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The 1-Hz fluorometer: a new approach to fast and sensitive long-term studies of active chlorophyll and environmental influences

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ABSTRACT: A new instrument for environmental monitoring, called a 1-Hz fluorometer, provides two modes of application. First, it enables a quantitative determination of algal concentrations down to 20 ng/l. Second, it can be used as a biosensor for changes in environmental conditions. The distinction between the signals from living chlorophyll-containing algae and other fluorescent material is achieved by using two modulated light-sources resulting in a mean fluence rate of 200 µE. The measuring light induces changes in chlorophyll fluorescence (yield) with a frequency of 1 kHz, and the actinic light modulates the redox state of the quenchers of PS II with a frequency of 1 Hz. This leads to a modulation of the yield which is detected by two phase-sensitive rectifiers (double correlation). Measurements from different sites in a river, and in the Baltic and North Seas, show that correction by the built-in simultaneously-measured attenuation is necessary in order to obtain values which are identical with those determined by a photometric analysis (Uvikon 860). This applies if the transmission becomes less than about 95 %. Suspensions of *Dunaliella salina* exposed to ammonia and phosphate were used for illustrating the usage for environmental monitoring. It is shown that this system can measure changes in the chlorophyll fluorescence of living algae caused by changes in concentration of ammonia down to 1 µg/l and of phosphate down to 10 µg/l.

INTRODUCTION

Chlorophyll fluorescence has become a convenient signal for monitoring the photosynthetic apparatus of plants (Hansen et al., 1991; Pasda et al., 1992; Schreiber & Schliwa, 1987; Schreiber et al., 1986; Schroeter et al., 1991). Simple devices cannot distinguish between living plant cells and other material which happens to emit red fluorescence. Others can give very detailed information about the function of the photosynthetic apparatus.

Schreiber's method, employed in the PAM-fluorometer, is a very sophisticated approach (Schreiber et al., 1986). It enables the quantitative determination of photochemical and non-photochemical quenching mechanisms. These results can be supplemented by the analysis under linearizing conditions, which facilitate evaluation by curve-fitting procedures and enable the monitoring of 7 different processes of the photosynthetic reaction scheme (Hansen et al., 1991, 1993).

For field application, very often an instrument is required which provides a compromise between simplicity and amount of available information. Such a device is the

1-Hz fluorometer here described. It can be used for determining concentrations of active chlorophyll and for monitoring environmental influences (Schroeter et al., 1991; Vanselow et al., 1992).

Another important feature is also the measurement of cells adapted to physiological fluence rates. The mean fluence rate of 200 µE applied in the 1-Hz fluorometer provides an estimate of the photosynthetic efficiency prevailing under common light conditions.

PRINCIPLE OF OPERATION

As with many other fluorometers (Schreiber et al., 1986; Hansen et al., 1991, 1993), the 1-Hz fluorometer employs two different light-sources: the measuring light and the actinic light (Table 1). The response to the measuring light is called yield. As the amplitude of the measuring light is constant, the yield f is a measure of the fluorescence F per irradiance I.

$$f = \frac{F}{I} = \frac{dF}{dI} \quad (1)$$

Equation 1 holds for fluorescence induced by measuring lights of high frequency (usually 1 to 100 kHz). The yield is used preferentially because it provides a more sensitive measure of the changes in the photosynthetic apparatus than the fluorescence itself does.

Table 1. Comparison of the properties of measuring and actinic light

Measuring light	Actinic light
high-frequency (1 kHz–1MHz)	low frequency
periodic signals	different time-courses allowed
acting as a probe	acting as input signal
without influence on kinetics	evoking kinetic responses
response is called yield	response is called kinetics

Figure 1 shows the set-up. The body of water to be investigated is pumped into a stainless steel cylinder, which is 4 cm long, with an inner diameter of 2.4 cm. The measuring light is provided by three LEDs (TLRA 190P, Toshiba) on the right-hand side of the cylinder, the actinic light by two LEDs on the left-hand side. A photodiode (S-3590, Hamamatsu) records the induced fluorescence. It is located behind a filter (RG 9, Schott, Germany) on the wall, in the middle of the cylinder. This location, and the filter, prevent the photodiode being exposed to direct illumination by the light-sources – which would cause overload of the current-voltage converter.

The irradiance of both light-sources is subject to modulation: periodic high-frequency (1 kHz to 1 MHz) changes for the measuring light (Fig. 2A), slower time-courses for the actinic light (Fig. 2B). The measuring light induces periodical changes of chlorophyll fluorescence. This yield is constant, if the sample does not show photosynthetic activity (similar to Fig. 2A). The actinic light influences the physiological status of the photosynthetic reaction chain, and evokes kinetic responses. This leads to a modulation of the amplitude (Fig. 2C) of the response to the measuring light (yield). Because of this,

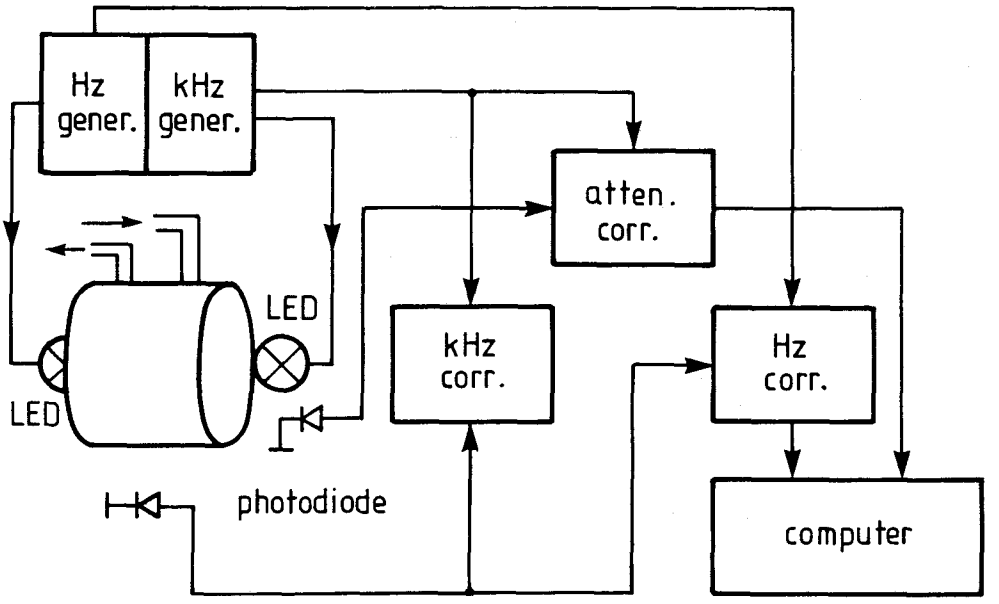

Fig. 1. Block-diagram of the 1-Hz fluorometer showing actinic- and measuring-light sources (LED TLRA 190P, Toshiba) located at opposite sides of the measuring-cell and the detector on a side wall. The light-sources are controlled by the computer. The signal from the detector (S-3590, Hamamatsu) is evaluated by double correlation, and the output is corrected for attenuation and evaluated by the computer

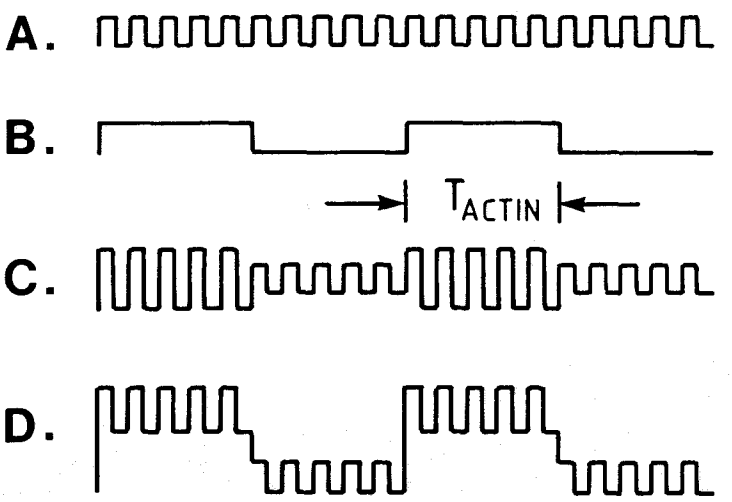

Fig. 2. Time-courses of the fluence rates of the measuring-light (A), the actinic light (B), the theoretical modulation of the yield (C) by the actinic light (B), the induced chlorophyll fluorescence (D)

the electronic apparatus has to extract the curve form of Figure 2C from the signal recorded by the photodiode (Fig. 2D), i.e. it has to ignore the component related to the direct effect of the actinic light.

This is achieved by the principle of double correlation. The two correlators in Figure 1 are phase-sensitive rectifiers with subsequent low-pass-filters. Rectification in the first correlator is done by switching the amplification factor of the correlator between +1 and −1, as controlled by the positive and the negative phase of the measuring signal. Thus, only the response to the measuring light adds up in the low-pass filter – whereas the direct response to the actinic light is eliminated by averaging.

The important feature is the indirect effect of the actinic light, i.e. the modulation of the amplitude of the yield (Fig. 2C) due to induced changes in the status of the photosynthetic apparatus (Fig. 3). This results in a modulation of the dc-component at the output of the first correlator.

The rectifier of the second correlator is controlled by the actinic signal. Because of this, only the changes in the yield induced by the actinic light can pass the second correlator. The output of the second correlator is zero, if the fluorescence is not changed by the actinic light (dead material). Otherwise it is a measure of the redox changes in the photosynthetic apparatus induced by the actinic light.

According to the above description, the measuring light has to act as a probe, i.e. under steady-state conditions it has to record the status of the photosynthetic apparatus, without itself exerting any influence. For kinetic investigations, this postulate can be fulfilled, since the frequency of the measuring light is much higher than that of the actinic light. Thus, it acts like a constant background light for the slower reactions (shown in Fig. 3 below) stimulated by the actinic light.

CORRECTION FOR ATTENUATION

Chlorophyll and debris attenuate the stimulating light and reduce the fluorescence on its way to the detector. Thus, a correction has to be applied, which compensates for the effect of the attenuation. For this issue, one of the three LEDs at the right-hand side of the cylinder in Figure 1 is used as a photodiode. It records the measuring light which is attenuated by the chlorophyll and the debris in the cylinder. The attenuation signal has the frequency of the measuring light, and is detected by cross correlation with the measuring signal in the attenuation correlator (see Figure 1: atten. corr.).

The output of the attenuation correlator is used to correct the measured fluorescence yield f_u as follows:

$$f = f_u \exp(\lambda (100 - d)) \quad (2)$$

The transmission d is given in percent of the unattenuated light. The factor λ has to be determined experimentally. This is done by adding increasing amounts of bentonite to the sample which has to be measured. As the real chlorophyll content remains unchanged after all the additions of bentonite, the measured transmission and the measured chlorophyll content can be used for the calculation of λ. This calibration routine accounts for the attenuation of the stimulating and the emitted light. Table 2 gives examples of this correction.

BIOPHYSICAL BACKGROUND OF THE 1-HZ RESPONSE

The inset of Figure 3 shows the response in chlorophyll fluorescence yield of a plant cell evoked by a step in the fluence rate of the actinic light (step response). Curve-fitting of this step response by a sum of exponentials

$$f(t) = \sum_{i=1}^{7} A_i \left(1 - \exp\left(-\frac{t}{\tau_i}\right)\right) \quad (3)$$

provides 7 time constants τ_i and 7 amplitude factors A_i, which can be assigned to 7 individual processes in the photosynthetic reaction scheme which are marked by the

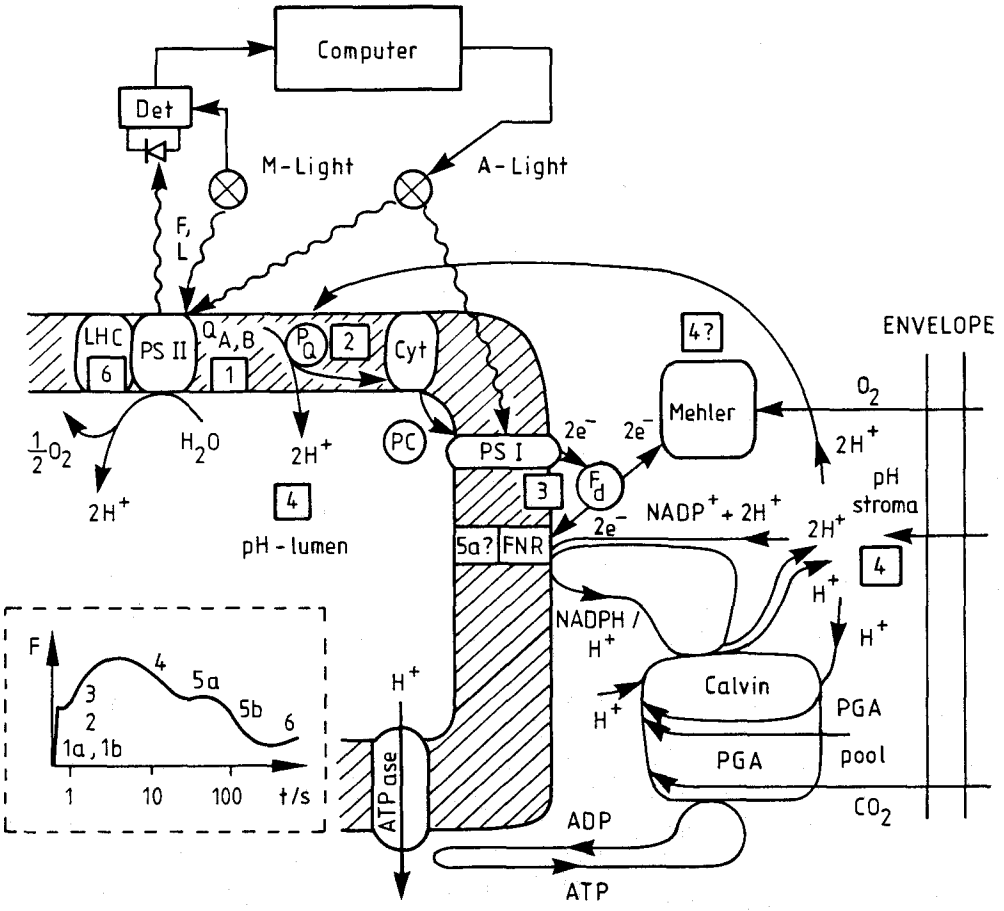

Fig. 3. Schematic presentation of the photosynthetic reactions determining the temporal behaviour of chlorophyll fluorescence in the range of about 1 s to 10 min. The numbers in the boxes correspond to those at the induction curve in the inset and in Eq. 3. The assignment as obtained from previous investigations (Hansen et al., 1991, 1993; Vanselow, 1993; Vanselow & Hansen, 1989) is as follows: 1: primary acceptor (Q) of photosystem II (PS II), 2: plastoquinone pool (PQ); 3: primary PS I acceptor pool X, 4: transthylakoid pH-gradient, 5a: unknown, speculation: FNR-reductase, related to Ca2+-uptake into the chloroplasts, 5b: unknown, 6: state-1 state-2 transitions via the light harvesting complex (LHC)

numbers at the induction curve and in the boxes (Dau & Hansen, 1989, 1990; Hansen et al., 1991, 1993; Vanselow 1993; Vanselow & Hansen, 1989; Vanselow et al., 1988, 1989).

The analysis of the time-course shown in Figure 1 requires plenty of time, experience and control experiments; for instance, the comparison with other signals, like absorption at 535 nm, or photoacoustically measured gas exchange (Dau & Hansen, 1989, 1990). This effort may be too time-consuming just for monitoring the density and physiological state of algal cultures. However, the background knowledge obtained from investigations like those which led to the scheme in Figure 1 is utilized in order to construct a reliable easy-to-operate instrument.

The principle of the 1-Hz fluorometer is based on the knowledge that the kinetic behaviour in the range of 1 sec is mainly determined by the changes in the redox-state of the primary quenchers Q_A and Q_B of photosystem II, the plastoquinone pool and the acceptor pools of PS I. This is the range of the time-constants τ_1 to τ_3 (Fig. 3; Hansen et al., 1991, 1993; Vanselow, 1993). These changes in redox-state occur only in living plant cells. Because of this, the fluence rate of the actinic light is modulated with a frequency of about 1 Hz.

CALIBRATION OF THE 1-HZ FLUOROMETER

As is obvious from Figure 3, the magnitude of chlorophyll fluorescence and also of the yield depends on many individual processes in the photosynthetic apparatus, which change their state whenever irradiance is changed (Schreiber et al., 1986; Hansen et al., 1991, 1993; Vanselow et al., 1989).

This also holds for the measurements with the 1-Hz fluorometer, as illustrated by Figure 4. Cells of *Dunaliella salina* were grown in artificial seawater (Guillard & Ryther, 1962) on the sill of a north-facing window in Büsum, and were subject to normal daylight conditions. The cells were then sucked into the measuring vessel shown in Figure 1. The exposure to the lights of the 1-Hz fluorometer induced changes in the photosynthetic apparatus which led to the time-course of the 1-Hz signal, shown in Figure 4.

The time-course displayed in Figure 4 raises the problem of when to take the reading of the 1-Hz fluorometer. However, Figure 4 shows that the reading of the 1 Hz fluorometer reaches a steady-state after the exposure to the fluence rate of the instrument within 40 sec. In order to allow for even slower kinetics, the reading of the 1-Hz fluorometer is taken 120 sec after exposure to the light of the measuring chamber.

In Figure 5, a calibration curve is given. A sample of cells of *Dunaliella salina* was taken. The concentration of Chl a was determined to be 90 µg/l, according to the method of Jeffrey & Humphrey (1975) by means of an Uvikon 860. This value was used to calibrate the output of the 1-Hz fluorometer. Linearity was then tested. For this purpose, the data points to lower concentrations being obtained by taking this sample of cells (data point at the right-hand side), and diluting it (in several steps) down to a concentration of 19 ng Chl/l. It is obvious that linearity holds over a wide range. However, the experimenter has to take care to use the same temporal range during calibration and during the experiment.

The problem of the adequate selection of the measuring protocol is related to the general question of what is actually recorded by the instrument. Uncontrolled parameters may be involved, resulting from the state of energy quenching (Schreiber & Neubauer,

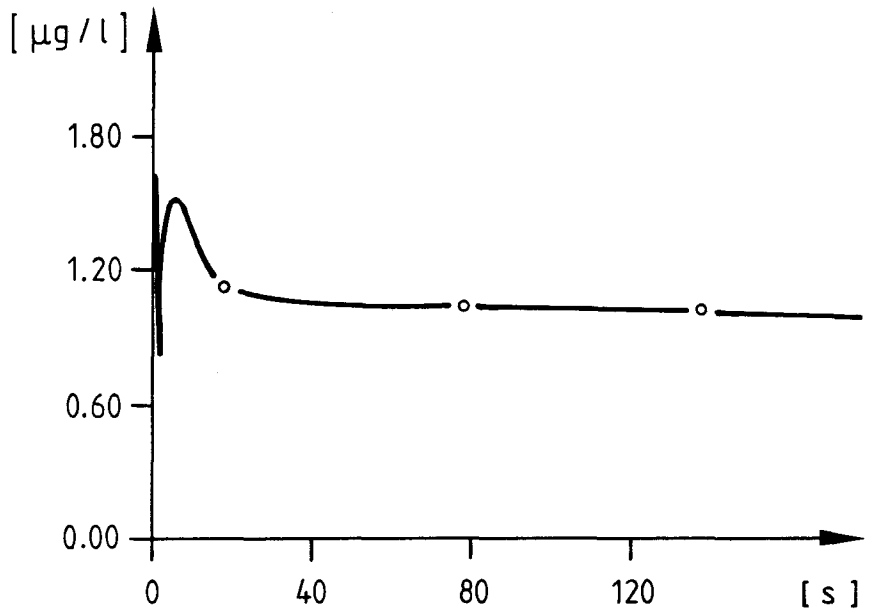

Fig. 4. Temporal behaviour of the output of the 1-Hz fluorescence after exposure to the light conditions of the instrument

1990; Dau & Hansen, 1990; Horton et al., 1991; Vanselow et al., 1988), state transitions (Dau & Canaani, 1992) or photoinhibition (Demmig & Winter, 1988).

The state of energy quenching can be set to a reproducible value, by keeping the cells in the dark for about 120 sec prior to the measurement and then in the light of the experimental vessel for additional 120 sec, because the time-constant of the changes in energy quenching (τ_4, Fig. 3) is less than 50 sec (Hansen et al., 1991, 1993).

Other influences are more difficult to control. Usually, the effect of state transitions (Dau & Canaani, 1992) is not big. However, if a major influence is expected, an equilibration period (exposure to the fluence rate of the 1-Hz fluorometer) longer than τ_6 in Figure 3, e.g. 20 min, should precede the measurement (Hansen et al., 1991, 1993).

Photoinhibition (Demmig & Winter, 1988) or accumulation of zeaxanthin (Bilger et al., 1989) is a more difficult problem. Recovery from photoinhibition or reversion of de-epoxidation can last a very long time. Thus, it is recommended to avoid photoinhibitory light regimes. If photoinhibition cannot be avoided, the experimenter should be aware of the fact that the instrument records only the non-photoinhibited portion of the investigated sample. This, however, holds for all fluorometers analysing the kinetics of chlorophyll fluorescence.

EXAMPLES OF APPLICATION

Samples of water were collected at the places listed in Table 2. The chlorophyll content was measured by the 1-Hz fluorometer, and the readings were compared with the results obtained by an Uvikon 860, according to the method of Jeffrey & Humphrey

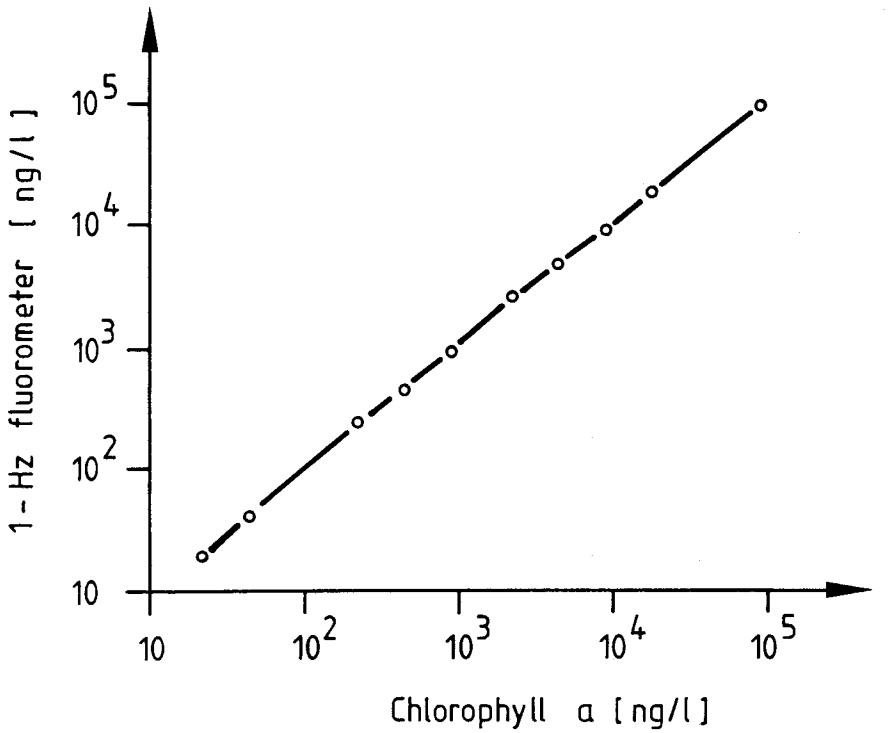

Fig. 5. Calibration curve. Chlorophyll a was determined by the Uvikon 860 (x-axis) and by 1-Hz fluorometer (y-axis)

(1975). In order to determine the amount of photosynthetically active chlorophyll, the values from the Uvikon 860 were corrected by the Genty parameter (Genty et al., 1989) with f_m being measured by flashes of 4000 µE. Table 2 shows that correction for attenuation is necessary. Column 1 gives the results of a chemical analysis (Uvikon 860) regarded as "true values"; column 2, the values obtained without correction for attenuation. Obviously, they become incorrect if higher algal densities (Eider) or mood (Büsum)

Table 2. Density of algal concentrations measured at different sites with different methods, illustrating the necessity of correcting the fluorescence reading by the simultaneously measured attenuation. Concentrations are given in µg Chl a/l

Site	Chemical analysis	Fluorescence without corr.	Corrected fluorescence	Transmission (4 cm)
Baltic Sea, Kiel	3.4	3.4	3.4	98 %
River Eider	13.0	12.5	13.2	93 %
Büsum Harbour	5.3	2.3	5.4	89 %
Büsum North Sea	5.9	2.1	5.9	86 %

result in transmissions less than about 95 %. However, the values in column 3 show that the corrected values come very close to those determined by the Uvikon 860.

THE 1-HZ FLUOROMETER AS ENVIRONMENTAL MONITOR FOR NUTRIENTS

Cells of *Dunaliella salina* were kept in a vessel on the window-sill. The artificial seawater (Guillard & Ruyther, 1962) was not renewed for 4 weeks in order to induce nitrogen and phosphate starvation. Then the experiments were done, according to the protocol as shown in Figure 4. Cells were sucked into the experimental vessel. The exposure to the lights of the 1-Hz fluorometer caused transient changes in the 1-Hz signal as described above. After a calibration phase of sufficient length (ca 1 h), NH_4Cl or K_2HPO_4 was added. The concentrations ranged from 1 to 100 µg/l (0.056 mmol m^{-3} to 5.6 mmol m^{-3}) in the case of ammonia, and from 10 to 1000 µg/l (0.11 mmol m^{-3} to 11 mmol m^{-3}) in the case of phosphate.

The addition of the nutrient resulted in a sharp upward peak and a slow, partially reversible downward deflection (right-hand side of Fig. 4).

Figure 6A shows some time-courses as induced by the addition of NH_4Cl of different concentrations. Significant changes in the 1-Hz signal are obtained at concentrations above 0.56 µM. The addition of the nutrient resulted in a sharp upward peak and a slow, partially reversible, downward deflection. The difference between the upper and lower maxima was taken as a measure of the effect of the nutrient.

From experiments of this kind, the dependence of the change in fluorescence yield on concentration was determined (Fig. 6B). The dose-effect curves show a nearly-linear dependence on the logarithm of the nutrient concentration.

The origin of the effect is known in the case of ammonia. Vanselow (1993) has shown that nitrogen salts induce a stimulation of electron transfer from PS I to the nitrogen metabolism in the chloroplast (related to τ_3 in Fig. 3). This leads to a photochemical quenching of chlorophyll fluorescence as indicated by the downward deflection in Figure 4. In addition, the electron flow to nitrogen which is related to a much lower consumption of ATP than that to the Calvin cycle causes an increase in the transthylakoid pH-gradient and thus to additional energy quenching. The effect of phosphate is different and still under investigation.

SUMMARY OF THE BENEFITS AND RESTRICTIONS OF THE 1-HZ FLUOROMETER

Benefits:

- no flash lamps; that means low power consumption, no bleaching light intensities
- LEDs as light-sources: long life-time
- high sensitivity (Chl a: 20 ng/l)
- wide linear range as shown in Fig. 5
- distinction between fluorescence of live and dead material
- measurement at naturally occuring light intensities

Restrictions:

- equilibration time of 2 or more minutes for the adaptation of the photosynthetic apparatus to the measuring light intensity

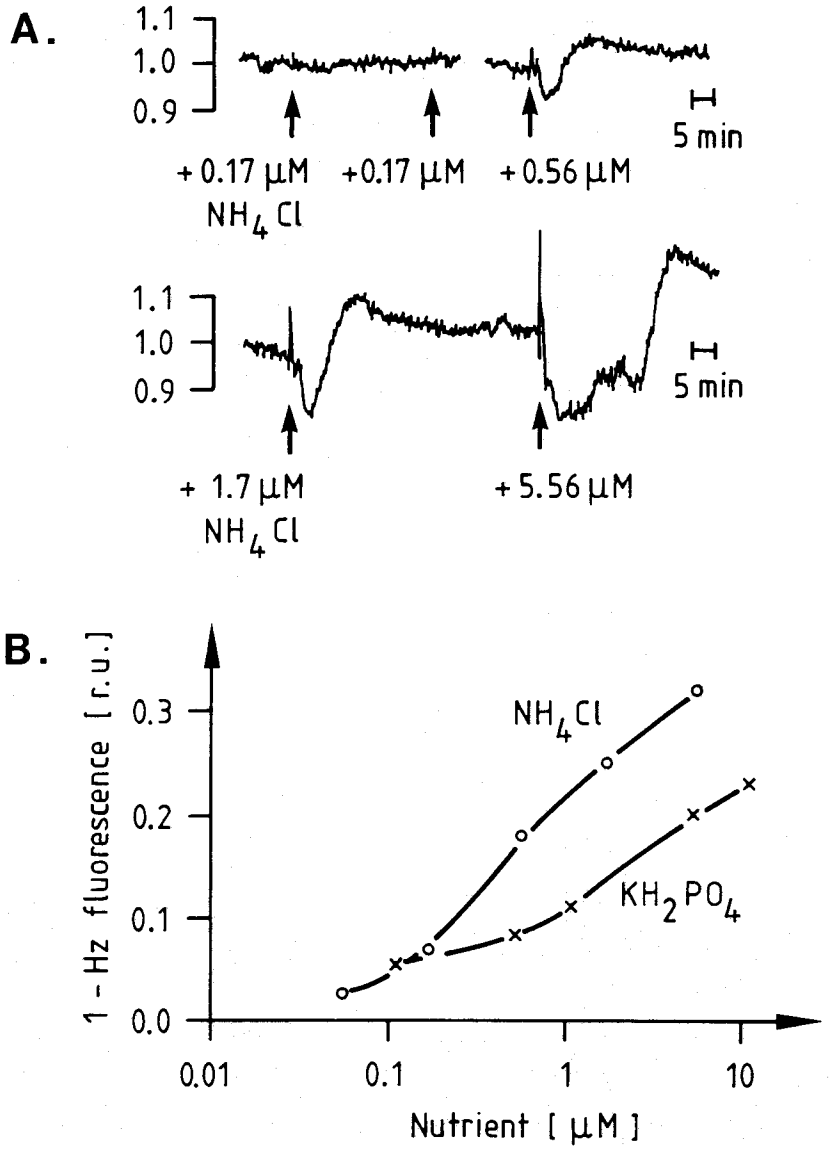

Fig. 6. Nutrient detection by the 1-Hz fluorometer for ammonia. A: Examples for the time-course of the responses to the addition of different concentrations of NH_4Cl. B: Dose-effect curve as obtained from the averages of three to five measurements at each concentration. The 1-Hz fluorescence is given in relative units (r.u.) as obtained by dividing the measured deflection (second upper peak – lower peak in [A]) by the steady-state value recorded before the addition

- photoinhibited cells are ignored as is all other non-living material
- calibration in cell number requires calibration by the same species as occurs in the investigated body of water.

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