In Vivo Fluorescence/chlorophyll $a$ ratio as an ecological indicator in oceanography*

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SUMMARY: This article reviews the main factors affecting the in vivo fluorescence versus chlorophyll relationships of phytoplankton and presents a case study based on data from three oceanographic cruises carried out, at different times of the year, in the Catllan-Balearic Sea. In all three surveys, the in vivo fluorescence/chlorophyll ratio of the upper euphotic layer samples presented a diel variability with a minimum at or before noon time. The relationships between the spatio-temporal distribution of this variability and characteristics of photosynthesis versus irradiance curves obtained during each cruise are discussed.

Key words: Phytoplankton, fluorescence to chlorophyll ratios, quenching, diel variability.

INTRODUCTION

Phytoplankton distributions present patchiness at a wide range of temporal and spatial scales. This feature has important implications in the functioning of the pelagic ecosystem (Margalef, 1958; Platt and Denman, 1980, Harris, 1980). Earlier studies on this subject (Bainbridge, 1957) were constrained by the lack of adequate sampling techniques. The development, in the late sixties, of equipment for continuous monitoring of in vivo fluorescence of chlorophyll (Lorenzen, 1966; Platt, 1972) allowed high resolution sampling of a phytoplankton biomass indicator (Platt and Denman, 1975, Estrada and Wagensberg, 1977). The application of this methodology to the study of vertical distributions, showed also a rich structure at different spatial scales (Derenbach et al., 1979).

The recording of fluorescence has provided meaningful information on phytoplankton distribution patterns in the sea (Harris, 1986). However, chlorophyll fluorescence varies in response to a series of environmental conditions in addition to chlorophyll concentration (Strickland, 1968; Blasco, 1973; Kiefer, 1973a; Loftus and Seliger, 1975), which is itself a poor index of biomass, because the chlorophyll/carbon ratio changes according to species, light regime and nutritional state, among other factors (Eppley, 1980). These limitations have not invalidated the use of the in vivo fluorescence technique, which continues to be the best choice for automatic measurement of an index of phytoplankton biomass, albeit with the need of frequent calibration (Harris, 1986). On the other hand, the study of the variability of the in vivo fluorescence (IVF) to chlorophyll $a$ (Chl) ratio ($R = \text{IVF}/\text{Chl}$) itself opened the way to interesting insights on ecological aspects of the aquatic ecosystem.

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The early fluorometric work on oceanography was carried out with filler fluorometers provided with a continuous light source of low intensity. At present, most of the instruments used in field surveys are based on single-flash excitation (Cunningham, this volume). The recent appearance of sophisticated fluorometry equipment such as modulated beam (PAM; Hofstraat et al., 1994) and multiple flash systems (pump-probe and fast repetition rate fluorometers; Kolber et al., 1990; Kolber and Falkowski, 1993; Greene et al., 1994) has allowed the application to phytoplankton of more flexible and powerful experimental techniques. In addition to the methods based on the above mentioned instruments, which measure actively stimulated fluorescence, other techniques have been based on the recording of natural (solar-induced or passive) fluorescence (Topliss and Platt, 1986).

The field use of modulated beam and multiple flash systems is still restricted due to technological and economic constraints. However, much information can be gained from careful operation of standard oceanographic fluorometers, in connection with additional determinations of parameters like extracted chlorophyll concentration. This article will review sources of variability in the in vivo fluorescence to chlorophyll ratio of phytoplankton and will present a case study, based on measurements obtained with single flash fluorometers in the Catalan-Balearic Sea (NW Mediterranean, Fig. 1).

Physiological basis of in vivo fluorescence variability

The photochemical conversion in eukaryotic cells occurs through the connection of photosystems I (PSI) and II (PSII). In PSII, the absorption of light produces oxidation of water, whereas in PSI, the absorption of light is used to reduce the intermediate metabolites involved in the fixation of CO₂ (Krause and Weis, 1991). Electrons are transferred between the two photosystems through a series of intermediate molecules. Fluorescence is generated basically in PSII, and has been used, together with oxygen production, in the measurement of the quantum yield of PSII photochemistry.

The fluorescence emission per unit chlorophyll, F, can be expressed (Lawlor, 1987; Geider et al., 1993a) as:

![Fig. 1. - Map of the sampling stations of the cruises FR91 (April 91), FR92 (October 92) and FR93 (June 93).](image-url)
where

\[ F = I \alpha' \Phi_f \]

\( I \) = incident photon flux density (quanta m\(^{-2}\) s\(^{-1}\))
\( \alpha' \) = chlorophyll-specific light absorption coefficient; it has the dimensions of a cross-section of chlorophyll \( a \) m\(^2\) (mg Chl\(^{-1}\))
\( \Phi_f \) = quantum efficiency of the fluorescence emission (dimensionless).

Both \( \alpha' \) and \( \Phi_f \) are wavelength-dependent; thus, their value will depend on the amount of quanta corresponding to each wavelength in the total radiant flux, \( I \). The adequate spectrally-averaged values will have to be considered in each case (Dubinsky, 1992).

The quantum efficiency of the fluorescence emission is determined (Butler, 1978; Krause and Weis, 1991) by the rate of loss, as fluorescence, of excitation energy of chlorophyll molecules, divided by the sum of the rates of all processes leading to de-excitation of chlorophyll (mainly thermal dissipation, spillover to Photosystem I and photochemistry). Fluorescence emission (\( F \)) will be minimal (\( F_m \)) in dark adapted cells, when all PSII centers are oxidised (“open”) and will reach a maximum (\( F_m^\infty \)) when all reaction centers are reduced (“closed”).

The photochemical yield of open PSII reaction centers (\( \Phi_{PSII} \)) can be related to fluorescence characteristics by the expression (Butler, 1978):

\[ \Phi_{PSII} = (F_m - F_v)/F_m \text{ or } F_v/F_m, \]

where \( F_v \) (variable fluorescence) = \( F_m - F_0 \).

**Variability of the in vivo fluorescence yield. Photochemical and non-photochemical quenching**

Environmental factors and the physiological state of the cells may alter significantly the in vivo fluorescence emission per unit of chlorophyll \( a \). Differences in chlorophyll absorption cross-sections may be caused by qualitative and quantitative changes in chloroplast pigment composition (Blasco, 1973; Falkowski and Kiefer, 1985) and self-shading within and between chloroplasts, contributing to “package effects” (Kiefer, 1973b; Kirk, 1983). The rate constant of fluorescence emission is supposed to vary little (Butler, 1978; Geider \textit{et al.}, 1993a), and spillover to PSI is generally assumed to be <10% (Malkin \textit{et al.}, 1986; Geider \textit{et al.}, 1993). Thus the major influences on \( \Phi_f \) will come from changes in the rate constants of photochemistry (\( k_h \)) and thermal dissipation (\( k_p \)). \( k_p \) may be affected by damage to PSII reaction centers, due to nutrient limitation or exposure to high light (Falkowski, 1992; Falkowski and Kolber, 1993). Rapid reversible reduction of the in vivo fluorescence yield under bright light may be caused by changes in \( k_p \) due to interactions between the xanthophyll cycle and the chlorophyll molecules of the antennae of PSII (Krause and Weis, 1991, Falkowski and Kolber, 1993, Oizilota and Yamamoto, 1994). Other mechanisms of dissipation of excess light energy could involve reversible changes in structure and turnover of certain PSII proteins (Critchley and Russell, 1994). The variations of in vivo fluorescence caused by the opening or closure of reaction centers are called photochemical quenching. In contrast, decreases in the quantum yield of fluorescence caused by factors that are not related to photochemistry have been called non-photochemical quenching (Kiefer and Reynolds, 1992). The term photoinhibition has been often used in relationship to a light-induced decrease in fluorescence yield; properly, this term should be reserved to deal with cases in which damage to PSII reaction centers occurs.

The effects of non-photochemical quenching on the relationships between fluorescence and photosynthesis have been discussed by Kiefer and Reynolds (1992). Based on a model of energy transformations in PSII, Kiefer and Reynolds suggested expressions to calculate the quantum yield of photosynthesis, using fluorescence measurements by the pump-probe technique. However, they recognized that there were still difficulties for testing of their hypotheses in the field.

The temporal changes in fluorescence characteristics of dark adapted cells (fluorescence induction curve) have been often used to measure variable fluorescence (\( F_v \)) yields and to derive parameters such as functional cross-sections of PSII for fluorescence or oxygen production. However, fluorescence induction methods are difficult to apply during steady state photosynthesis (Falkowski \textit{et al.}, 1986). As an alternative, Falkowski \textit{et al.} used the pump and probe technique to investigate the effects of continuous background irradiance on fluorescence yield and oxygen production of unicellular eukaryotic algae. They found that 5-30% of the change in variable fluorescence (the difference between the fluorescence yield induced by the pump and probe flashes) occurred at low irradiances, with negligible oxygen production, and suggested that this effect was due to the formation of transmembrane chemical or electrical potentials. At irradiances exceeding...
360 μE m⁻² s⁻¹ for chlorophyll c-containing organisms and 830 μE m⁻² s⁻¹ for chlorophytes, the fluorescence yield after both the probe and pump flashes declined very rapidly. Up to the highest irradiance tested (about 1600 μE m⁻² s⁻¹), this non-photochemical fluorescence quenching was not accompanied by a decrease in oxygen production.

**Effect of nutrients on the in vivo fluorescence yield**

Blasco (1973), working with continuous cultures of *Skeletonema costatum*, found that R (IVF/Chl) increased with nutrient limitation. In batch cultures, R was constant when the populations grew exponentially, but increased when the cells became deficient in nitrate or phosphate. Similar conclusions were reached by Kiefer (1973b), who reported that *in vivo* fluorescence of chlorophyll a was stronger in nitrogen-starved than in nitrogen-enriched cells of *Thalassiosira pseudonana* (then quoted as *Cyclotella nana*) in continuous cultures. Later studies using pump and probe techniques have shown that the efficiency of PSII photochemistry decreased with nitrogen and iron limitation, while the absorption cross section of PSII increased (Geider et al., 1993a). These can be explained by the loss of certain proteins in the reaction center of PSII, which reduces the efficiency of conversion of light energy to photochemical energy and causes the emission of a larger energy fraction as fluorescence (Falkowski et al., 1992).

**Taxonomic variability of the in vivo fluorescence yield**

Apart from photoacclimation, species-specific variability in pigment composition, chloroplast structure or other characteristics of the cells may produce changes in fluorescence properties. This was already observed in early laboratory studies (Strickland, 1968; Blasco, 1973) and has been confirmed by later work (Vincent, 1983; Cunningham, this volume). Harris (1980) measured the fluorescence response of single cells under the fluorescence microscope and observed that at an excitation energy of 70 Wm⁻², the fluorescence yield of diatoms was rapidly bleached, while this did not happen with chlorophytes. Species-related variations have been found also in natural phytoplankton populations (Kiefer, 1973a; Estrada, 1974; Loftus and Seliger, 1975), but in field observations it may be difficult to separate taxonomic from physiologic effects. Although differences in chloroplast structure may explain some features of certain groups, it is not clear whether phylogenetic generalizations can be made; at least in connection with work using standard oceanographic fluorometers.

**Variability of the IVF/Chl ratio in the field. The Catalano-Balearic sea, a case study**

One of the most important sources of variability of R in field studies is the inverse relation between fluorescence yield and ambient light intensity (Kiefer, 1973a; Loftus and Seliger, 1975; Uehlinger, 1985; Strass, 1990). Surveys in the Baja California (Blasco, 1973) and NW African upwelling (Estrada, 1974) areas have shown significant differences between day and night values of R for surface populations. Another source of variability may be the presence of chlorophyll degradation products. Analyses of the correlation between R on one side and phaeophytin a (or other chlorophyll derivatives) on the other led Kiefer (1973a) and Loftus and Seliger (1975) to conclude that these pigments did not con-

<table>
<thead>
<tr>
<th>Cruise</th>
<th>Date</th>
<th>N</th>
<th>r²</th>
<th>a</th>
<th>b</th>
<th>Np</th>
<th>Ip</th>
<th>zp</th>
<th>Ip</th>
<th>zp</th>
<th>Lm</th>
<th>zm</th>
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<td>04/01-21/01</td>
<td>95</td>
<td>0.89</td>
<td>0.013</td>
<td>0.891</td>
<td>5</td>
<td>755±123</td>
<td>63±8</td>
<td>90±39</td>
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<td></td>
</tr>
<tr>
<td>FR92</td>
<td>15/10-92</td>
<td>74</td>
<td>0.85</td>
<td>0.042</td>
<td>0.397</td>
<td>7</td>
<td>416±137</td>
<td>69±14</td>
<td>142±41</td>
<td>10-20</td>
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</tr>
<tr>
<td>FR93</td>
<td>1/6/93-29/93</td>
<td>264</td>
<td>0.89</td>
<td>0.001</td>
<td>0.411</td>
<td>5</td>
<td>879±52</td>
<td>59±12</td>
<td>322±60</td>
<td>20-30</td>
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tribute significantly to variations of in vivo fluorescence in the photic zone. However, it would be interesting to expand these results with modern techniques of pigment separation.

In the earlier studies, the in vivo fluorescence measurements were obtained by pumping water through a fluorometer on board or by measuring chlorophyll and fluorescence of discrete water samples. In recent years, an extensive data set of R values has been obtained in several cruises conducted by the Institut de Ciències del Mar of Barcelona, using a combination of profiling in situ fluorometer (Seatech) readings with measurements of chlorophyll concentration following acetonic extraction on water sampled, simultaneously or in close succession, using oceanographic bottles. A detailed study of these data will be provided by Marrasè et al., in prep. The surveys considered here were carried out in the Catalano-Balearic Sea (NW Mediterranean, Fig. 1), at different times of the year, between 1991 and 1993 (Table 1). In FR91 and FR92, the fluorescence profiles and the water samples were obtained in successive casts. In FR93, the fluorometer was attached to a CTD and a Rosette sampler.

Preliminary statistical analyses revealed that, while the overall regression of IVF on Chl was highly significant within each set of data, the variability of the residuals with respect to time of the day and depth appeared to show coherent patterns. Thus, the analysis of the data was pursued using the relative error (FRE) of observed fluorescence (IVF) vs fluorescence estimated (IVF̃) from its linear regression on chlorophyll concentration (Chl). As we were particularly interested in ascertaining the variability of IVF in relationship with the environmental light conditions, we decided to consider night time measurements as representative of dark adapted conditions and to take the night time values (ambient irradiance = 0) as the basis for the IVF vs Chl regressions.

The following expressions were calculated separately for each cruise:

\[
IVF \text{ night} = a + b \text{ Chl}_{\text{night}}
\]

where \(a\) = ordinate intercept, \(b\) = regression coefficient only with the IVF and Chl data pairs corresponding to night time measurements.

The predicted fluorescence, IVF̃, corresponding to Chl using the regression coefficients obtained for night time measurements (previous equation) is IVF̃ = a + b Chl; and

\[\text{FRE} = [(\text{IVF}-\text{a}) - (\text{IVF̃}-\text{a})]/(\text{IVF}-\text{a})\]

where FRE is the relative error of IVF̃ with respect to IVF. Thus, FRE represents the relative deviation of an observed IVF value from that (IVF̃) expected from the Chl concentration at 0 ambient irradiance.

The number of night time data points, explained variance \(r^2\), intercept and regression corresponding to each cruise are given in Table 1. The total number of data points ranged was 315 in FR91, 285 in FR92 and 914 in FR93.

The diel variability of the FRE profiles within each cruise was visualized by means of an interpolation program using an inverse of the distance method. The data noise was reduced by calculating, for each sampling interval of 10 m in depth and 3 hours...
in time, the mean ($F_{RE}$) of all $F_{RE}$ values comprised in it. These $F_{RE}$ provided the basis to plot the isolines shown in Figs. 2-4. Using the global regression of IVF on Chl (day + night values) to evaluate $F_{RE}$, instead of the regression for the night values, gave qualitatively similar results. As can be seen in the figures 2 and 3, in FR91 and FR92, $F_{RE}$ starts to decline at about 4:30 and 8 GMT respectively, reaches its minimum value at local noon (12 GMT), at depths between surface (FR91) and 10 m (FR92) and recovers in the afternoon. In FR93, the pattern is similar but the minimum $F_{RE}$ is deeper and occurs at about 8 GMT rather than at noon. In both FR92 and FR93, the increase of $F_{RE}$ in the afternoon seems to be somewhat slower than its decrease in the morning. A relative maximum of $F_{RE}$ was found at depths of 30-40 m, at least during part of the day. Less marked $F_{RE}$ minima appeared to occur below the deep chlorophyll maximum (DCM), between the end of the night and the beginning of the morning; they were centered around 80 m in FR91, 70-80 m in FR92 and 50-60 m in FR93.

In all the cruises, the chlorophyll distribution presented a deep chlorophyll maximum, between 40 and 50 m. It was specially marked in FR93, between 4 and 14 GMT (data not shown), although it was not clear if this enhancement was just a result of spatial heterogeneity or responded to a diel variability in the chlorophyll concentration. The surface phytoplankton was dominated by flagellates (including abundant haptophytes in FR91 and FR93), dinoflagellates and coccolithophorids (Margalef and Estrada, unpublished data). In general, diatoms were scarce and occurred only near the DCM (specially at
some stations of FR92); silicoflagellates were abundant in the lower euphotic zone during FR93.

The possibility of diel changes in stratification of the upper water layer's, due to heating during the day, was explored by means of depth-time graphs of the temperature data (Figs. 2-4). No diel temperature patterns were apparent in the pooled data set. The increased temperature, in FR93, of the upper 5 m of water between 6 and 15 GMT and 17 and 22 GMT, was probably due to spatial variability.

In the natural environment, the vertical distribution of photoacclimation parameters of phytoplankton reflects the combined result of physiological processes tending to produce variations in depth and vertical mixing in the water column tending to homogenize vertical differences (Lewis et al., 1984). Although the spatio-temporal resolution of our data is limited by the averaging process, examination of Figs. 2-4 may be illustrative. The distribution of \$F_{m}'\$ in FR91 (Fig. 2) follows the pattern that can be expected if the time scale of photoacclimation is faster than the time scale of vertical motions. In FR92 (Fig. 3), \$F_{m}'\$ seems to show some degree of vertical homogenization in the upper 20 m. The case of FR93 (Fig. 4) is more difficult to interpret.

As \$F_{m}'\$ represents the relative deviation of measured IVF with respect to the expected IVF at 0 irradiance (night time), a negative \$F_{m}'\$ for a particular group of data points indicates a lower mean fluorescence yield per unit chlorophyll (i.e., a lower mean R) than that expected at 0 irradiance. Thus, the diel \$F_{m}'\$ fluctuations shown by our data can be compared with the diel variability of R described by Kiefer (1973a), Estrada (1974) and Loftus and Seliger (1975). In our data set, the lack of clear diel stratification patterns (Figs. 2-4) indicates that diel fluorescence variation was mainly influenced by light. This contrasts with the observations of Vincent et al. (1984) and Neale (1987), who found that the decrease of surface fluorescence in lake Titicaca was closely coupled to the development of stratification, and did not recover until the night, partly because vertical mixing brought to the surface non-photonhindered algae and partly due to physiological recovery. The diel pattern of decline and fast recovery of \$F_{m}'\$ (reflecting the decline and recovery of the fluorescence yield per unit chlorophyll) in our cruises suggests the operation of photo-inhibitory mechanisms such as those involving the xanthophyll cycle (Olazola and Yamamoto, 1991) or the metabolism of PSII proteins (Crichtley and Russell, 1994).

![Graphs showing photosynthesis versus irradiance relationships for the primary production stations of the different cruises. Curve fits of 7-10 points, using the model of Platt et al. (1980).](image)

Fig. 5. – Photosynthesis versus irradiance relationships for the primary production stations of the different cruises. Curve fits of 7-10 points, using the model of Platt et al. (1980).

Primary production was estimated at several stations of each cruise (Estrada, unpublished data), using the \(^{14}C\) technique. At each station a series of bottles from the surface and the DCM depth were placed in deck incubators and incubated at different light levels for 2 hours. A summary of environmental data and photosynthetic parameters of surface phytoplankton is given in Table 1. These experiments, carried out around mid-day, do not permit analysis of diel changes, but do provide some infor-
mation about the photoacclimation characteristics of phytoplankton irradiance, $I_1$ (calculated as the ratio between the light-saturated chlorophyll $a$-specific rate of photosynthesis and the initial slope of the P-I curve) was relatively low during FR91 and FR92 and high during FR93 (Table 1). This finding may be related to a greater degree of high light acclimation of the phytoplankton community during the latter cruise, in which both incident irradiance and stratification were stronger than in the others. As can be seen in Figs. 2-4, the diel effect on fluorescence quenching appeared to be limited to depths receiving noon irradiances exceeding $I_n$ (Table 1). However, in all cruises, the onset of fluorescence quenching took place slightly before the irradiance at the surface ($I_s$) soared to exceed $I_1$ (5-7 GMT for FR91, 6:30-9:30 GMT for FR92 and 4-6 GMT for FR93). This finding supports the view of non-photochemical quenching processes as part of regulatory mechanisms, involved not only in protection, but also in the fine-tuning of light reactions (Crichtle and Russell, 1994). An interesting observation was the lack of coincidence between the minimum fluorescence yield and local noon in FR93, suggesting either a decrease of non-photochemical quenching or the initiation of some process with opposite effects on the fluorescence yield. A possible speculation is that the early recovery of fluorescence in this cruise was related to the high-light acclimated state of the phytoplankton during that cruise.

The causes of the apparent maximum of $FRE_m$ at 40-50 m depth are not clear. Perhaps this mid-depth maximum was related to physiological or ecological changes at the DCM, which coincides with the nutrient (Estrada et al., 1993) and is associated with increased phytoplankton biomass and chlorophyll content per cell (Latasu et al., 1992). It is also difficult to explain the deep $FRE_m$ minima, which occurred at average depths close to the 1% light level in FR92 and FR93, and somewhat deeper (between the 1% and the 1%e. light level) in FR91 (Table 1). Even at these low irradiances, a light effect, acting on a shade-adapted phytoplankton community, cannot be completely ruled out.

Conclusion and perspectives

Photoacclimation-induced changes of IVF to chlorophyll ratios are found in natural populations in situ, under wide ranges of environmental irradiances and physiological conditions. Improved understanding of these phenomena should provide valuable insights on mechanisms regulating algal responses to light.

The study of IVF yields implications for the interpretation of fluorescence data in large-scale field surveys, whether obtained by means of in situ or remote sensing techniques. Remote (active or passive) fluorescence sensors have been used mainly to map chlorophyll distributions, but the possibility of applying remote sensing of passive fluorescence to obtain large-scale estimates of primary production has also been suggested (Topliss and Platt, 1986). Data on IVF variability obtained by means of standard oceanographic equipment, may provide valuable complementary information.

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