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Autofluorescence and other optical properties as tools in biological oceanography

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ABSTRACT

Bulk fluorescence measurements have been popular in algal culture studies and in oceanographic and limnological applications. Usually, fluorescence is interpreted as an indicator of chlorophyll concentration or phytoplankton biomass, but sometimes measurements of fluorescence can be related to physiological properties of phytoplankton, such as responses to light. Now that *in situ* fluorometers are being deployed routinely with optical packages, there is active interest in interpreting the relationships between fluorescence, beam transmission, diffuse attenuation, and the physiological characteristics of phytoplankton. Flow cytometry offers the potential to extend these interpretations to the scale of individual cells. It may be difficult to compare measurements of fluorescence, however, because instruments differ greatly in excitation irradiance and time scale of measurement. With this in mind, we examined the short-term responses of a marine diatom to bright light, comparing different instruments (SeaTech *in situ* fluorometer, Turner Designs fluorometer, EPICS flow cytometer, FACS Analyzer, SeaTech beam transmissometer) while making concurrent measurements of photosynthesis *vs* irradiance and absorption spectra. Each fluorometer yielded somewhat different information, yet all showed a similar pattern of inhibition after exposure. One instrument, the *in situ* pulsed fluorometer, could show rapid changes of fluorescence immediately after large shifts of irradiance. Beam attenuation did not decline with the bright light treatment, nor did the specific absorption of chlorophyll. Photosynthetic efficiency was reduced after exposure to bright light, but the capacity for photosynthesis in high irradiance increased at the same time. These results are preliminary: nonetheless they support some interpretations of fluorescence/beam attenuation ratios, clarify some aspects of photosynthetic response to bright light, and suggest that flow cytometry may be useful for assessing physiological heterogeneity in phytoplankton assemblages.

1. INTRODUCTION

The fluorescence of chlorophyll is one of the principal measurements for studying biological processes in the upper ocean. Fluorescence is at best an imprecise indicator of chlorophyll concentration; however, variability of fluorescence per unit chlorophyll can be corrected for and interpreted[1]. Variations of fluorescence yield can sometimes be related to photosynthesis[2,3], physiological state[4,5], cellular pigment concentration[5], and taxonomic characteristics[6,7]. These processes and properties are responsive to physical forcing in the ocean. In order to study physical-biological coupling in the upper ocean it is therefore necessary to interpret patterns of fluorescence in terms of biological processes. In doing this it is important to determine to what extent different measurements of fluorescence are comparable and to examine the relationships between fluorescence and other optical measurements such as beam transmission and diffuse attenuation.

1.1. Differences between instruments that measure fluorescence

Fluorescence can be stimulated and measured in many ways (e.g., *in situ* fluorometry[8], flow-through fluorometry[9], discrete sample *in vivo* fluorometry[10], flow cytometry[11], and LIDAR[12]). To obtain information on the status of the photochemical apparatus, samples can be manipulated prior to measurement (e.g., pump-and-probe fluorometry[13], DCMU-enhanced fluorescence[3,14,15], induction-curve measurements[16]). Solar-induced fluorescence is measured with no manipulation of the sample at all[17]. Each type of measurement has its own qualities and interpretations, many of which have not been fully explored.

The fluorescence of chlorophyll *in vivo* is variable with time and excitation irradiance (Fig. 1) and the temporal scales for measurement and the excitation intensities of various fluorometers range over several orders of magnitude (Fig. 2). Clearly, there is reason to conclude that various instruments that measure fluorescence might yield distinct information about biological processes in the ocean. The newly-introduced fluorometric instruments that offer promise for advancing biological oceanography (flow cytometers, *in situ* fluorometers, laser fluorometers[18], LIDAR) are quite different from the bench-top fluorometers on which many interpretations of fluorescence have been developed. How comparable are different measurements of fluorescence?

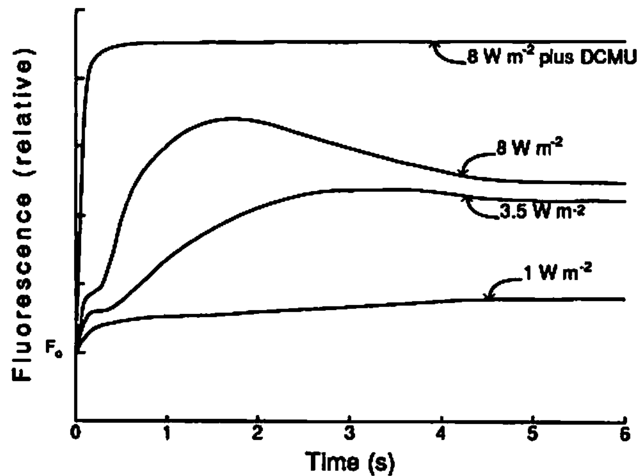

Figure 1. Variability of fluorescence with time and excitation irradiance (redrawn from Krause and Weiss 1984)[19]. The variability with time is called fluorescence induction and is related to photosynthesis. The photosynthetic inhibitor DCMU blocks non-cyclic photosynthetic electron flow. Photosynthetic effects are thereby eliminated and fluorescence rises rapidly to a maximum. Curves such as these are frequently interpreted in physiological studies[3].

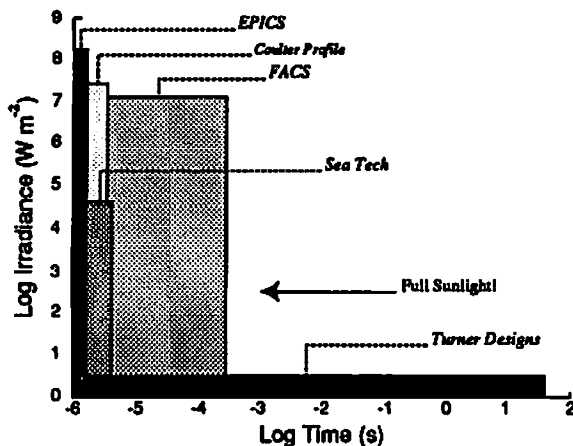

Figure 2. Excitation irradiance and time of exposure and detection for different instruments that measure fluorescence. Rough estimates. Note the log scale.

1.2. Relationships between fluorescence and other optical properties in the upper ocean

In many studies, spatial and temporal variations of fluorescence in the ocean are interpreted in terms of chlorophyll concentration or phytoplankton biomass. If chlorophyll concentration is determined, or if samples are enclosed, it is sometimes possible to interpret spatial or temporal patterns of fluorescence yield[1,4], but resolution is restricted by the limitations of discrete sampling. It would be better to relate fluorescence to other relevant measurements, such as beam transmission and diffuse attenuation, which are made on a comparable scale. Efforts to do this are well underway[20,21,22,23]. In our opinion, these efforts can be enhanced by developing further the theoretical[24] and experimental bases for interpreting the relationships between fluorescence, absorbance, transmissivity, and the physiological status of marine phytoplankton.

Fluorescence, transmissivity, diffuse attenuation, and primary productivity are bulk properties of near-surface waters in the open ocean that are primarily attributable to individual phytoplankton cells. Heterogeneity in the phytoplankton is the key to ecological succession and to concurrent changes in the optical properties of the water. The heterogeneity within a parcel of water can be assessed by flow cytometry. In order to relate optical properties of individual cells to the optical properties of bulk water, it is useful to compare diverse measurements made on the same parcels of water. We describe here some experiments in which fluorescence was measured several ways during exposure of a phytoplankton suspension to bright light. Absorbance, beam transmission, chlorophyll and photosynthesis were also measured. The results may be of some use for interpreting patterns of fluorescence in the upper ocean and for using flow cytometry for assessment of physiological heterogeneity in the phytoplankton.

2. RESPONSES OF A MARINE DIATOM TO BRIGHT LIGHT

2.1. Experimental method

2.1.1. Experimental organism. A culture of the marine diatom *Thalassiosira pseudonana* (clone 3H) was grown in f/2 media[25] at 20°C under fluorescent lighting (photosynthetically active photon flux density, PPFD = 40 $\mu mol \cdot m^{-2} s^{-1}$; 12 h:12 h light:dark cycle). Several hours into the light period, the exponential-phase culture was transferred to an experimental vessel for observation during exposure to bright light and subsequent recovery.

2.1.2. Experimental vessel. The transfer was made with a Masterflex tubing pump into a 20-liter polycarbonate vessel filled with filtered seawater (Whatman GF/F). A second head on the pump was used to withdraw water from the large vessel at the same rate, so a constant volume (which was stirred continuously) was maintained. The vessel contained a 25 cm pathlength SeaTech beam transmissometer (660 nm) and a SeaTech *in situ* fluorometer. The zinc anti-corrosion blocks were removed from the transmissometer. Both instruments were rinsed very well before the experiment and were blanked in filtered sea water and checked in deionized water before and after the experiment. During the experiment, fine bubbles accumulated slowly on the upper surface of the transmissometer, changing the response slightly. The effect of the bubbles was corrected for by clearing the surface and adjusting the record, assuming linear changes of transmission between cleanings.

After transfer, the phytoplankton suspension was maintained in ambient laboratory lighting (about 20 $\mu mol \cdot m^{-2} s^{-1}$). At time zero, two banks of tungsten-halogen lamps were illuminated, providing light of about 3000 $\mu mol \cdot m^{-2} s^{-1}$. Illumination was not uniform, but it was bright enough to induce measurable responses. Water-filled heat filters and external cooling coils were used to maintain the temperature near 20°C. The lights were later extinguished and recovery was observed.

2.1.3 Sampling. The tubing pump was used to remove samples. As the algal suspension was removed, an equal amount of filtered seawater was pumped into the vessel. Measurements are corrected for dilution as appropriate. Aliquots were taken for flow cytometry (EPICS flow cytometer and FACS Analyzer), and measurements of chlorophyll concentration[26], dark-adapted *in vivo* fluorescence ± DCMU (F, F_d) using a Turner Designs 10-005R fluorometer[3], absorption spectra of material retained on a GF/F filter[27], and photosynthesis *vs* irradiance (P-I)[28]. The P-I relationship was described by fitting the data to the model of Platt *et al.*[29] using the derivative-free method in the NLIN procedure of SAS. The error on P_{max} was calculated from the output[30].

2.2. Results and discussion

2.2.1. Fluorescence in situ and beam attenuation. Measurements were made *in situ* for more than 3.5 h with the bright lights on from 90 min to 162 min (Fig. 3). The *in situ* fluorometer showed rapid inhibition and recovery of fluorescence with large changes within 30 s. The initial increase of fluorescence in bright light was not observed in preliminary experiments, but was repeated in a subsequent exposure at 245 min (not shown). Beam attenuation changed slowly during the experiment and showed no rapid response to the bright-light exposure.

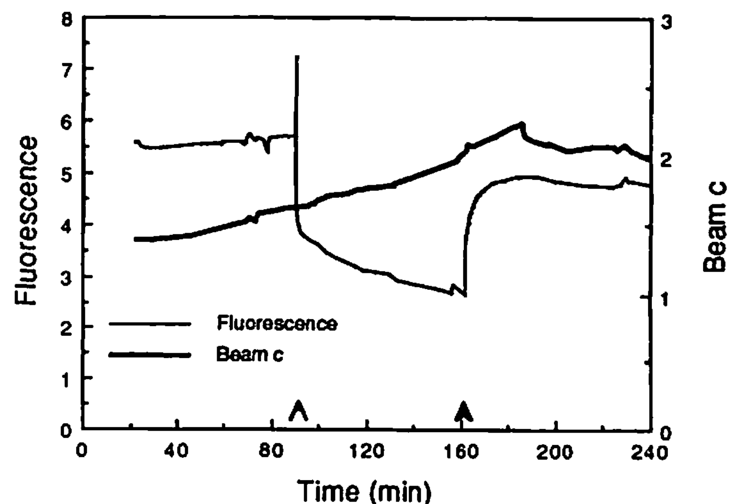

Figure 3. Measurements made *in situ* on a suspension of the marine phytoplankter *Thalassiosira pseudonana*. Exposure to bright light was from 90 min to 162 min. Light line: fluorescence (relative units, linear scale). Heavy line: beam attenuation , c (m^{-1}) Outlined arrow: lamps illuminated. Filled arrow: lamps extinguished.

2.2.2. Fluorescence. Fluorescence was measured in five different ways (Fig. 4). The inhibition of fluorescence by bright light was clearly discernible in each record, but the magnitude of response differed between instruments. The largest decline, about 50%, was recorded by the *in situ* fluorometer followed in order by F_d, F, FACS fluorescence per cell, and EPICS fluorescence per cell.

Only the *in situ* fluorometer could measure responses on a time scale of seconds, so it is difficult to compare rigorously the kinetics of each type of fluorescence response. Nonetheless, it seems clear that the measurements made on discrete samples assessed processes that change on a time scale on the order of one hour[31,32], whereas the *in situ* fluorometer might observe a more rapidly-changing component as well (Fig. 4). The distinction seems clearest during the recovery phase: the *in situ* measurement rebounded much more rapidly than the others.

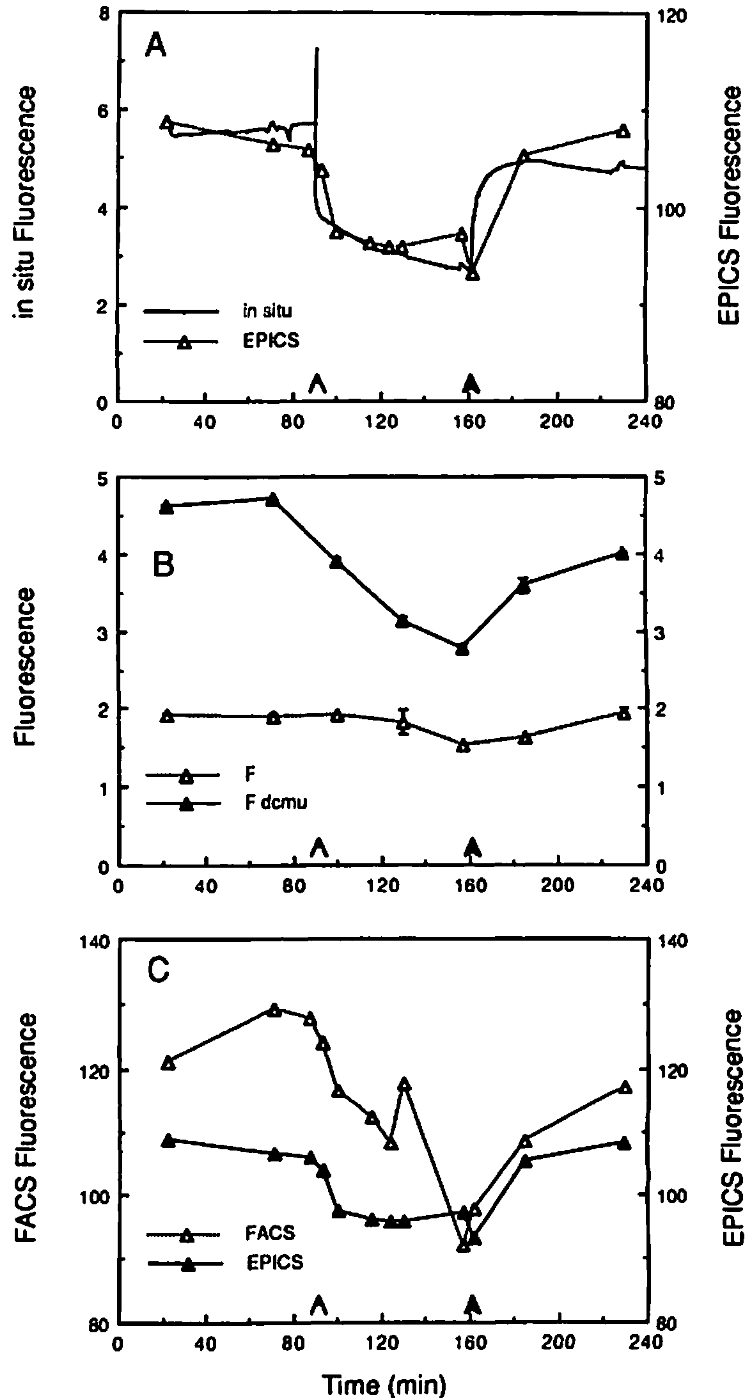

Figure 4. *Thalassiosira pseudonana*: different measures of fluorescence during exposure to bright light and subsequent recovery. Outlined arrow: lamps illuminated. Filled arrow: lamps extinguished. A. *In situ* fluorescence and EPICS fluorescence per cell (linear scale). Note that scaling is adjusted so that kinetics can be visually compared. B. Fifteen-minute dark-adapted fluorescence (±s.e.) as measured with a Turner Designs fluorometer in the absence (F) and presence (F_d) of the photosynthetic inhibitor DCMU. C. Fluorescence per cell, comparing measures from the EPICS flow cytometer and FACS Analyzer (linear scale).

2.2.3. Beam attenuation. The decline of fluorescence in bright light was not accompanied by a comparable change in beam attenuation (Fig. 3). This experimental result validates the interpretation that beam transmission is a relatively conservative property to which fluorescence can be normalized when interpreting apparent photoinhibition of fluorescence near the sea-surface. Attenuation did change significantly over several hours, however. The nature of such changes of beam attenuation should be understood in order to interpret diel or longer-scale patterns in the ratio of fluorescence to transmissivity.

The change in beam attenuation can be compared with other properties of the phytoplankton. Chlorophyll concentration was nearly constant during the experiment (fig. 5A). The concentration of cells, determined microscopically with a precision no better than ±10%, increased about 20% during the first 200 minutes. The chlorophyll-specific attenuation coefficients changed little (Fig. 5A). Scattering by the phytoplankton cells, as measured by the EPICS flow cytometer, showed at best a small proportional change over the course of the experiment, although the temporal pattern of forward angle light scatter was nearly the same as that of beam attenuation (Fig. 5B).

A more thorough experimental assessment of the relationships between beam transmission and other optical properties of phytoplankton suspensions is warranted. The influence of bacteria must be assessed and cellular scattering should be treated quantitatively. The results presented here are nonetheless useful in showing that large changes in fluorescence were not accompanied by changes of absorption. In some large phytoplankton, though, chloroplast migration can lead to a substantial reduction of absorbance after exposure to bright light[4].

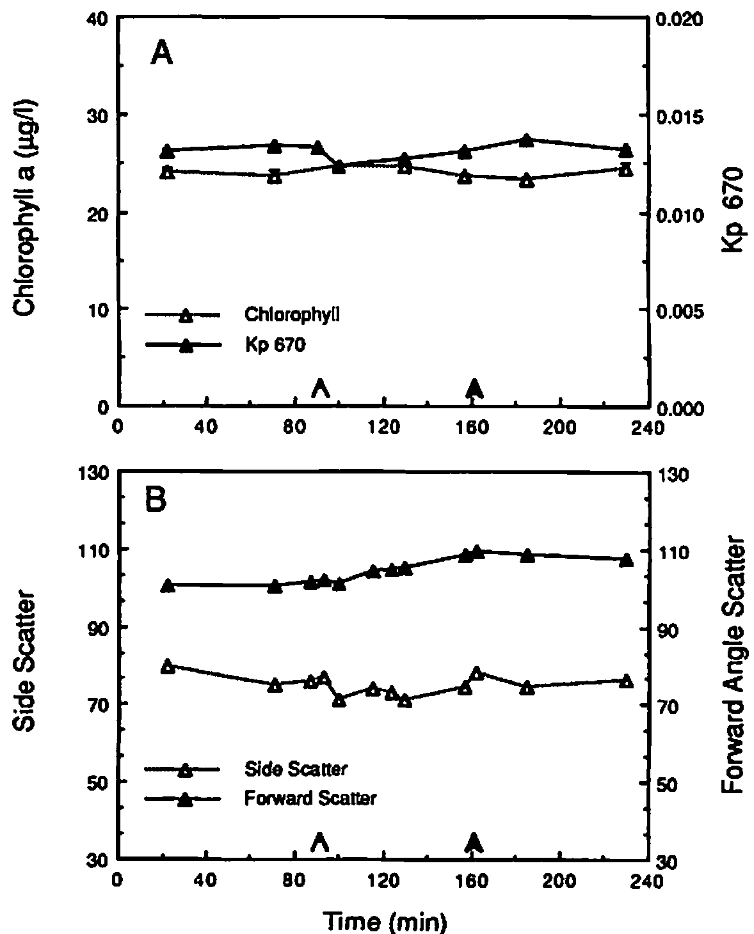

Figure 5. *Thalassiosira pseudonana*: responses during exposure to bright light. Exposure from 90 min until 162 min. Outlined arrow: lamps illuminated. Filled arrow: lamps extinguished. A. Chlorophyll *a* concentration (µg·l⁻¹) and attenuation coefficient at 670 nm (K_p670: $m^2 \cdot mg\,Chl^{-1}$). The shapes of the absorption spectra changed very little over the course of the experiment. B. Side scatter (90°) and forward angle light scatter, population means for cells, EPICS flow cytometer.

2.2.4 Photosynthesis. Photosynthetic performance changed after the suspension was exposed to bright light. The capacity for photosynthesis (generally equated with P_{max}) did not decline during this experiment, but photosynthetic efficiency (here indicated by α, the initial slope of the P-I curve) was reduced by about 1/3 after after the lights were turned on (Fig. 6A). The change is roughly consistent with the determinations of fluorescence increase on addition of DCMU, an indicator of photosystem II activity[3] (Fig. 6B). The reduction of photosynthetic efficiency in bright light has been observed in nature and in the lab and has been compared with fluorescence response[31]. When inhibition is severe, photosynthetic capacity also declines and inhibition

of photosynthesis at ambient irradiance is observed[32,33]. During this experiment, inhibition was not so severe[34]. The inhibition of fluorescence in bright light apparently did reflect a decline in photosynthetic performance, but because efficiency was reduced rather than capacity, photosynthesis in bright light actually increased, perhaps as a manifestation of adaptation to high PPFD[35] (Fig. 6C).

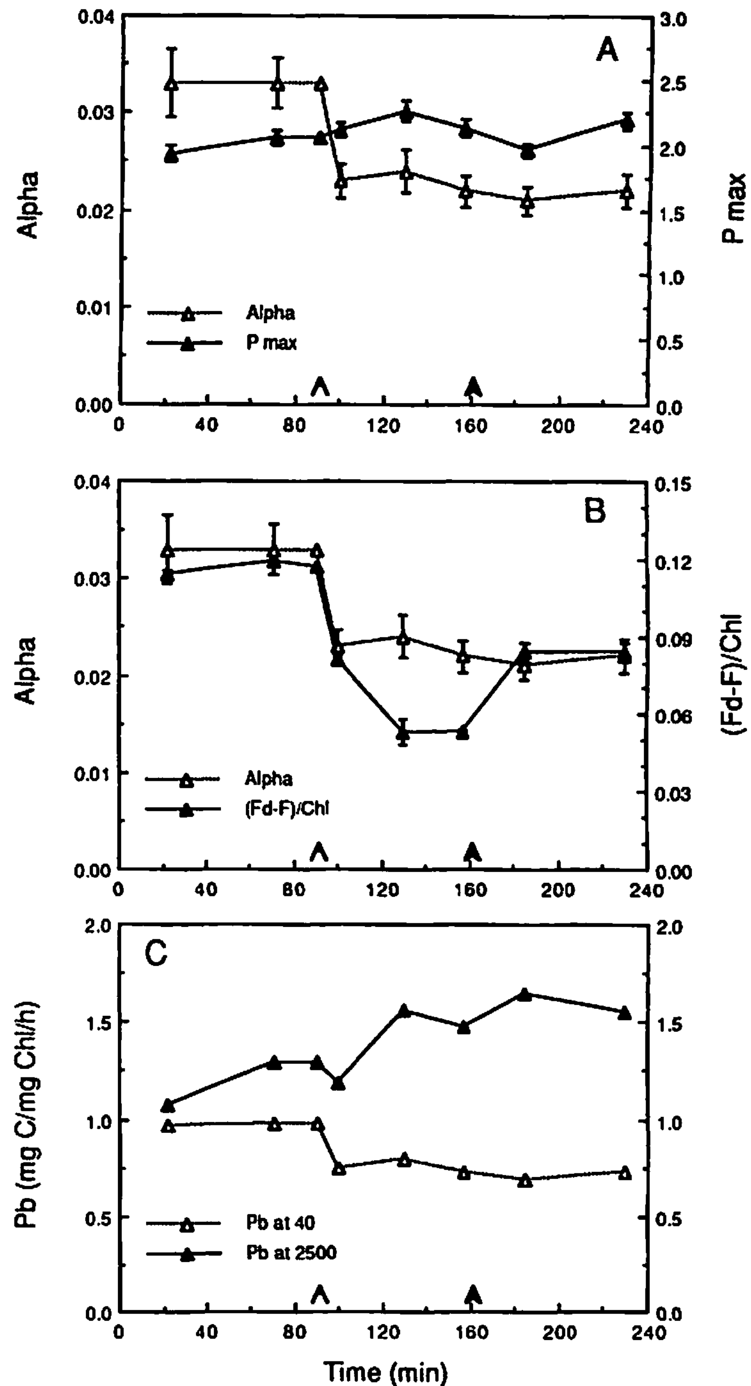

Figure 6. *Thalassiosira pseudonana*: photosynthetic performance and fluorescence parameters. Outlined arrow: lamps illuminated. Filled arrow: lamps extinguished. **A.** Photosynthetic parameters: α (g C·(g Chl *a*)$^{-1}$·h^{-1}) · (μmol·m^{-2}·s^{-1})$^{-1}$, the initial slope of the P-I curve; P_{max} (g C·(g Chl *a*)$^{-1}$·h^{-1}), maximum photosynthesis. Error bars are ± s.e. for 36-point curves, incubated for 20 min. **B.** Comparison of α and the photosynthetic parameter (F_d - F)/Chl. Error bars are ± s.e. **C.** Photosynthesis (Pb: mg C·mg Chl a^{-1}·h^{-1}) at 40 and 2500 μmol·m^{-2}·s^{-1}, calculated from the P-I curves. Note that "photoinhibition" can have several meanings and that during adaptation one aspect of photosynthetic performance can increase while another declines.

3. CONCLUSIONS

Modern oceanographers have a number of new instruments at their disposal, many of which measure optical properties attributable to phytoplankton. Interpretations of the measurements often rely on assumptions about how measurements from the field relate to those made in the lab with different instruments. It is extremely useful to verify such assumptions. We have taken a step in this direction, describing a few relationships between fluorescence, beam transmission, absorbance, and the photosynthetic characteristics of phytoplankton. Each fluorometer yielded somewhat different information, yet all showed a similar pattern of inhibition after exposure. The flow cytometers showed this response in individual cells[24]. One instrument, the *in situ* pulsed fluorometer, could show rapid changes of fluorescence immediately after large shifts of irradiance. Beam attenuation did not decline during the bright light treatment, nor did the specific absorption of chlorophyll. These results are relevant to studies of vertical mixing[25] and the optical characteristics of phytoplankton: they indicate that the *in situ* fluorometer and beam transmissometer are very useful when used together[20-22]. Photosynthetic efficiency declined after exposure to bright light, but the capacity for photosynthesis in high irradiance increased at the same time. Thus, equivalence of photoinhibition of fluorescence and photoinhibition of photosynthesis cannot be assumed[26]. Our results are preliminary: nonetheless they support some interpretations of fluorescence/beam attenuation ratios, clarify some aspects of photosynthetic response to bright light, and suggest that flow cytometry may be useful for assessing physiological heterogeneity in phytoplankton assemblages.

4. ACKNOWLEDGEMENTS

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5. REFERENCES

1. J.J. Cullen, "The deep chlorophyll maximum: comparing vertical profiles of chlorophyll *a*," Can. J. Fish. Aquat. Sci. 39, 791-803 (1982).
2. B.B. Prézelin and A.C. Ley, "Photosynthesis and chlorophyll *a* fluorescence rhythms of marine phytoplankton," Mar. Biol. 55, 295-307 (1980).
3. W.F. Vincent, P.J. Neale and P.J. Richerson, "Photoinhibition: algal responses to bright light during diel stratification and mixing in a tropical alpine lake," J. Phycol. 20, 201-211 (1984).
4. D.A. Kiefer, "Chlorophyll *a* fluorescence in marine centric diatoms: responses of chloroplasts to light and nutrient stress," Mar. Biol. 23, 39-46 (1973).
5. J.S. Cleveland and M.J. Perry, "Quantum yield, relative specific absorption and fluorescence in nitrogen-limited *Chaetoceros gracilis*," Mar. Biol. 94, 489-499 (1987).
6. S.I. Heaney, "Some observations on the use of the *in vivo* fluorescence technique to determine chlorophyll *a* in natural populations and cultures of freshwater phytoplankton," Freshw. Biol. 8, 115-126 (1978).
7. G.P. Harris, "The relationship between chlorophyll *a* fluorescence, diffuse attenuation changes and photosynthesis in natural phytoplankton populations," J. Plankton Res. 2, 109-127 (1980).
8. R. Bartz and R.W. Spinrad, "Low power, high resolution, *in situ* fluorometer for profiling and moored applications," Proc. SPIE 925 (1988).
9. C.J. Lorenzen, "A method for the continuous measurement of *in vivo* chlorophyll concentration," Deep-Sea Res. 13, 223-227 (1966).
10. W.F. Vincent, "Mechanisms of rapid photosynthetic adaptation in natural phytoplankton communities. II. Changes in photochemical capacity as measured by DCMU-induced chlorophyll fluorescence," J. Phycol. 16, 568-577 (1980).
11. C.M. Yentsch, P.K. Horan, K. Muirhead, Q. Dortch, E. Haugen, L. Legendre, L.S. Murphy, M.J. Perry, D.A. Phinney, S. A. Pomponi, R.W. Spinrad, M. Wood, C.S. Yentsch and B.J. Zahuranec, "Flow cytometry and cell sorting: a technique for analysis and sorting of aquatic particles," Limnol. Oceanogr. 28, 1275-1280 (1983).
12. F.E. Hoge and R.N. Swift, "Airborne simultaneous spectroscopic detection of laser-induced water Raman backscatter and fluorescence from chlorophyll *a* and other naturally occuring pigments," Appl. Opt. 20, 3197-3205 (1981).
13. P.G. Falkowski, K.D. Wyman, A.C. Ley and D.C. Mauzerall, "Relationship of steady state photosynthesis to fluorescence in eukaryotic algae," Biochem. Biophys. Acta 849, 183-192 (1986).
14. J.J. Cullen and E.H. Renger, "Continuous measurement of the DCMU-induced fluorescence response of natural phytoplankton populations," Mar. Biol. 53, 13-20 (1979).
15. S. Roy and L. Legendre, "Field studies of DCMU-enhanced fluorescence as an index of *in situ* phytoplankton photosynthetic activity," Can. J. Fish. Aquat. Sci. 37, 1028-1031 (1980).

16. S. Demers, J.-C. Therriault, L. Legendre and J. Neveux, "An *in vivo* fluorescence method for the continuous *in situ* estimation of phytoplankton photosynthetic characteristics," Mar. Ecol. Prog. Ser. 27, 21-27 (1985).
17. B.J. Topliss and T. Platt, "Passive fluorescence and photosynthesis in the ocean: implications for remote sensing," Deep Sea Res, 33, 849-864 (1986).
18. D.A. Phinney and C.S. Yentsch, "Three color laser fluorometer for studies of phytoplankton fluorescence," Proc. SPIE 925 (1988).
19. G.H. Krause and E. Weiss, "Chlorophyll fluorescence as a tool in plant physiology. II. Interpretation of fluorescence signals," Photosynthesis Res. 5, 139-157 (1984).
20. H. Pak, "Characteristic variations of bio-optical properties in the central North Pacific," Proc. SPIE 637, 314-320 (1986).
21. T.D. Dickey, D.A. Siegel, A. Bratkovitch and L. Washburn, "Optical features associated with thermohaline structures," Proc. SPIE 637, 308-313 (1986).
22. J. Marra, "Ocean processes and their optical properties," (Abtract) EOS 68, 1703 (1987).
23. B.G. Mitchell and D.A. Kiefer, "Optical prediction of photobiological properties during Racer," (Abtract) EOS 68, 1685 (1987).
24. R.W. Spinrad, "Use of the specific beam attenuation coefficient for identification of suspended particulate material," Proc. SPIE 637, 135-147 (1986).
25. R.R.L. Guillard , "Culture of phytoplankton for feeding marine invertebrates," in *Culture of Marine Invertebrate Animals*, W.L. Smith and M.H. Chanley, eds., Plenum Press, New York (1975).
26. T.R. Parsons, Y. Maita and C.M. Lalli, *A Manual of Chemical and Biological Methods for Seawater Analysis*, Pergamon Press, New York (1984).
27. D.A. Phinney and C.S.Yentsch, "The relationship between phytoplankton concentration and light attenuation in ocean waters," Proc. SPIE 637, 321-327 (1986).
28. M.R. Lewis and J.C. Smith, "A small volume, short incubation method for measurement of photosynthesis as a function of incident irradiance," Mar. Ecol. Prog. Ser. 15, 141-149 (1983).
29. T. Platt, C.L. Gallegos and W.G. Harrison, "Photoinhibition of photosynthesis in natural assemblages of marine phytoplankton," J. Mar. Res. 38, 687-701 (1986).
30. R.F. Davis, M. Zhu and J.J. Cullen, "Measurement of primary production in turbid waters," Submitted to Limnol. Oceanogr. (1988).
31. P.J. Neale, "Algal photoinhibition and photosynthesis in the aquatic environment," in *Photoinhibition*, D. Kyle, C.J. Arntzen and B. Osmond, eds., Elsevier, Amsterdam (1987).
32. J.J. Cullen and M.R. Lewis, "The kinetics of algal photadaptation in the context of vertical mixing in the sea," Submitted to J. Plankton Res. (1988).
33. J. Marra, "Effect of short-term variation in light intensity on photosynthesis of a marine phytoplankter: a laboratory simulation study," Mar. Biol. 46, 191-202 (1978).
34. E. Sakshaug, S. Demers and C.M. Yentsch, "*Thalassiosira oceanica* and *pseudonana*: two different photoadaptational responses," Mar. Ecol. Prog. Ser. (in press) (1988).
35. M.L. Lewis, J.J. Cullen and T. Platt, "Relationships between vertical mixing and photadaptation of phytoplankton: similarity criteria," Mar. Ecol Prog Ser. 15, 141-149 (1984).
36. P. Falkowski and D.A. Kiefer, "Chlorophyll *a* fluorescence in phytoplankton: relationship to photosynthesis and biomass," J. Plank. Res. 7, 715-731 (1985).