PHOTOSYNTHETICA 37 (2): 249-254, 1999

Measurements of phytoplankton of sub-nanomolar chlorophyll concentrations by a modified double-modulation fluorometer

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Abstract

A modification of the double-modulation fluorometer is described that allows measuring very dilute phytoplankton samples. The high sensitivity is achieved by increasing the sample volume and by collecting the fluorescence from the large volume by an integrating sphere. The sensitivity of the instrument increased approximately proportionally to the volume of the sample. A further improvement of the sensitivity was achieved by replacing the PIN photodiode of the earlier versions by a photomultiplier. The instrument was used to measure fluorescence induction, F_0 and F_m parameters, and Q_A^- reoxidation kinetics at concentrations at and below 100 pM chlorophyll.

Additional key words: fluorescence; *Phaeodactylum tricornutum*; *Pseudococcomyxa* sp.; *Synechococcus marinus*.

Introduction

The world's oceans form a very dynamic environment. Large variations in irradiance,

Received 3 May 1999, *accepted* 17 June 1999.
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Abbreviations: Chl - chlorophyll; F_0 and F_m - fluorescence emission levels measured with Q_A acceptor oxidized (F_0) or reduced (F_m); F_v - variable fluorescence ($F_m - F_0$); PhAR - photosynthetically active radiation 400-700 nm; PS2 - photosystem 2.
Acknowledgements: The research has been supported by the INCO-COPERNICUS grant of European Commission (IC15CT96-0105) and by the matching grant of Ministry of Education of the Czech Republic (OK-271). N.D. was supported by Alfred Wegener Institute of Polar and Marine Research (publication No. 1656). Support from the *P.S. Instruments*, Brno is also gratefully acknowledged.

temperature, and nutrient availability occur both in time and place and influence the physiological status of the phytoplankton and, eventually, the primary productivity of the ocean as the key element of the global climate. Understanding of these fundamental processes largely depends on our ability to evaluate the photosynthetic capacity of phytoplankton in its natural environment. The instrumentation serving to assess the photosynthetic activity of the phytoplankton is frequently based on the measurements of the chlorophyll (Chl) fluorescence emission (Kolber and Falkowski 1993, Olaizola *et al.* 1996).

The major difference between most of the fluorometers used in the photosynthesis research and the fluorometers designed to be applied in ocean research is in the higher sensitivity of the latter that allows measurements with very dilute phytoplankton samples. In the open ocean, phytoplankton concentrations are generally low. Greene *et al.* (1994) reported concentrations of 0.1-0.4 mg m^{-3} on a transect in the eastern equatorial Pacific. Olaizola *et al.* (1996) found 0-0.5 mg m^{-3} on a transect across the Atlantic Ocean. Global maps of Chl concentrations in the upper ocean, derived from satellite images, show that Chl concentrations are seldom higher than *ca.* 2 mg m^{-3} (Falkowski and Raven 1997).

Also important is the capacity of the instruments to discriminate between the various processes contributing to the measured integral value of the Chl fluorescence. The most elemental requirement is to discriminate between the minimal fluorescence (F_0) that is usually measured in the dark adapted cells and the maximal fluorescence (F_m) that is usually measured with cells in which both the primary electron acceptor Q_A and the plastoquinone pool are reduced by a long pulse of a bright light (Krause and Weis 1991, Schreiber *et al.* 1998). The transient from F_0 to the fluorescence levels corresponding to fully or partially reduced Q_A elicited by a continuous irradiation is called fluorescence induction or Kautsky effect. It is also frequently measured to obtain information on the kinetics of the reduction of Q_A and the PQ^- pool and on the efficiency of the photosystem PS2 (Govindjee 1995). The decay of the fluorescence after a single-turnover flash reflects the re-oxidation of Q_A^-. A combination of the steady-state and kinetic parameters is needed for a reliable interpretation of the observed values.

Here, we report a modification of a commercially available double-modulation fluorometer that results in a substantially increased sensitivity. The potential of the instrument was tested with two marine and one freshwater phytoplankton species diluted to several hundreds of cells per cm^3. The sensitivity was increased without loosing the capacity of the instrument to measure kinetics of fluorescence transients with a resolution up to 10 μs.

Materials and methods

Algae: The algae were grown in a water-jacketed glass cylinder of 500 cm^3 volume and 4.5 cm internal diameter. The cultures were stirred by a continuous flow of air bubbles and irradiated by two cool-white fluorescence tubes (18 W, *Tungsram*, Hungary). The suspensions were continuously diluted to keep the density stable.

The marine diatom *Phaeodactylum tricornutum* Bohlin, strain Cough from CCMP, Bigelow Laboratory for Ocean Sciences, W. Boothbay Harbor, USA was grown at a density of *ca.* 1 μM Chl (*a*+*c*) irradiated by 80 μmol m^{-2} s^{-1} of PhAR, at a temperature of 16 °C in F/2 artificial seawater. The green freshwater alga *Pseudococcomyxa* sp., Wydrzycka 1981/GC-10 from the Culture Collection of Autotrophic Microorganisms (Třeboň, Czech Republic), was grown at 25 °C and 300 μmol m^{-2} s^{-1} in the liquid growth medium (Šetlík *et al.* 1981). The cyanobacterium *Synechococcus marinus* Butcher, strain WH 5701 from Woods Hole Oceanographic Institution, MA, USA was grown in F/2 artificial seawater at 20 °C and 30 μmol m^{-2} s^{-1} of PhAR.

Double-modulation fluorometer: The instrument was constructed by modifying the double-modulation fluorometer produced by *P.S. Instruments* (Brno, Czechia). The PIN photodiode of the standard instrument was replaced by the *R2228* photomultiplier in the *E990-07* socket/divider that was connected to the *C4900-01* high-voltage power supply (all components from *Hamamatsu*, Japan). The photomultiplier was placed in a laboratory-made housing with a custom made optical filter of 700 nm central wavelength and 30 nm FWHM bandwidth (*Corion*, Franklin, MA, USA). The standard sample compartment accommodating a fluorescence cuvette (10×10 mm of 2 cm^3 internal volume) was in most experiments replaced by a spherical glass cuvette of 5 cm internal diameter (*ca.* 50 cm^3 volume) enclosed in 2" *Spectralon* integrating sphere (*Labsphere*, North Sutton, NH, USA). Two opposite ports of the integrating sphere were used to pass measuring and actinic flashes from two LED units similar to those described in Trtílek *et al.* (1997) and Nedbal *et al.* (1999). The first LED unit was populated exclusively by *DH-08* orange LEDs (*Hewlett-Packard*, USA) out of which seven diodes generated measuring flashes (20 μs duration) and the remaining sixty diodes were used to generate actinic flashes. The excitation wavelength was limited by a dichroic filter (*FL-650, Andover Corp.*, Salem, NH, USA). The second LED unit was populated by seven orange *DH-08* and forty-eight red *HLMP-8103* diodes (*Hewlett-Packard*, USA), all generating actinic flashes. The remaining twelve blue diodes (*NSPB500S, Nichia*, Japan) were used to produce a continuous actinic radiation. The LED units were powered by 3 independently programmable lines controlled by the *FluorWin* program (*P.S. Instruments*, Brno, Czechia) that defined the energy of the flashes. The timing and duration of the flashes were defined in a *FluorWin* experimental protocol and executed by a microprocessor in the Control Unit of the fluorometer. Values were collected by the standard (100 kHz, 16 bit) input channel and stored in the memory of the Control Unit (300 kB SRAM) before being transferred to computer. Artifact signals originating from the measuring radiation that leaked through the optical filters with the integrating sphere filled by the growth medium were always subtracted from signals obtained with algae using the same protocols.

Results and discussion

The Kautsky effect of the green alga *Pseudococcomyxa* sp. was measured in the standard fluorescence cuvette and in the large integrating sphere (Fig. 1). The

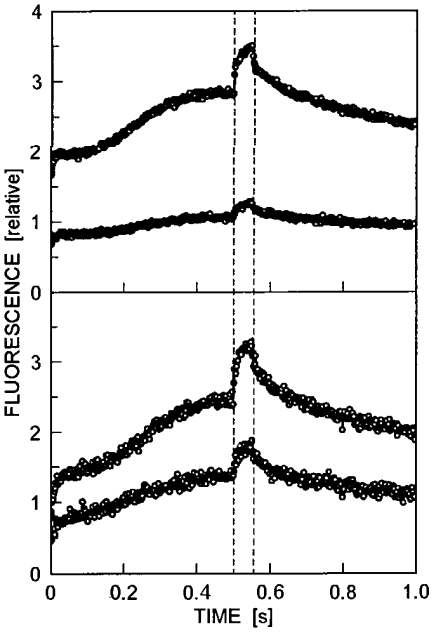

Fig. 1. Fluorescence induction in the suspensions of *Pseudococcomyxa* sp. measured using a standard 10×10 mm fluorescence cuvette (*top*) or in a 2" integrating sphere (*bottom*). The suspensions were diluted to the concentrations of 7 and 1.75 (*top*) or 220 and 110 (*bottom*) nM Chl (*a*+*b*) (*ca.* 6 300 and 1 500 or 200 and 100 mg m^{-3}, respectively). The sample volume was 2 cm^3. The actinic effects were elicited by strong measuring flashes (80 % relative power) given at a frequency 500 flashes per s. The photomultiplier voltage was 600 V. The actinic radiation was supplemented between 0.50 and 0.55 s by 26 single-turnover saturating flashes that were given at the frequency of 1 flash per 2 ms. Fluorescence was measured 1 ms after each actinic flash. The flashes transiently reduced PQ pool and fluorescence increased to F_m. The dashed lines show the time interval when the actinic flashes were given.

fluorescence was emitted from *ca.* 14.0 or 3.5 pmol Chl (*a*+*b*) (56 000 or 14 000 algal cells) in a 2 cm^3 sample in the small fluorescence cuvette and from *ca.* 13.3 and 6.6 pmol Chl (*a*+*b*) (about 53 000 or 26 000 cells) in a 50 cm^3 sample in the large integrating sphere cuvette. The integrating sphere was able to collect radiation efficiently from a volume that was much larger than the volume of the standard

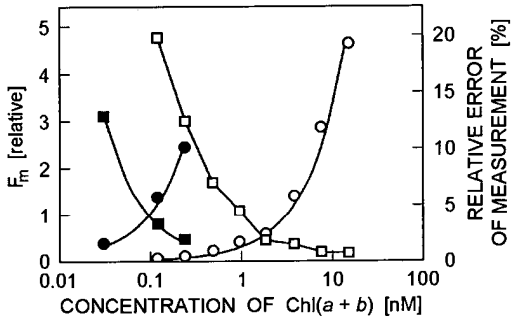

Fig. 2. Comparison of the F_m fluorescence signal (*circles*) obtained for different concentrations of Chl (*a*+*b*) in *Pseudococcomyxa* sp. when measured in the standard fluorescence cuvette (*open symbols*) and in the integrating sphere (*closed symbols*). The curves marked by *squares* show the respective relative error of the F_m measurement.

fluorescence cuvette. The use of the integrating sphere is further justified by Fig. 2 that demonstrates a shift towards high sensitivities and low relative errors in the sphere compared with the small volume cuvette.

The F_0 and F_m parameters and the F_v/F_m ratios measured for three different Chl concentrations (Fig. 3) show that the F_v/F_m ratio can be measured down to the concentration of 30 pM Chl (*a*+*b*) that roughly corresponds to 7 200 cells in the 50 cm^3 sample volume (*ca.* 140 cells per cm^3). Typical relative error of the F_m determination at this concentration was 12 %.

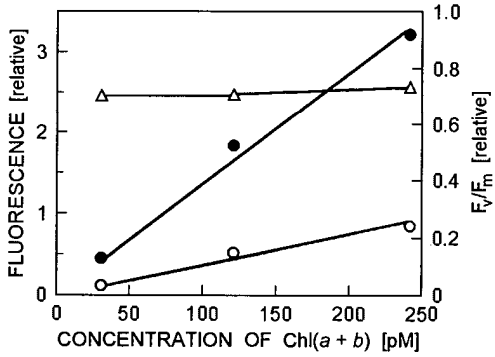

Fig. 3. The F_0 (○), F_m (●), and F_v/F_m (Δ) parameters measured for three chlorophyll (Chl) (*a*+*b*) concentrations of *Pseudococcomyxa* sp. in the integrating sphere using protocol as in Fig. 1. The lowest concentration of 30 pM Chl (*a*+*b*) (*ca.* 27 μg m^{-3}) corresponds roughly to 1.8 pmol Chl (*a*+*b*) contained roughly in 7 200 cells.

For the green alga *Pseudococcomyxa* sp., the cyanobacterium *S. marinus,* and the diatom *P. tricornutum*, 30–50 pM concentrations of total Chl yielded F_0 emission with relative measuring error lower than 10 % (Fig. 4). The F_v/F_m ratio was constant in the measured range of concentrations with variations caused by a relative error lower than 7 %.

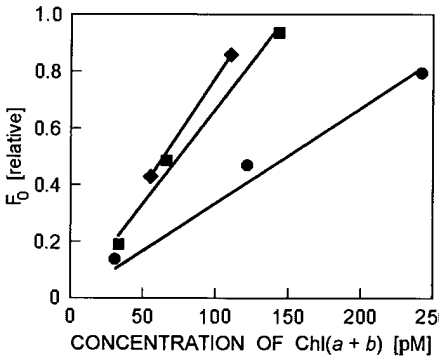

Fig. 4. The F_0 fluorescence measured for various concentrations of total chlorophyll in *Pseudococcomyxa* sp. (●), in *Phaeodactylum tricornutum* (■), and in *Synechococcus marinus* (◆). All measurements were done in the integrating sphere.

The fluorescence transients accompanying reduction of the primary quinone acceptor Q_A by a single-turnover-saturating flash (30 μs, 100 % power of all LEDs) in *P. tricornutum* are shown in Fig. 5 for three different Chl (*a*+*c*) concentrations. The experiment demonstrated the capacity of the instrument to measure fluorescence transients of dilute algal samples with a high time-resolution.

The experiments showed that the employment of an integrating sphere in the collection of fluorescence photons offers a possibility to increase substantially the sensitivity of fluorometers while keeping a high time resolution. Here, the sensitivity was roughly proportional to the sample volume in the range between 2 and 50 cm^3.

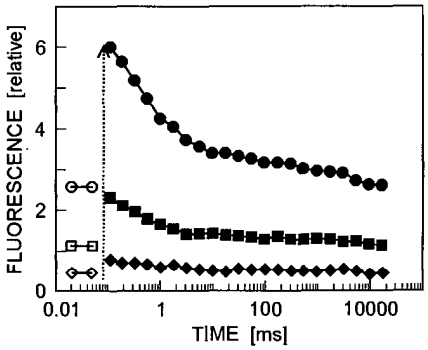

Fig. 5. The fluorescence transient elicited in *Phaeodactylum tricornutum* by a single-turnover saturating flash (*arrow*). The diatoms were diluted to 1 nM (*circles*), 0.5 nM (*squares*), or to 0.13 (*diamonds*) nM Chl (*a+c*). The *open symbols* represent the F_0 level. The first point was recorded 100 μs after the actinic flash. The measurements were done in logarithmic time series with 4 measurements per decade.

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