PUMP-DURING-PROBE FLUOROMETRY OF PHYTOPLANKTON: GROUP-SPECIFIC PHOTOSYNTHETIC CHARACTERISTICS FROM INDIVIDUAL CELL ANALYSIS

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ABSTRACT

Saturating-flash fluorescence techniques are used to monitor the state of the photosynthetic apparatus in phytoplankton under natural conditions. At present these are bulk water measurements, which produce estimates of average properties of all the fluorescent particles present in a sample. Here we describe an improved approach for single-cell measurements of phytoplankton.

We have combined individual-cell "pump-during-probe" (PDP) measurements of chlorophyll (Chl) fluorescence induction on the time scale of 30 to 100 microseconds [1, 2] with flow cytometric (FC) characterization of each cell, to obtain population-specific photosynthetic characteristics. The results provide information about the potential quantum yield of photochemistry (Φ_p), the fraction of functional reaction centers (f), and the functional absorption cross section for photosystem 2 (σ_{PS2}).

Key words: phytoplankton, photosynthesis, fluorescence, technique, pump-during-probe, induction

1. CHL FLUORESCENCE INDUCTION AND PHOTOSYNTHETIC CHARACTERISTICS

The biophysical background of the PDP approach is discussed in [1, 2]. To a first approximation, fluorescence induction at the time scale of 30-100 μs under supraoptimal light intensity I can be described as

$$\Phi_f(t) = (\Phi_m^{-1} - (\Phi_m^{-1} - \Phi_o^{-1})\, e^{-\alpha t})^{-1}, \qquad (1)$$

where Φ_o and Φ_m are initial and maximum values of Chl fluorescence yield, and α is the rate constant of closing of PS2 reaction centers (RCs, $\alpha = \sigma_{PS2} I$). The magnitudes of Φ_m, Φ_o and α can be retrieved using nonlinear regression of a measured PDP induction curve to equation (1), and the photosynthetic characteristics can be estimated as

$$\Phi_p = (\Phi_m - \Phi_o)/\Phi_m, \qquad f = (\Phi_{p\,max}^{-1} - 1)/(\Phi_p^{-1} - 1), \qquad \sigma_{PS2} = \alpha / I. \qquad (2)$$

The maximum PS2 photochemistry efficiency for phytoplankton, $\Phi_{p\,max}$, is known to be 0.65 [3].

2. THE EXPERIMENTAL TECHNIQUE

We measure the time course of chlorophyll fluorescence yield during a 100 μs excitation flash provided by a weak 488 nm argon ion laser whose beam passes through an electro-optical modulator, while a strong laser beam for measuring conventional FC parameters is blocked by an electromechanical shutter (Figs. 1, 2). A cell detector based on an infrared diode laser triggers the PDP and subsequent FC measurements. Since both analog and photon counting detection techniques are used, cells ranging in size from <0.7 μm (prochlorophytes) to >30 μm can be assayed.

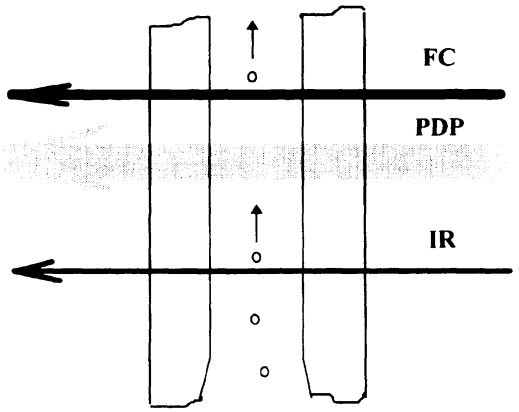

Fig 1. Schema of the flow cell with the IR, PDP and FC laser beams.

The optical setup (Fig. 2) includes an Ar ion laser (488 nm, 350 mW), splitters S1 and S2 (20% transmission), an electro-mechanical shutter (100 μs opening time) which is normally closed to prevent distortions in PDP induction, an electro-optic modulator (1 μs opening time) which produces a 100-μs PDP pulse, and an infrared (785 nm, 50 mW) laser. Spherical lenses (L1, L2, f = 80 and 100 mm) and cylindrical lenses (L3-L10, f = 50 mm) are used to shape the beams.

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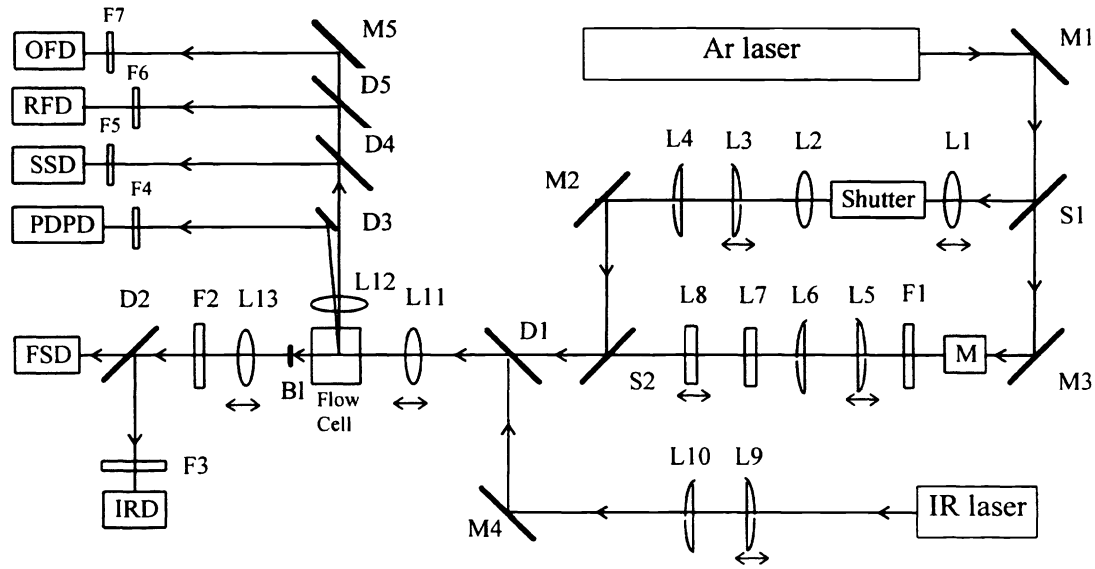

Fig. 2 Schema of the optical part of the PDP flow cytometer.

The achromatic lens L11 (f = 38 mm) provides focusing of the IR, PDP and conventional FC beams as presented in Fig.1. Lenses L7 and L8 increase the vertical divergence of the PDP beam, resulting in vertical widening of the PDP laser spot in the flow cell and an almost rectangular PDP "pulse" with opening of the modulator. Translations of L3, L5 and L8 allow independent adjustment of the horizontal size of the PDP and FC laser spots. Dichroic filter D1 serves to direct the IR beam to the flow cell. Lens L12 (from a Becton-Dickinson FACScan) and spherical lens L13 (f =30 mm) are used for collection of side and forward optical signals from the the flow cell. Blue and IR forward scattered (FS) signals are split by dichroic filter D2 and detected by a photodiode (FSD) and an avalanche photodiode (IRD), respectively. Neutral density filters F1 and F2 are used for adjustment of the PDP beam and FS signals to appropriate levels. Dichroic filters D3, D4, D5, and a mirror M5 provide splitting of the side optical signals and, after filtering with F4, F5, F6 and F7 filters, direction to PMT detectors for PDP (PDPD), side scattering (SSD), red fluorescence (RFD), and orange fluorescence (OFD).

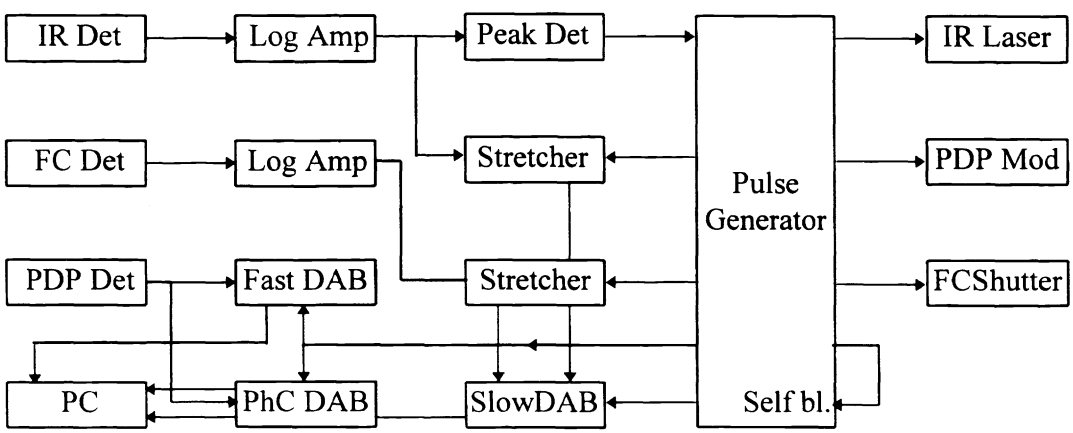

Fig. 3 Simplified schema of the PDP FC electronics.

A simplified schema of PDP FC electronics is presented in Fig. 3. It consists of the IR triggering channel, a conventional FC measuring part, and the PDP measuring channel. Since FC channels for measuring forward scattering, side scattaring, orange and red fluorescence are almost identical, we show just one "FC" channel in Fig. 3. 4.5-decade logarithmic amplifiers (Log Amp) are used to maximize the dynamic range in both IR and FC channels. Stretcher modules catch and hold the peak amplitudes of cell-produced pulses in IR and FC channels. An 8-channel 100-KHz ADC computer board (Slow DAB) measures these amplitudes on triggering from a timing pulse generator. This generator also provides resets of the stretchers after each measuring cycle, self-blocking to avoid triggering by new cells during event processing, blocking the IR laser to reduce background when measuring the PDP and FC response, pulsed opening of the PDP modulator (PDP Mod) and the FC beam shutter (FC shutter), and triggering of data acquisition in the PDP channel. This channel includes a PMT (PDP Det), a 60-Mhz analog ADC (Fast DAB) and time-resolved photon counting (PhC DAB, 2-μs resolution) boards installed (along with the Slow DAB) in a 100-MHz Pentium personal computer (PC). Fig. 4 shows a timing diagram of the system.

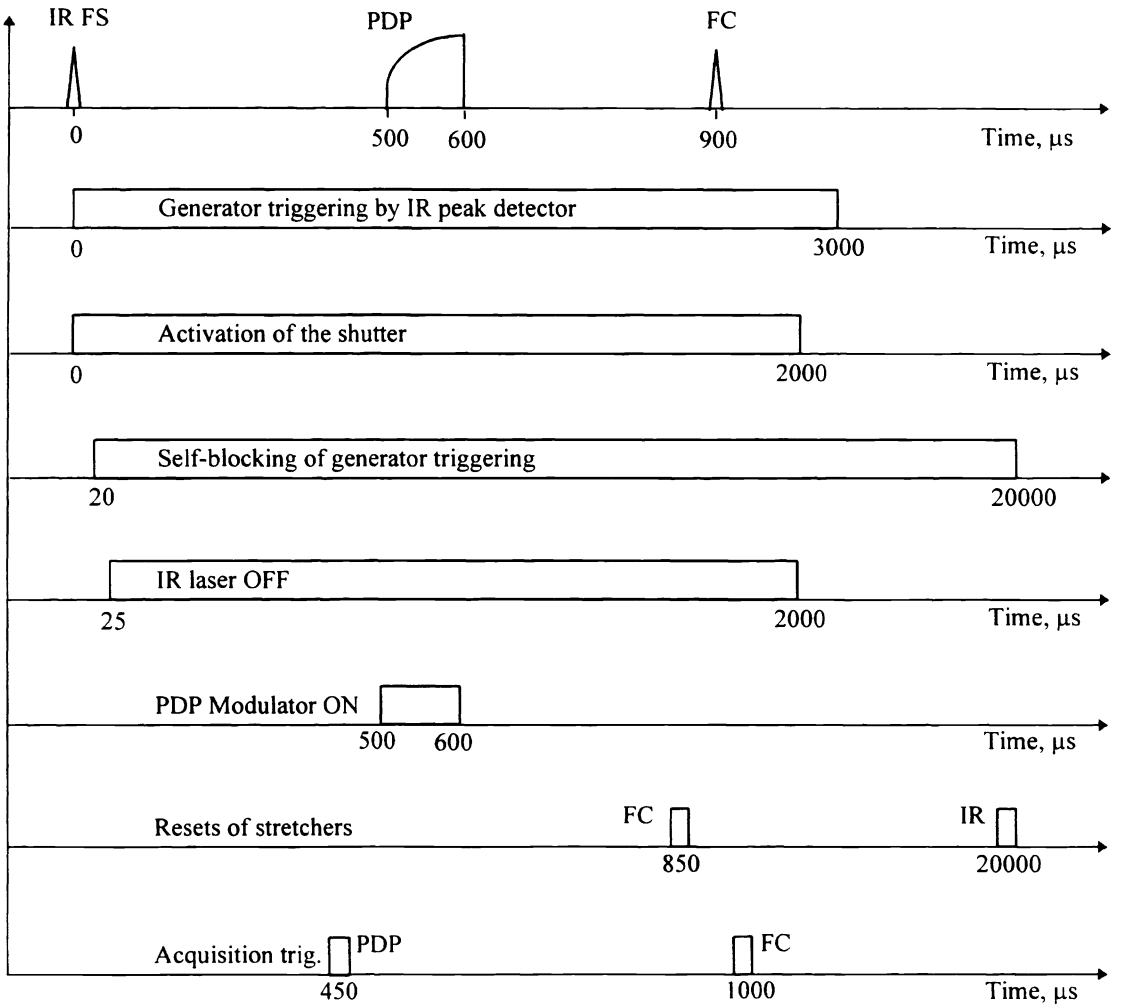

Fig. 4 Timing diagram of the PDP electronics.

A specially developed data acquisition software runs under Windows and allows real-time control of Fast DAB, Slow DAB, PhC DAB, and storage of both the PDP induction curve and FC data for each cell in the PC memory and on the hard drive. Due to substantial amount of information to be acquired (about 1.6 Kbyte per cell) , we use a CD recording system for permanent data storage after finishing the measurement. Typically we accumulate 1,000 to 25,000 events from each sample. An analysis of conventional FC data allows specification of cells or cell groups in the sample for retrieving individual or group-specific photosynthetic characteristics. In analog mode, either individual cell curves (for cells > 5 μm) or curves averaged over a group of cells can be reconstructed; in photon counting mode data from hundreds to thousands of cells are accumulated for each PDP induction curve.

We measure the fluorescence time course of red-fluorescing latex microspheres, added to the sample, to monitor the shape of the excitation "pulse", i.e. the light intensity profile in the PDP area within the core of the flow cell. The PDP induction curves are then normalized to this profile to compensate for deviations from the "ideal" rectangular shape. The intensity of the PDP laser beam was adjusted with neutral-density filters (F1 in Fig. 2) to obtain cell fluorescence rise times of approximately 30-100 μs, and the rate of sample introduction was adjusted to ensure that only one cell was in the PDP beam at any given time.

3. LABORATORY TESTS OF THE PDP APPROACH

The results of preliminary laboratory tests of the PDP approach were presented in (Olson et al. 1995, 1996). In particular, for six species of phytoplankton grown under different conditions of light intensity and nutrient depletion, estimates of the quantum yield of photochemistry in PS2 by PDP measurements made on individual cells were well correlated with estimates derived from DCMU-enhancement measurements of bulk samples (Fig. 5). Part of the measurements was made with a microscope-based PDP system (Olson et al. 1995, 1996). No obvious differences were observed between the two PDP instruments or among the six species tested. These results indicate that the microscope- and flow cytometer-based PDP techniques, applied to individual cells, provide reliable information about the photosynthetic characteristics of phytoplankton.

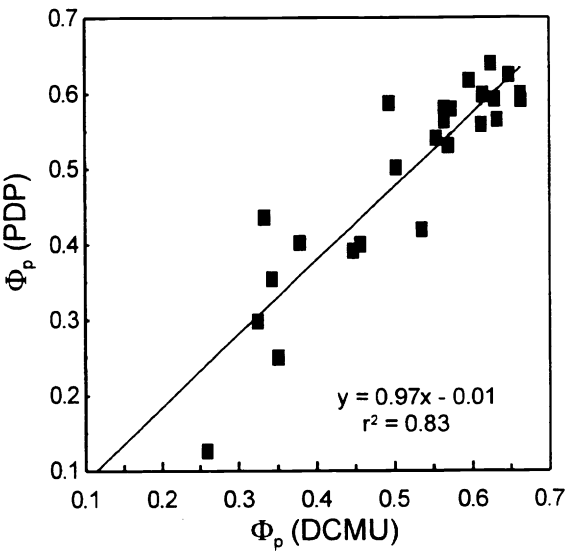

Figure 5. Correlation between PDP -based estimates of Φ_p and estimates derived from bulk measurements of DCMU enhancement of fluorescence.

We have recently tested the use of a compact solid-state green laser (100 mW, 532 nm) in the PDP FC system. Although we observed an apparent decrease in the efficiency of Chl fluorescence excitation (due to the lower absorption cross-section as compared to the blue region of the spectrum), the green laser appeared to be suitable for both FC and PDP measurement of relatively big (>1 μm) cells. In Fig. 6 we present results of a sample containing a mixture of *Dunaliella tertiolecta* (5-7 μm cells, DT) and *Nannochloris sp.* (2-3 μm cells, N). 1-μm red beads (BD) were used for monitoring the PDP profile in the flow cell. This profile was used to normalize PDP intensity profiles for the two species (panel B in Fig. 6). Processing of the PDP induction curves (see Fig. 6-C) was done based on equations (2). Due to unfavorable nutrient conditions both species indicated moderate photosynthetic performance:

	DT	N
Φ_p	0.51	0.38
f	0.56	0.32

In fact, just one third of PS2 reaction centers were active in the case of *Nannochloris sp.*, and about 50% for *Dunaliella tertiolecta.*

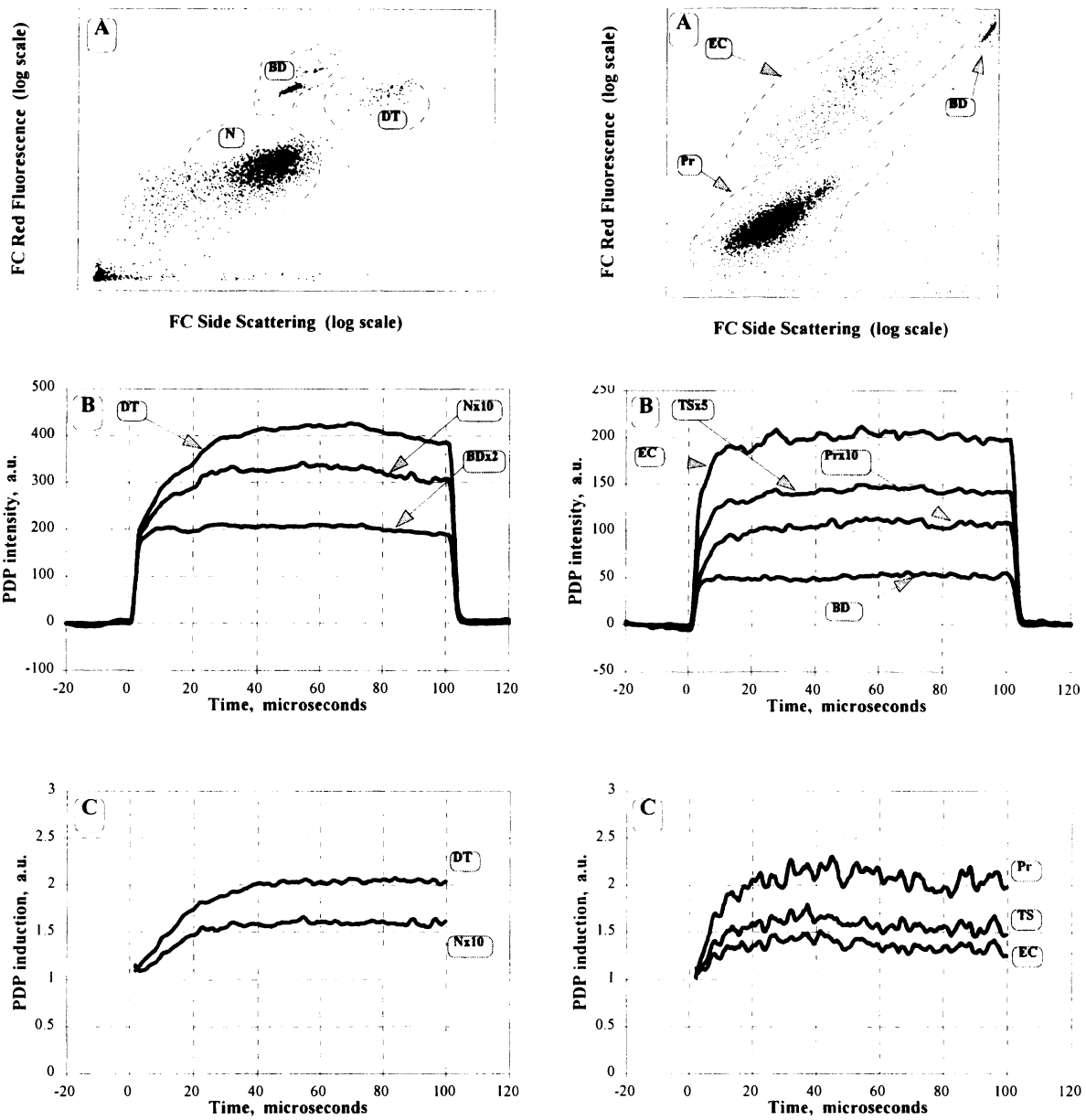

Fig. 6 PDP FC measurement of a mixture of *Dunaliella tertiolecta* (DT) and *Nannochloris sp.* (N), and 1-μm red beads (BD). The solid-state green laser (100 mW, 532 nm) was used. Panel **A**: FC red fluorescence vs. FC side scattering (5,000 events); **B**: PDP intensity profiles for different sample componetnts, reconstructed based on individual PDP curves; panel **C**: PDP induction curves for two species, obtained by normalizing corresponding PDP intensity profiles to BD PDP profile.

Fig. 7 Analysis of group-specific PDP induction curves for a natural sample of sea water (279 Oceanus Cruise, June 1996, Gulf Stream, Chl maximum at 80 m). Panel **A**: prochlorophytes (**Pr**) and small eukaryotic phytoplankton (**EC**) are well defined along with 2-μm Nile Red beads. Panel **B**: PDP intensity profiles for different sample components. Panel **C**: PDP induction curves for two phytoplankton groups (**Pr** and **EC**) and a curve averaged over these two groups (**TS**).

4. FIELD APPLICATION

The operation of the PDP flow cytometer was tested at sea during R/V Oceanus cruise 279 (June 1996). An example of group-specific PDP induction curves for natural populations is presented in Fig. 7. Note the change in relative intensity of red fluorescence per particle between beads and phytoplankton cells in the FC (upper panel) and PDP (middle panel) modes. This can be explained by strong saturation of Chl phytoplankton fluorescence in the powerful FC laser beam. Normalization of the group-specific PDP induction profiles to the PDP profile for beads (middle panel) allows us to obtain the phytoplankton group-specific PDP induction curves (lower panel) and photosynthetic characteristics presented in the table:

	Prochlorophytes	Eukaryotic cells	Total sample
PS2 efficiency, Φ_p	0.52	0.31	0.42
Fraction of active RCs, f	0.59	0.27	0.43
Abs. cross-section, a.u.	0.16	0.11	0.14

The photosynthetic functional state of the smallest phytoplankton cells (**Pr**) appeared to be better than that of the larger eukaryotic group (59% vs. 27% of functional RCs) in the same sample, and the **TS** column indicates intermediate values, as would be obtained with techniques based on bulk measurements. This example illustrates how group-specific analysis of photosynthetic characteristics can improve evaluation of the "health" of the phytoplankton community.

5. CONCLUSION

By combining pump-during-probe measurements of individual phytoplankton cells with conventional flow cytometric measurements of light scattering and fluorescence, we can obtain information about the photosynthetic characteristics of different groups of cells in natural populations. This capability will be valuable in investigations of the regulation of phytoplankton growth and productivity; for example, it should help to elucidate the responses of phytoplankton of different size classes to nutrient limitation.

6. REFERENCES

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