method (Bradbury and Baker 1981) the so-called 'saturation pulse method' was developed (Quick and Horton 1984, Dietz et al. 1985, Schreiber et al. 1986) which allows separation of different forms of fluorescence quenching. This has opened the way for rapid assessment of photosynthesis yield and capacity in situ by fluorescence measurements. Weis and Berry (1987) and Genty et al. (1989) first showed that the relative rate of photosynthetic electron flow can be determined from fluorescence parameters alone. In the meantime, their findings have been confirmed by numerous researchers (see e.g. Sharkey et al. (1988), Harbinson et al. (1990), Seaton and Walker 1990).

In practice, fluorescence quenching analysis by the saturation pulse method requires a special modulation technique, which allows to selectively monitor fluorescence *yield* with weak measuring light under vastly varying background light conditions. Applying high-frequency pulse-modulated light-emitting diodes (LEDs) and a

selective window amplifier, the so-called PAM Fluorometer was developed (Schreiber 1986, Schreiber et al. 1986) which tolerates non-modulated light more than 10^6 times stronger than the integrated measuring light. This system has been successfully used in many studies, in particular with intact leaves and isolated chloroplasts (for reviews, see Horton and Bowyer 1990, Walker 1992, Schreiber and Bilger 1993, Schreiber et al. 1993). On the other hand, practical use of the PAM Fluorometer for quenching analysis with algae and cyanobacteria has been rather limited. The reason for this is mainly of technical nature: The wavelength range of sufficiently strong LEDs is restricted to the red spectral region. Hence, with the presently available instrumentation, quenching analysis of organisms with different excitation and emission properties has been problematic. Furthermore, under natural conditions microalgae occur at low cell densities with chlorophyll concentrations ranging around 1–50 μg/l, which is far below the detection limit of presently available modulation fluorometers.

Here we report on a newly developed PAM Fluorometer which employs a Xe-discharge flash lamp instead of an LED as pulse modulated measuring light source. This system displays great flexibility in both excitation and emission wavelengths. Due to the superior intensity of the Xe-flashes, a very high sensitivity can be reached, which allows to monitor variable fluorescence even in extremely dilute samples, as e.g., encountered in natural waters of lakes, rivers and oceans.

Description of the new measuring system

Figure 1 shows a block diagram of the new measuring system which consists of the following essential components:

1. Bulb type Xe-flash lamp (EG&G, type FX-134), with flat front window.
2. Main control unit housing the flash driver and synchronous pulse signal amplifier (laboratory built).
3. Filterset for flash excitation light, in standard applications consisting of a 650 nm short-pass filter (Balzers DT Cyan special) and a blue glass filter (Schott BG 39).
4. 10 × 10 mm quartz rods (optical quality, Kindl GmbH) for guiding the excitation light to the cuvette and the fluorescence to the detector.
5. 10 × 10 mm cuvette (Hellma), mirrored at the side opposite to entrance of excitation light.
6. Filterset for separating fluorescence emission from stray measuring light, in standard applications consisting of a non-fluorescing

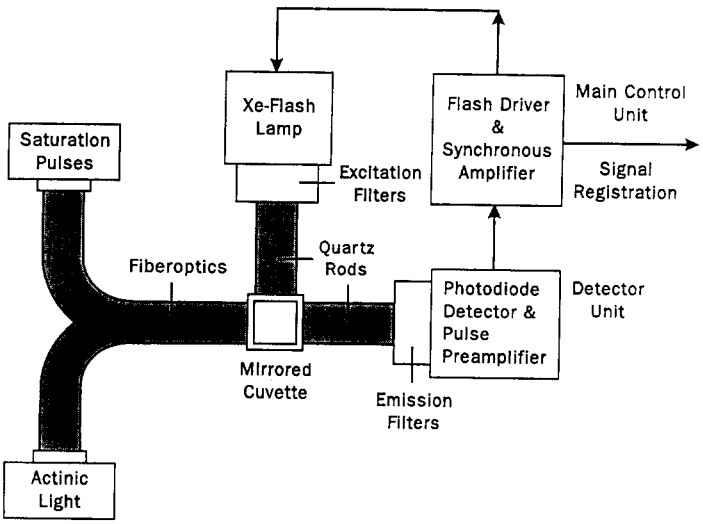

Fig. 1. Block diagram of the Xe-PAM measuring system in its standard optical configuration for measuring variable chlorophyll fluorescence with broad excitation and emission bands. See text for details.

green glass filter (Schott GG10) and a low-fluorescing red glass filter (Schott RG 645).
7. Detector unit housing a PIN-photodiode (Hamamatsu S 3590-01) and a pulse pre-amplifier (laboratory-built).
8. Branched fiberoptics (Walz) connecting to various light-sources, with the fibers being statistically mixed at the common end for homogenous sample illumination.
9. Actinic light source to drive photosynthesis, consisting of an LED lamp (Walz, type 102L in conjunction with the PAM-102 unit) or a halogen lamp fiber illuminator (Schott KL 1500).
10. Saturation pulse lamp (Walz, type FL 103, operated in conjunction with the PAM-103 unit), equipped with a 650 nm short-pass filter (Balzers DT Cyan special).

In standard applications, chlorophyll fluorescence is excited by flashes of blue-green light (ranging from 400 to 560 nm), covering the absorbance peaks of Chl *a* and Chl *b*, and chlorophyll fluorescence is detected over its whole emission range (650–800 nm). This is at variance to the normal PAM Chlorophyll Fluorometer (Schreiber 1986) which employs 650 nm LED measuring light and detects fluorescence at wavelengths above 710 nm, thus sacrifying the main emission peak around 685 nm.

Flash excitation is applied either at low (16 Hz) or at high (130 Hz) repetition rate. In the present report only applications using 16 Hz are described. Only at low frequency the integrated measuring light intensity is sufficiently low to allow measurement of the minimal fluorescence yield, F_o, and of variable fluorescence, F_v. Figure 2 shows the intensity profile of the Xe-flashes serving as pulse modulated measuring light. The peak intensity at the sample site, as measured with a calibrated PIN-photodiode, is approx. 200 times higher than the intensity of the LED pulses produced by the ED-101 emitter-unit of the normal PAM Fluorometer at maximal setting. The measuring principle of selective pulse signal amplification is similar to that employed with the normal PAM Fluorometer (Schreiber 1986). Signal detection is synchronised with Xe-flash triggering. Fluorescence and reference signals are selectively sampled during 1 μs periods during and shortly after the Xe-flashes, respectively. This provides for exceptional insensitivity to large, non-modulated background signals (see Schreiber et al. 1986).

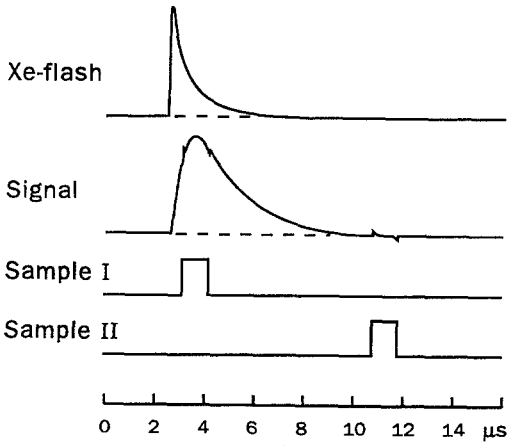

Fig. 2. Intensity profile of the Xe-flash serving as measuring light and signal pulse shape at the output of the pulse preamplifier. Flash triggering is initiated by a high-voltage ignition pulse at time zero. The flash is fired with a delay of approx. 3 μs. Two signals are sampled, sample I at the plateau of the pulse signal and sample II approx. 8 μs later, serving as a reference. The difference between sample I and sample II is registered.

System performance

In Fig. 3, fluorescence induction curves of intact spinach chloroplasts at chlorophyll concentrations of 20 μg/l and 2 μg/l are shown, as measured with the new Xe-PAM Fluorometer in the standard configuration described in Fig. 1 at a flash frequency of 16 Hz. The induction curve at 20 μg Chl/l displays all the features known from measurements with the standard PAM Chlorophyll Fluorometer at approx. 1000 times higher chlorophyll concentrations (see e.g. Neubauer and Schreiber 1989). Even after another 10-fold dilution to 2 μg Chl/l, an induction curve with quenching analysis can be measured, although now an overlapping background signal becomes apparent and the signal/noise ratio is decreased. The dotted line indicates the signal level displayed by the suspension medium (main components sorbitol and Hepes-buffer) alone. Actually, with pure H_2O the background signal is about half of that with the suspension medium (not shown).

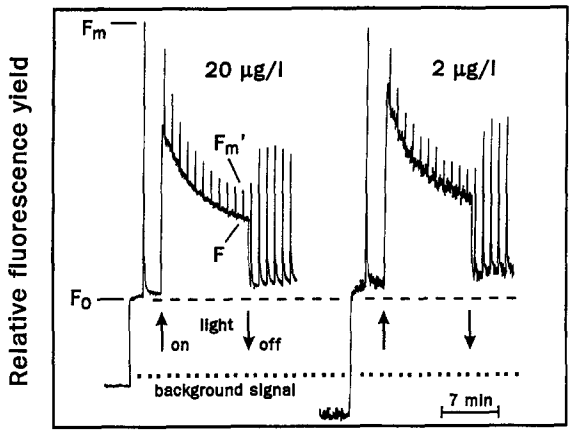

Fig. 3. Dark–light induction curves of intact spinach chloroplasts at chlorophyll concentrations of 20 μg/l and 2 μg/l, as measured with the newly developed Xe-PAM Fluorometer. Actinic red light (Schott RG 630) at an intensity of 30 $\mu E\, m^{-2}\, s^{-1}$. Saturation white light (1700 $\mu E\, m^{-2}\, s^{-1}$) was applied repetitively in pulses of 3 s duration. The minimal fluorescence yield, F_o, corresponds to the level monitored briefly after onset of measuring light. The maximal fluorescence yield, F_m, is measured by application of a saturation pulse before actinic illumination. Fluorescence yield, F, varies in the course of the induction curve. During illumination the maximal fluorescence yield, F'_m, which can be induced by a saturation pulse, is lowered with respect to F_m. It rises again upon light-off.

The complete dependence of the fluorescence signal vs. chlorophyll concentration is depicted in the double logarithmic plot of Fig. 4A. To avoid time dependent fluorescence changes, a stock chloroplast suspension at 500 μg Chl/ml was preilluminated in the presence of 5×10^{-5} M DCMU and 10 mM NH_2OH (Bennoun and Li 1973). Hence, maximal fluorescence yield, F_m, was recorded. This presentation reveals non-linearity due to fluorescence reabsorption by the chloroplasts at concentrations exceeding 5×10^{-6} M (i.e. approx. 5 μg/ml). The apparent non-linearity below 5×10^{-9} M results from the increasing relative contribution of the background signal (here: main component DCMU, which gives a 10 times higher signal than pure water). This is more clearly expressed by the linear plot in Fig. 4B which covers the range up to 10 nM. Extrapolation to zero chlorophyll reveals a background signal equivalent to the fluorescence signal (F_m) of a sample at 2.5 nM chlorophyll.

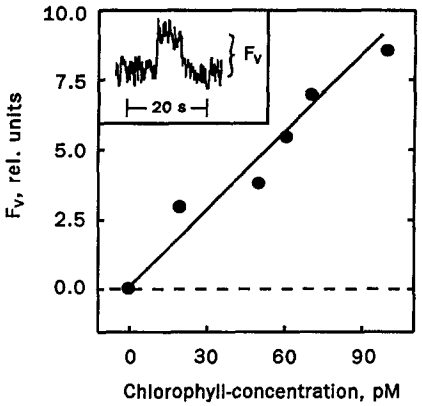

Fig. 5. Variable fluorescence of intact chloroplasts at chlorophyll concentrations approaching the detection limit with single measurements. Variable fluorescence was induced by a 5 s pulse of saturating white light. Inset: original trace at 20 pM chlorophyll (i.e. approx. 0.02 μg Chl/l).

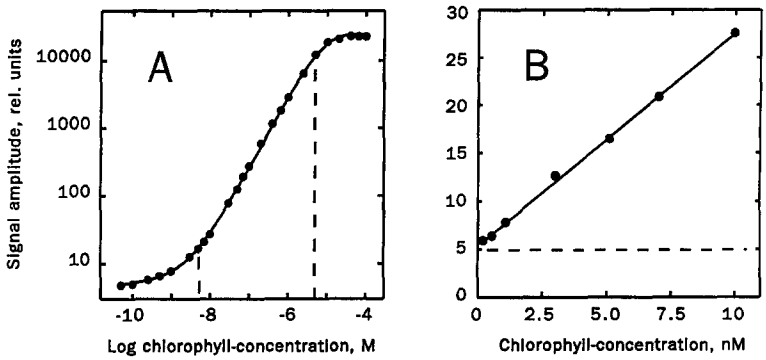

Fig. 4. Dependence of the signal amplitude on chlorophyll concentration in a suspension of intact chloroplasts inhibited by preillumination in the presence of DCMU and NH_2OH. (A) Double logarithmic plot, displaying non-linearity below approx. 5×10^{-9} M and above approx. 5×10^{-6} M (see text). (B) Linear plot for the concentration range below 10^{-8} M. The dotted line corresponds to the signal amplitude measured in the absence of chloroplasts. For further details, see text.

The existence of an unavoidable background signal puts a limit to quantitative determination of the F_o-level and, hence, of F_v/F_m or PS II quantum yield. This limitation can at least in part be overcome by determination of the background signal using the sample filtrate with an appropriate millipore filter. Whereas satisfactory quenching analysis presently requires at least 1 μg Chl/l, the detection limit of variable fluorescence (i.e. of active chlorophyll) is substantially lower. In the present configuration, without signal averaging the minimal chlorophyll concentration which can be detected by F_v-induction with a pulse of saturating light amounts to approx. 20 pM (see Fig. 5). Notably, for this determination only 1 ml suspension is required, i.e., 20 femto mol of photosynthetically active chlorophyll can still be detected. Furthermore, assuming a ratio of 500/1 between chlorophyll and electron transport chains, with a 1 ml sample the detection limit for light induced F_v corresponds to the closure of 0.04 femto mol PS II reaction centers. With signal averaging, larger volumes and more sophisticated cuvette geometries even lower detection limits should be possible.

Applications in hydrobiology

A major application of the new Xe-PAM Fluorometer can be envisaged for the study of phytoplankton in natural waters like lakes, rivers and oceans, which display chlorophyll concentrations ranging from approx. 50 μg/l to 1 μg/l. An essential advantage of the new instrument with respect to conventional fluorometers is its specificity to *active* chlorophyll. In practice, not the fluorescence signal as such but the *variable* fluorescence induced by a saturation pulse is a measure of the concentration of active chlorophyll participating in photosynthetic energy conversion and of the relative yield of photosynthetic energy conversion at given environmental conditions.

Figure 6 shows an example of a chlorophyll fluorescence induction curve with the unicellular alga *Scenedesmus obliquus D3* at 10 μg Chl/l. Repetitive saturation pulses are applied for quenching analysis. In the steady state, even at

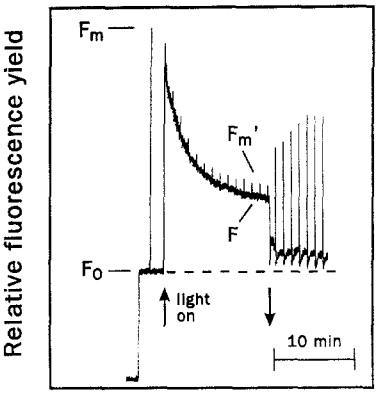

Fig. 6. Dark–light induction curve of *Scenedesmus obliquus D3* diluted to 10 μg Chl/l. Actinic red light (peak 650 nm) was applied at an intensity of 330 μE m^{-2} s^{-1} using an LED source (Walz, 102-L).

this low chlorophyll concentration, the effective quantum yield can be determined from the expression $\Phi_{PS\,2} = \Delta F/F'_m$ (Genty et al. 1989). On the basis of such yield determinations at different light intensities a light saturation curve of the relative electron transport rate can be derived (see Fig. 8).

For comparison, in Fig. 7 the dark–light induction curve of natural phytoplankton contained in the water of the river *Main* is displayed. The signal quality is sufficiently good to allow fluorescence quenching analysis and, in particular, determination of effective PS II quantum yield, $\Delta F/F'_m$. In Fig. 8, the light saturation

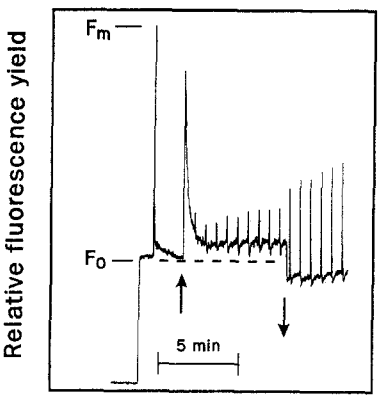

Fig. 7. Dark–light induction curve of natural phytoplankton contained in water of the river *Main*. Actinic red light (Schott RG 645) was applied at an intensity of 1000 μE m^{-2} s^{-1} with a fiberilluminator (Schott KL 1500). The water was collected on October 17, 1992, in Würzburg-Heidingsfeld.

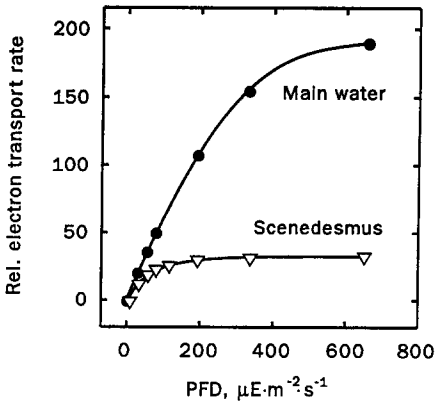

Fig. 8. Light-saturation curves of apparent relative electron transport rate determined by fluorescence quenching analysis. Comparison of a *Main* water phytoplankton sample (see also Fig. 7) and a *Scenedesmus obliquus D3* suspension at 100 μg Chl/l (see also Fig. 6). Photosynthetically active radiation (PAR) was measured in $\mu E\, m^{-2}\, s^{-1}$ with a Licor Quantum Sensor (Model LI-189). Relative electron transport rate is calculated from the product of PAR and the effective PS II quantum yield determined as $\Delta F/F'_m = (F'_m - F)/F'_m$ (for nomenclature see legend to Fig. 1).

characteristics of the *Main* water phytoplankton sample and *Scenedesmus* are compared. The ordinate represents $PAR \times \Delta F/F'_m$, which provides a relative measure of electron transport rate (Genty et al. 1989, Seaton and Walker 1990, Schreiber et al. 1993). It is apparent that light saturation of the natural phytoplankton occurs at much higher light intensities than with *Scenedesmus*. This is a consequence of the vastly differing light intensities to which these organisms were adapted during growth (day light reaching 1000 $\mu E\, m^{-2}\, s^{-1}$ for the phytoplankton as compared to 35 $\mu E\, m^{-2}\, s^{-1}$ fluorescent light for *Scenedesmus*).

The induction pattern displayed by the *Main* water sample (Fig. 7) differs from that of *Scenedesmus* (Fig. 6). This is due to the fact that the phytoplankton consists of a mixture of different algal groups, including chlorophyceae, cyanophyceae, xanthophyceae, cryptophyceae and phaeophyceae. Present knowledge on the fluorescence characteristics of these various types of algae is rather limited. However, it is clear that, due to fundamental differences in antenna organisation and overall physiology, there are also principle differences in fluorescence behaviour (see e.g. Papageorgiou and Govindjee 1968, Caron et al. 1987, Wilhelm et al. 1990, Büchel and Wilhelm 1990, Lee et al. 1990). Therefore, the mere assessment of variable fluorescence, although being advantageous when compared to conventional methods, can only be considered a first step in the analysis of phytoplankton by the Xe-PAM Fluorometer. In future work advantage must be taken of the possibility to select narrow excitation and emission bands with this instrument. In this way, it should be possible to distinguish between algae with different pigment organisation, provided the fluorescence behaviour of each alga group has previously been characterized by basic research using pure cultures.

Figure 9 compares induction curves of *Synechococcus* PCC 7942 and *Scenedesmus obliquus D3* measured with blue excitation (400–460 nm, mainly exciting Chl *a*) and green excitation (530–

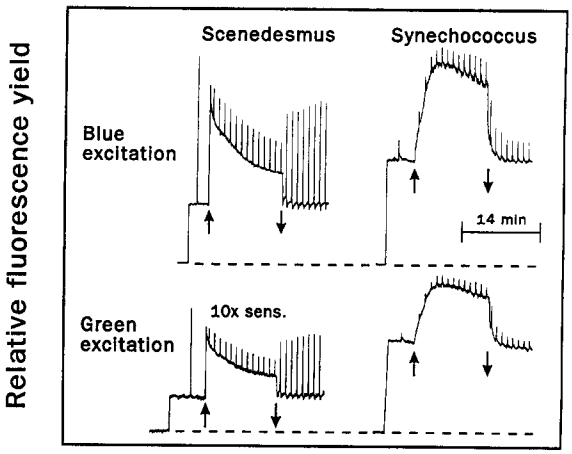

Fig. 9. Comparison of dark–light induction curves of the chlorophycea *Scenedesmus obliquus D3* and the cyanophycea *Synechococcus PCC 7942* measured with blue or green excitation. Red actinic illumination (Schott RG 645) at an intensity of 75 $\mu E\, m^{-2}\, s^{-1}$. Excitation bands were selected with interference filters (Balzers K-40 for blue and Balzers K-55 for green light). Chlorophyll concentrations: 100 μg/l with *Scenedesmus* and 120 μg/l with *Synechococcus*. Note: With green excitation a 10 times higher sensitivity was used for measuring the displayed induction curve of *Scenedesmus*. The ratios of F_v(*Scenedesmus*)/F_v(*Synechococcus*) are 1.3 and 0.12 for blue and green excitation, respectively. For both organisms identical intensities of the two types of excitation light was used. F_v here denotes the maximal light induced fluorescence change.

580 nm), which in the cyanobacteria preferentially excites phycocyanin. It is apparent that with blue excitation the ratio of F_v(*Scenedesmus*)/F_v(*Synechococcus*) is much higher than with 560 nm excitation. It should be noted that with green excitation the *Scenedesmus* signal was very low and had to be recorded at 10 times higher sensitivity to become comparable to the *Synechococcus* signal. Hence, by using green excitation light it should be possible to minimize the contribution of chlorophyceae to the overall fluorescence response and in this way to assess other types of algae, like cyanophyceae, which possess absorption bands in this spectral region. This example may serve to illustrate a potential approach analysing not only chlorophyll content, but also alga composition and activity by the Xe-PAM Fluorometer.

Other envisaged applications

In view of the exceptional sensitivity, selectivity and flexibility of the new instrument a number of other possible applications besides chlorophyll fluorescence measurements can be envisaged. These include:

1. Measurement of NADPH and NADH fluorescence in vivo and in vitro (Duysens and Amesz 1957, Schreiber et al., in preparation).
2. Recording of 9-aminoacridine fluorescence (or of other fluorescent amines) to assess transthylakoidal proton gradients (Schuldiner et al. 1972).
3. Measurement of pyranine fluorescence (or of other fluorescent dyes) to assess the pH in various leaf compartments, as introduced by Heber and co-workers (see e.g. Yin et al. 1990).

Potential application of the Xe-PAM are not restricted to fluorescence measurements. By use of appropriate filter combinations and depending on the position of the quartz rods connecting to the Xe-flash lamp and the detector (see Fig. 1), in principle also measurements of 90° or 180° light scattering as well as of absorbance changes of intrinsic components (like P515 or zeaxanthin) or of added substances (like ferricyanide or methyl purple) are possible. However, for these applications it is necessary to correct for unavoidable fluctuations in flash intensity which amount to approx. $\Delta I/I = 3 \times 10^{-2}$. When flash intensity is measured and the resulting signal applied for signal correction via a ratio amplifier, the noise is reduced to approx. $\Delta I/I = 5 \times 10^{-4}$ (Schreiber et al., in preparation).

Conclusions

The newly developed Xe-PAM fluorometer extends the range of practical applications of the chlorophyll fluorescence quenching analysis by the saturation pulse method. With this instrument, it is now possible to monitor variable fluorescence yield and the relative yield of photosynthetic electron transport in chloroplast and algae suspensions at chlorophyll concentrations more than 1000 times lower than with presently available equipment. Hence, the method now even can be applied to phytoplankton in natural waters like lakes, rivers and oceans. It may be expected that this development will have considerable impact on phytoplankton research in limnology and oceanography. Furthermore, due to considerable flexibility in the choice of excitation and emission wavelengths, this new instrument should provide the experimental basis for a thorough analysis of variable fluorescence characteristics of different algae classes in comparison with standard measurements of photosynthetic activity, as e.g., polarographic detection of O_2-exchange. In this way, the eventual goal of a quantitative assessment of phytoplankton photosynthetic activity and, hence, primary biomass production by fluorescence measurements appears in reach.

Acknowledgements

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