Chapter 2
In Situ Measurement of Variable Fluorescence Transients

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1 Introduction

Chlorophyll variable fluorescence provides considerable insight into the photosynthetic physiology of plants and algae, in particular the structure and function of Photosystem II (PSII). A longstanding method for measuring variable fluorescence relies on the addition of DCMU, an herbicide, which blocks electron flow through PSII and eliminates photochemistry as a quencher of fluorescence (Malkin and Kok 1966; Trebst 1980). When the photochemical pathway is blocked by DCMU a sample's fluorescence yield F is greater than when it is not blocked, and this variable fluorescence difference between F measured before and after the addition of DCMU is a valuable indicator of photochemistry in photosynthetic organisms. Unfortunately the DCMU method is not well suited for use in the field. It is possible to measure variable fluorescence using DCMU on discrete or continuous samples of natural phytoplankton assemblages (e.g., Cullen and Renger 1979; Roy and Legendre 1979; Vincent 1981) but it is difficult to do so *in situ* under the ambient light and nutrient conditions that phytoplankton experience in the natural environment.

An alternative is to force F from its minimum to its maximum photochemically using brief actinic flashes of light. Several variations of this basic approach have been developed to date including the pump and probe fluorometric method (Mauzerall 1972; Falkowski et al. 1986), the pulse amplitude modulation (PAM) method (Schreiber 1986), the fast repetition rate (FRR) method (Kolber and Falkowski 1992; Kolber et al. 1998), the pump during probe (PDP) method (Olson et al. 1996), and several others that are functionally similar (e.g., Koblížek et al. 2001; Fuchs et al. 2002; Gorbunov and Falkowski 2004; Johnson 2004; Chekalyuk and Hafez 2008). At least two of these methods – pump and probe fluorometry and FRR fluorometry – have been performed directly on phytoplankton assemblages in the natural aquatic environment using specialized submersible instruments (e.g., Kolber and Falkowski 1992; Antal et al. 2001; Fujiki et al. 2008). These *in situ* "variable fluorometers" are now widely used in oceanographic and lacustrine field research in large part because they are easy to operate and because they readily integrate into standard platforms for profiling or towing instrumentation. These fluorometers have greatly advanced our ability to examine phenomena such as the vertical structure of primary production in the ocean (e.g., Boyd et al. 1997; Melrose et al. 2006), the distribution of photophysiology over meso- and basin scales (e.g., Behrenfeld and Kolber 1999; Holeton et al. 2005), succession in natural assemblages (e.g., Strutton 1997; Suggett et al. 2001), and seasonal and inter-annual cycles in primary production (e.g., Corno et al. 2006; Kaiblinger and Dokulil 2006; Suggett et al. 2006; Fujiki et al. 2008). The ease of operating and deploying these instruments is somewhat deceiving, however, because proper stimulation, measurement, and interpretation of variable fluorescence kinetics is anything but simple.

A number of physiological, optical, instrumental, and computational factors can each introduce considerable error into those photophysiological properties of PSII that can be estimated from variable fluorescence

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transients $F(t)$, sometime in subtle and counterintuitive ways. Some of these sources of error have been examined before (e.g., Cullen and Davis 2003; Laney 2003; Laney and Letelier 2008) but many have not. In the best case scenario such errors can be identified, minimized, or even made negligible by carefully examining variable fluorescence techniques and the resulting measured fluorescence transients. In the worst case scenario, however, these errors remain unexamined and unquantified and have an unknown effect on PSII photophysiological estimates. This introduces uncertainty into any metabolic or ecological inference drawn from these estimates and thus robust interpretation of variable fluorescence transients in a physiological, metabolic, or ecological context requires accurate *in situ* stimulation and measurement of $F(t)$, as well as the accurate application and fitting of an appropriate biophysical model. This ultimately requires that the dominant sources of error in $F(t)$ be identified and their effects estimated.

A primary goal of this discussion is to provide an overview of some of the primary factors specific to *in situ* measurement of $F(t)$ that introduce error into the estimates of phytoplankton PSII photophysiology derived from variable fluorescence transients. Many aspects of the natural aquatic environment can potentially alter measured $F(t)$ considerably, as can issues related to how *in situ* variable fluorometers are typically deployed in field studies (Table 1). Expert users have composed guidelines for using particular *in situ* instruments in the field (e.g., Suggett DJ, Moore CM, Oxborough K, and Geider RJ, 2005, personal communication) and developed software for identifying sources of artifact or bias in measured $F(t)$ transients (Laney 2002), yet at present a general review of *in situ* measurement of variable fluorescence remains lacking. This is partly due to an inherent difficulty with identifying or quantifying many of the artifacts that arise when measuring $F(t)$ *in situ*, as it is not always easy to determine which of several factors may introduce the largest error or even gauge the extent to which any single factor affects $F(t)$. A secondary goal of this overview is to identify avenues for minimizing or perhaps even eliminating altogether the influence of some of these environmental or operational factors. Although this overview focuses on phytoplankton assemblages primarily, many of the factors examined here may also be encountered when using variable fluorescence approaches on other aquatic photoautotrophs such as benthic algae, corals, and seagrasses.

Table 1 Some environmental and operational factors that can introduce error into estimates of PSII photophysiology when using variable fluorescence techniques *in situ*. Also noted is whether or not each particular factor may be more or less relevant when measuring variable fluorescence in aquatic systems that are classified optically as Case II or Case I

Sources of error	Case II vs. Case I
Environmental	
Presence of other fluorophores/absorbers/scatterers (e.g., Chekalyuk and Hafez 2008)	Case II > Case I typically
Algal assemblage taxonomic composition (e.g., Suggett et al. 2004)	Assemblage specific
Heterogeneity of PSII properties within a sample (e.g., Suggett et al. 2004)	Assemblage specific
Vertical structure of phytoplankton biomass (e.g., Corno et al. 2006)	Case II ≈ Case I typically
Signal degradation by scattered ambient sunlight	Acts deeper in Case I
Operational	
Relative motion of water sample past instrument	Deployment specific
Shading of the sample by instrument	″
Orientation of instrument vis-à-vis light field	″

A third goal of this overview is to identify some of those similarities and encourage the examination of these factors in aquatic photosynthesis research beyond that concerning phytoplankton alone.

2 Phytoplankton Variable Fluorescence *In Situ*

2.1 Dynamical Protocols for Stimulating Variable Fluorescence

The *in vivo* fluorescence of chlorophyll molecules in living organisms is a very different phenomenon than that of isolated chlorophyll molecules in solution. In some sense there is no such thing as *in vivo* "chlorophyll" fluorescence because *in vivo F* is not determined by chlorophyll molecules alone but by a complex system of pigments and proteins whose yield is determined by its inherent structure and by its immediate photochemical state. All of the different optical methods listed earlier (e.g., pump-and-probe, FRR, PDP, etc.) use a brief actinic flash of light to stimulate this system and photochemically close all functional PSII reaction centers. Because this system is dynamical,

this flash forces F to rise from some initial level F_o to a maximal, saturated level F_m. For this discussion the yields F_o and F_m will be used in the most general sense and subtleties in nomenclature as to whether or not they are measured in the dark- or light-regulated state, e.g., as discussed elsewhere by Kromkamp and Forster (2003) and in Chapter 1 (Cosgrove and Borowitzka) will be neglected for simplicity.

A brief review of some different variable fluorescence protocols may be instructive. The pump-and-probe fluorometric approach uses a single actinic "pump" flash short enough and intense enough to saturate all PSII reaction centers almost instantaneously. As a result F is forced very quickly from F_o to F_m, on the scale of one to several microseconds. Measurements of F using weak probe flashes before and after this strong pump flash provide measurements of F_o and F_m which are used to compute the variable fluorescence yield as F_v/F_m, where $F_v \equiv F_m – F_o$. With other protocols the light in this actinic flash is distributed over a longer time scale, on the order of tens of microseconds in the case of FRR or PDP fluorometry to up to hundreds of thousands of microseconds in the case of PAM fluorometry. The same amount of photons delivered over a longer period effectively results in a slower rate of excitation for this dynamical system, and consequently the apparent F rises gradually from F_o to F_m with kinetics $F(t)$ that reflect dynamical process related to PSII light harvesting and electron transport. These kinetics apparent in this $F(t)$ transient are physiologically more informative than the near-instantaneous transition from F_o to F_m induced by a single pump-and-probe measurement, which is partly why most *in situ* variable fluorometers used in field studies are those that close PSII gradually and not instantaneously (i.e., most are FRR fluorometers instead of pump-and-probe instruments).

Because this gradual closure of PSII is inherently a manipulation of a dynamic physiological system it should be evident that there is no single "correct" protocol for stimulating variable fluorescence in order to obtain a physiologically meaningful fluorescence transient. Rather, any number of different protocols can be designed to elicit dynamical responses that emphasize particular photophysiological aspects of interest. A very simple idealized protocol is shown in Fig. 1 in which a single light flash of uniform intensity is used to saturate PSII in a phytoplankton sample over a time scale of order 100 μs. The initial saturation portion of this protocol is similar in principle to the PDP approach used by Olson et al. (1996) and Koblížek et al. (2001). In this protocol the subsequent relaxation of F following this actinic flash, from F_m back down toward F_o, is monitored periodically over the many-ms time scale of a single photochemical turnover using weak light flashes that (ideally) only negligibly re-close PSII that are gradually reopening.

Fluorescence yield F is, strictly speaking, not the same as the observed fluorescence emission EM but for the purposes of this discussion EM and F will be considered equivalent for simplicity and to maintain consistent notation with prior treatments of fluorescence excitation and emission (Laney and Letelier 2008). A measured variable fluorescence transient $EM(t)$ only becomes useful in a physiological or ecological context if its kinetics can be robustly interpreted using a physiological model. A simple physiological model for the transient in Fig. 1 is one of a cumulative one-hit Poisson function that describes this ideal fluorescence transient $EM(t)$ in terms of its initial F_o, its final F_m, and a mean functional cross section of individual PSII (σ_{PSII}).

$$F(t)_{t<100\mu s} = F_o + (F_m - F_o)\cdot(1 - e^{-\sigma_{PSII}\cdot\int_0^t Edt}) \tag{1}$$

More complex physiological models can include a term to account for other factors that cause this saturation phase of $F(t)$ to deviate from a simple cumulative one-hit Poisson such as sharing of exciton energy among individual PSII (Joliot and Joliot 1964; Paillotin 1976; Laney 2003). Since the relaxation kinetics of $F(t)$ (right panel) reflect the action of a pool of electron acceptors (e.g., Q_A and others downstream) these are often described with one or several exponential decay constants which may or may not be weighted (e.g., Kolber et al. 1998), e.g.,

$$F(t)_{t>100\mu s} = F_o + (F_m - F_o)\cdot e^{-\frac{t}{\tau}} \tag{2}$$

2.2 The Practical Relevance of the Single-turnover Time Scale In Situ

The idealized fluorescence emission transient that would be stimulated by the protocol shown in Fig. 1 serves as a useful model for empirically interpreting the variable fluorescence kinetics measured in an

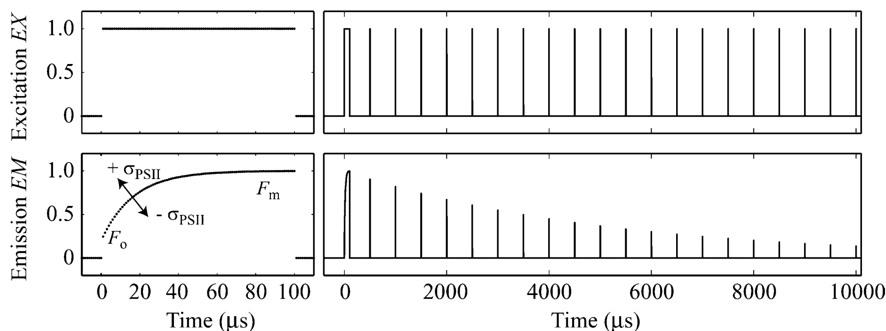

Fig. 1 An example of a simple, idealized variable fluorescence protocol that uses a single light flash of many tens of microsecond in duration (*EX*(*t*), *top left*) to gradually drive fluorescence emission *EM*(*t*) from an initial level F_o to a final saturated level F_m (*bottom left*). This saturation flash is the first in a longer train of shorter flashes that measure the relaxation in PSII photochemistry from this saturated state (*right panels*)

idealized phytoplankton assemblage. The kinetics of actual assemblages, however, are more complex than this idealization suggests. Under physiologically realistic irradiances a small number of photosystems are always open for photochemistry and thus absolute saturation is never achieved *sensu stricto*. This reflects the continual reopening of photochemically closed photosystems by downstream acceptors, even at the very beginning of the saturation portion of the excitation protocol. Thus the relaxation kinetics of a PSII population are always and unavoidably convolved with those of photochemical saturation during the actinic flashes.

If the energy delivery rate needed to saturate phytoplankton of a given species growing under particular conditions is already known, or if there is time to determine this iteratively using trial and error *in situ*, then this convolution effect can be minimized considerably and variable fluorescence can be stimulated in a way that results in *F*(*t*) very similar to those in Fig. 1. Yet, when performing variable fluorescence measurements *in situ*, especially when profiling vertically through assemblages of strongly dissimilar taxonomic compositions, it is not always possible to know ahead of time which particular protocol is appropriate, or even if there is a single protocol which will stimulate appropriate *F*(*t*) across strongly differing assemblages. An example of this effect is shown in Fig. 2, a fluorescence transient measured in the top few meters of the highly oligotrophic North Pacific subtropical gyre 100 nautical miles northeast of Oahu. In this region surface phytoplankton assemblage are dominated by cyanobacteria such as *Synechococcus* and deeper assemblages are more typically dominated by nano- or picoeukaryotes. The former are inherently difficult to saturate photochemically with the blue-green excitation wavelengths used by available *in situ* variable fluorometers (Suggett et al. 2001; Raateoja et al. 2004; Kaiblinger and Dokulil 2006) whereas the light harvesting apparatus of the latter will typically saturate in a shorter period of time given the same excitation wavelengths and delivery rates. If only a single excitation delivery rate is to be used when profiling an instrument vertically through these two different phytoplankton assemblages it cannot be so large that the deeper assemblages saturate too quickly, which would make their variable fluorescence kinetics difficult to interpret. Therefore a delivery rate is chosen in which the near-surface assemblages are saturated over a somewhat longer time scale, which may begin to overlap with the time scale of relaxation processes. This may result in an apparent decrease in *F* during the latter part of the actinic saturation flash (e.g., Fig. 2 left panel between 200 and 450 µs) and possibly only near-saturation of *F* instead of full saturation. As long as this phenomenon is recognized, physiological models for *F*(*t*) that include Q_A reoxidation or other processes that drive *EM* relaxation can be used to interpret these measured transients (e.g., Kolber et al. 1998, Eq. 9). An added complication in this particular example is that these near-surface cyanobacteria assemblages are presumably acclimated to the high-irradiance, upper optical depths and thus their light-harvesting apparatus may exhibit enhanced photochemical turnover rates (e.g., Kana and Glibert 1987; MacIntyre et al. 2002). These faster rates would exert even more influence on *EM* during the latter part of a longer actinic flash and contribute to an even greater

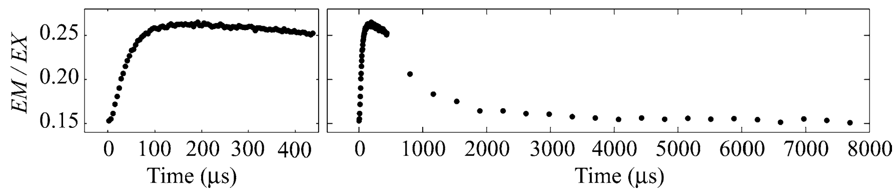

Fig. 2 An example variable fluorescence transient showing the effect of photochemical relaxation during longer time scales of photochemical saturation. Here, a longer-than-usual train of excitation flashlets is required to saturate (or near-saturate) a phytoplankton sample over ~450 µs, as the excitation source is not bright enough to do so more rapidly in these surface, oligotrophic phytoplankton samples. The time scale of this longer excitation train overlaps that of the relaxation processes in PSII which introduce an apparent decrease in fluorescence yield during the latter half of the saturation transient. Even if the actual emission did not attain the theoretical F_m, this parameter can still be retrieved by using a more appropriate dynamical model of PSII physiology than the simple one shown in Fig. 1, provided that the relaxation time scales are known accurately

apparent decrease during this period. The saturation kinetics of the fluorescence transient in Fig. 2 can be better described by an equation that incorporates both the saturation and relaxation process that are operating on the several-hundred microsecond time scale of this saturation protocol.

$$F(t) = F_o + (F_m - F_o)\cdot(1 - e^{-\sigma_{PSII}\cdot\int_0^t Edt}) \cdot \sum_{i=1}^{n} \alpha_i e^{-\frac{t}{\tau_i}} \quad (3)$$

The need to use such a representation when measuring $F(t)$ *in situ* on assemblages with strongly different photo-physiologies underscores the importance of considering the measurement time scale when using variable fluorescence methods *in situ*.

The fact that $F(t)$ *in situ* is measured on unconstrained samples of phytoplankton involves a somewhat similar convolution and is also of importance. Laboratory measurements of variable fluorescence are typically made on small sample volumes that are fixed in space relative to the volume that is illuminated by the excitation flash, e.g., in a cuvette. When the sample is constrained in this manner it is generally possible to stimulate and record fluorescence transients over virtually any time scale that is desired, including those of "multiple turnovers" much longer than those of in Figs. 1 and 2. These protocols with longer time scales (e.g., Kolber et al. 1998; Kromkamp and Forster 2003; Gorbunov and Falkowski 2004), which also include those of PAM fluorometry (Schreiber et al. 1993, 1995), would be difficult to make robustly *in situ* because phytoplankton samples in those situations are typically unconstrained and thus can move through the illuminated sample volume during the period in which variable fluorescence transient is being stimulated and recorded.

This effect is not easy to assess empirically but can be estimated using numerical models. The results of a simple simulation is shown in Fig. 3, where $F(t)$ is stimulated on a spatially uniform phytoplankton sample by an instrument moving along this sample while stimulating with the protocol shown in Fig. 1. Any degree of motion of sample past this volume means that some of the cells that were initially in the sample volume at the beginning of the excitation protocol have exited the sample volume, being replaced by new cells that arrived into the volume at some point in time $t > 0$. Thus $F(t)$ appears to change over time simply due to relative motion of sample and instrument. This simulation examined three different cases of relative motion: one with no relative motion of the sample past the instrument, one with an instrument passing through a uniform volume of phytoplankton at a vertical profiling rate of 60 m min^{-1} (100 cm s^{-1}, or 1×10^{-5} cm µs^{-1}), and one with an instrument being towed through this volume at 10 knots (≈ 5 m s^{-1}, or 0.5×10^{-3} cm µs^{-1}). In each case the instrument is given a sample volume having a characteristic length of 1 cm. It is apparent that with these model parameters relative motion between instrument and sample has no discernable effect on the saturation kinetics (left column) when profiling vertically at typical rates, and only a negligible effect is seen in the apparent relaxation kinetics. Thus a variable fluorescence protocol like the one in Fig. 1 would likely remain robust when profiling a variable fluorometer vertically through the water column at these rates. However, in the case where the same instrument is towed behind a ship at a typical speed of 10 knots, this relative motion would introduce a considerable apparent effect on the relaxation scale kinetics (right column) that would

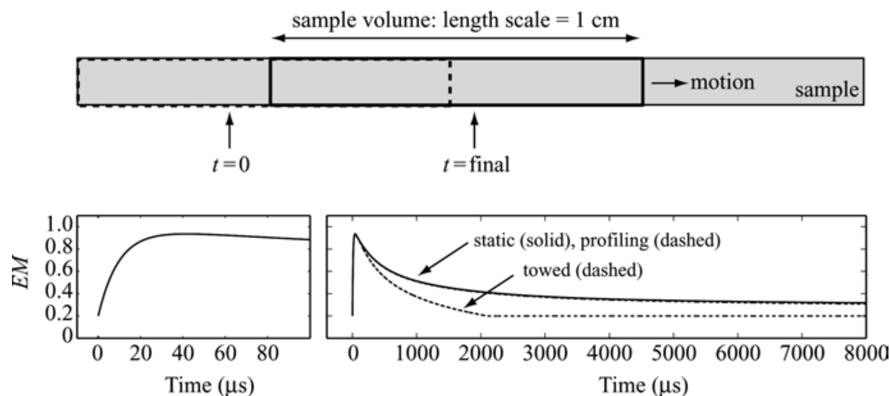

Fig. 3 Results of a simulation examining how relative motion between a phytoplankton sample and an instrument's sample volume affects a measured fluorescence transient *EM* on scales of photochemical saturation and relaxation. In this simulation an idealized sample window of length scale 1 cm (top diagram) stimulates and observes $F(t)$ in a uniformly distributed sample, while the instrument moved lengthwise (here, to the right) through the sample volume during the measurement. The fluorescence transients computed by this model (bottom panels) reflect the expected behavior of *EM* in the absence of any relative motion (solid trace) and when the instrument moves relative to the sample (dashed traces) at 1e-5 cm μs^{-1} (typical of a vertically profiled instrument) and 5e-4 cm μs^{-1} (typical of a towed instrument). Towing an instrument through a given water mass will result in *EM* transients that suggest a much faster rate of photochemical relaxation, an artifact due to the relative motion of the instrument past the sample

consequently cause the estimated electron throughput rates of these phytoplankton to be overestimated.

A specific conclusion from this modeling exercise is that studies that deploy variable fluorescence instruments on towed vehicles (e.g., Aiken et al. 1998; Berman and Sherman 2001; Allen et al. 2002; Moore et al. 2003; Melrose 2005) should exercise caution when estimating photochemical relaxation time scales from these apparent fluorescence transients. For completeness it should be noted that the same effect of relative motion may apply to variable fluorescence measurements performed on high-volume flows such as those made by attaching variable fluorometers to continuous seawater supplies on ships, depending on the flow rate and the characteristic length scales of the sample volume. A similar effect might be noted when measuring variable fluorescence on coral or benthic samples, e.g., using a fiber-probe variable fluorometer, if the instrument's sensing head moves substantially relative to the sample during the time scale of the transient measurement. Beyond these specific conclusions, a more general one is that typical degrees of relative motion between sample and instrument *in situ* may make it difficult to use multiple turnover protocols on small volumes of unconstrained phytoplankton if there is any meaningful degree of relative motion between instrument and sample. Seawater handling solutions might be devised to circumvent this limitation and thus perform multiple turnover measurements robustly *in situ*, e.g., using a stop-flow approach or similar, but these avenues have yet to be well explored.

2.3 Issues Related to the Marine Light Field

One obvious benefit of measuring variable fluorescence *in situ* is that the photophysiological properties of phytoplankton are assessed in the actual light fields in which these microbes are growing. This capability is especially important for studies aimed at estimating primary production from PSII physiology, given that most of the relevant photophysiological properties are extremely sensitive to the ambient light intensity and that any change in the ambient light field will cause these properties to change rapidly from their *in situ* values. However, measurement of phytoplankton $F(t)$ in the ambient marine light field can be complicated by several factors related to the light field itself and to the manner in which the instrument that is used to measure $F(t)$ also changes the ambient light conditions of the phytoplankton under study.

A direct effect of the underwater light field is through the scattering or redirection, into the instrument's fluorescence detector, of some portion of the ambient

light field that spectrally overlaps the chlorophyll fluorescence bandwidth. This effect would be particularly pronounced in samples taken at midday in the top optical depth and would be difficult to distinguish from other sources of apparent fluorescence that affect the $F(t)$ baseline, such as the background fluorescence from dissolved organic matter. This effect can be mitigated to some extent by physically blocking the ambient sunlight from entering the emission detector or by restricting the solid angle observed by the fluorescence detector, both of which act to reduce the relative contribution of solar scatterance. Another common strategy for avoiding this source of artifact is to reject very near-surface measurements of $F(t)$. Yet it is not easy to determine a threshold depth at which this effect becomes negligible, or what its magnitude would be when it is non-negligible. A strategy of rejecting very near-surface measurements may be unwise regardless if the physiological responses of interest occur predominantly in the well-lit, top optical depth (see Chapter 6 – Suggett et al.) as is the case when variable fluorescence measurements are used to inform remote sensing studies of ocean color.

If this ambient scatterance were effectively constant during a variable fluorescence measurement then its effect on $F(t)$ could be considered static over the measurement time scale and could potentially be corrected for using some independent estimate of ambient irradiance, such as from a PAR sensor. The corrective framework proposed by Laney and Letelier (2008) would be one way to incorporate these measurements of ambient irradiance, using a network diagram like the one shown in Fig. 4. It is also possible that the underwater light field cannot be considered invariant on these short time scales ($\approx$ 10 ms) in some cases, especially over the small spatial scales of the samples of interest ($\approx$ 1 cm), for example in the top optical depths where surface wave focusing may be considerable (Stramska and Dickey 1998; Zaneveld et al. 2001). In that situation the same or a similar network diagram may be used, which includes a description for how short-term fluctuations in ambient sunlight may introduce nonrandom fluctuations in $F(t)$ on the measurement time scale.

Engineering approaches such as restricting the view angle of the instrument, or numerical approaches such

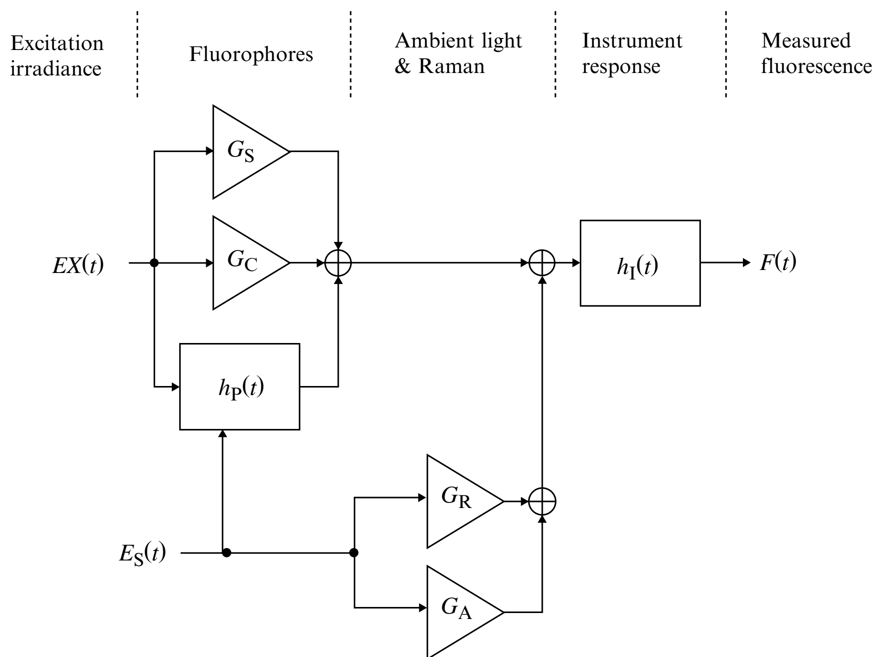

Fig. 4 A dynamical model showing how a time-varying incident solar irradiance $E_s(t)$ affects both the photosynthetic physiology of the sample $h_P(t)$ and contributes elastic and Raman scattering (G_A and G_R) to the measured fluorescence transient. Such a dynamical representation may be necessary to examine the effects of certain factors that may be time-variant on the time scales of a fluorescence transient measurement (Laney and Letelier 2008)

as dynamical corrective frameworks, can both potentially decrease an instrument's sensitivity to ambient solar scatterance and its effect on measured $F(t)$. Yet there still remains an unavoidable physiological impact of the instrument shading the phytoplankton sample under study, from some solid angle of the ambient light field. Because *in situ* variable fluorometers are large compared to the volume of water they sample, the phytoplankton being examined experience a transient in ambient irradiance at some point before the actual $F(t)$ measurement itself. Simply pointing the instrument "up" toward the surface will not eliminate this effect completely, as a non-negligible amount of the underwater light field has a upward component. This instrument shading effect may or may not affect the photophysiology of the sample meaningfully, but this depends on the degree of the shading, the directional structure of the underwater light field at a given depth, the geometry of the instrument and sample volume, and the time scales with which this shading acts on the cells passing through the instrument's (shaded) sample volume.

For a profiling instrument that is relatively free from ship shading a rough estimate of the time a phytoplankton experiences this ambient irradiance transient before the actual $F(t)$ measurement might be on the order of a second or less. The photophysiological effect on cells passing through this modulated ambient light field will presumably not be seen in photosynthetic responses that have time scales of a few seconds or so (Horton and Ruban 2005) but they may be apparent in processes such as those in the thermal phase (e.g., Samson et al. 1999) whose characteristic time scales are closer to this perturbation in ambient irradiance. This effect has not been examined in detail and so its actual impact on measured $F(t)$ in realistic light environments *in situ* is difficult to predict. An analysis approach similar to that used to examine relative motion between sample and instrument may potentially provide some estimate of the degree of this effect in actual $F(t)$ measurements.

2.4 Apparent Effects Resulting from Assemblage Composition

The analysis of variable fluorescence transients typically involves two assumptions, (a) that the phytoplankton sample of interest can be idealized as a population of individual PSII, and (b) that any physiological property of these PSII can be reasonably well represented by the value observed in a bulk fluorescence transient measurement on a volume containing a large number of cells. As was demonstrated earlier, gross differences in the taxonomic composition of a natural phytoplankton assemblage can introduce complications when using variable fluorescence methods *in situ*, in vertical profiles, if those different assemblages are examined using the same excitation protocol. Yet taxonomic factors can also be important in any single sample volume if there is a significant difference in PSII photophysiologies among cells in the sample volume, either between-species or within-species or both. With the simple $F(t)$ transient of Fig. 1 it can be shown that measurements of F_m and F_o from a single phytoplankton assemblage of differing photosynthetic physiologies will accurately reflect the average F_m and F_o of all cells in the sample but that bulk estimates of σ_{PSII} made on an assemblage do not reflect the average cross section of the individual cells (Fig. 5). With the latter, the total fluorescence transient of the bulk assemblage during the saturation phase is larger than what would be expected of an assemblage of the same number of cells with cross sections of the average of all cells in the bulk volume. The sum $F(t)$ of all cells in the sample will saturate faster than the transient that is computed from the arithmetic average of σ_{PSII} among all cells and as a result the functional cross section of a mixed assemblage will appear larger than the average cross section of all cells.

This predicted weighting of σ_{PSII} toward cells of larger functional cross section is supported empirically by laboratory binary mixing experiments performed with cultures (Suggett et al. 2004), but such studies are unfortunately uncommon and so the effect of mixed assemblages on PSII properties such as F_o, F_m, σ_{PSII} (and other physiological aspects such as PSII connectivity and relaxation time constants) remains largely unexamined. Laboratory and field studies can help elucidate this effect in natural assemblages and the degree to which it may affect the PSII properties of interest. Direct measurement of the taxonomic composition of micro- and nanophytoplankton assemblages in conjunction with $F(t)$, such as was done by Olson and coworkers using a flow cytometric and microscopy approach (Olson et al. 1996) may provide important insight into the degree which taxonomic variability affects measurements of $F(t)$ by examining these transients in individual cells.

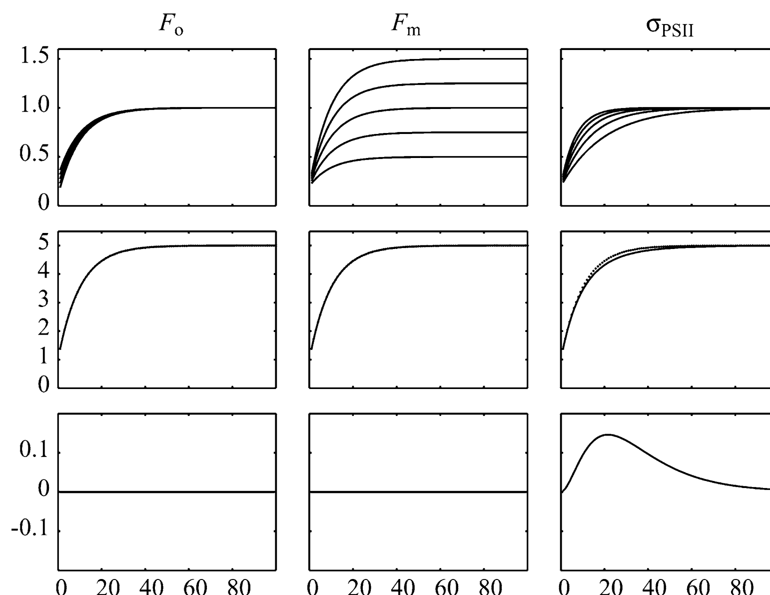

Fig. 5 Model results showing how the distribution of photophysiological parameters among individual cells in a phytoplankton assemblage affects the variable fluorescence transient of the bulk assemblage. For each of these three parameters (F_o, F_m, and σ_{PSII}), the top row shows the transient behavior of five individual cells. The second row shows the sum of these five transients (dashed lines) as well as their average, scaled by a factor of 5 (solid). For F_o and F_m there is no difference between the scaled average and the sum (bottom row), indicating that these properties are conservative. For σ_{PSII} the scaled average is less than the sum, particularly during the initial part of the saturation phase, indicating that it is not conservative in bulk

2.5 *Effects Due to Optical Properties of Natural Waters*

In situ measurement of $F(t)$ by definition involves stimulating and measuring variable fluorescence transients in natural, environmental water samples. Natural waters typically contain a number of optically active constituents that, depending on the optical design of the instrument itself, can either add photons to or subtract photons from the $F(t)$ transient that is being stimulated and measured. These photons may come from the fluorescence of colored dissolved organic matter (CDOM) including the degradation products of chlorophyll, or from particulate or molecular scattering of the excitation flash itself back into the emission detector. The amount of scatterance or non-algal fluorescence that reaches the instrument is itself decreased by the absorption properties of water and other optically active constituents, again such as dissolved organic matter. Water also has several Raman bands that couple excitation irradiance at ≈ 545 to 565 nm into the chlorophyll fluorescence wavelengths around 685 nm (Bartlett et al. 1998).

Since these same factors typically affect shipboard measurements of variable fluorescence in the same way as they do *in situ*, a number of workers have already examined these factors and their effect on F (Fuchs et al. 2002; Cullen and Davis 2003; Laney 2003; Laney and Letelier 2008). In most *in situ* situations these sources and detriments of variable fluorescence are unavoidable and so all *in situ* measurements of $F(t)$ will contain some degree of bias due to them. When working with discrete samples of natural assemblages in the laboratory or shipboard there is typically more opportunity to determine how these various factors affect the measured $F(t)$, but when working *in situ* there is generally little opportunity to make comparable assessments. Thus gauging the relative effect of these disparate optical properties of natural water samples is a fundamental challenge that is not easily addressed.

Methodological or instrumentation approaches may provide the most direct ways to identify the effect of these non-phytoplankton sources of apparent F and determine how best to correct for them. If the apparent variable fluorescence of filtered natural waters can be obtained, either through intermittently sampling filtered water using automated valves and pumps, or by performing duplicate vertical profiles with and without

filters, appropriate corrective procedures can be identified for removing the influence of dissolve contributors to apparent *F* (Laney and Letelier 2008). A different approach, recently described by Chekalyuk and Havez (2008), merges a PDP-like fluorometric protocol with a laser source in order to measure directly the contribution of scatterance and CDOM fluorescence in a wide range of natural waters. Generally speaking the effects of CDOM and particulates will be stronger in estuarine and coastal waters compared to the open ocean but even in dense open ocean blooms CDOM absorption can be large enough to warrant attention (Nelson et al. 2007). This issue of how the non-variable fluorescence contributors to $F(t)$ vary in time and space in natural waters remains largely unexamined and should be one of the main focuses of future *in situ* investigations.

3 Conclusions and Future Directions

The appropriate *in situ* use of extant or future variable fluorescence techniques is not simply a matter of performing standard laboratory protocols in the aquatic environment. Rather, proper *in situ* application of variable fluorescence methods requires careful attention to a number of operational and environmental factors that are not encountered in the laboratory, many of which have not been well examined and are not currently possible to assess directly. Simulations and models provide a means to estimate the effect of some of these factors, and characterization studies under controlled circumstances can also shed light on their magnitudes. The current challenge with using variable fluorescence methods *in situ* isn't so much a technical one of performing such measurements or a physiological one of appropriately interpreting measured $F(t)$ kinetics, but rather one of determining how different sources of apparent *F* contribute to measured transients and how to eliminate their effect on the photophysiological properties of interest.

To some readers the challenges that these various factors introduce may bring to mind the comment by Holzwarth and colleagues who questioned somewhat rhetorically whether or not it was time to "throw away" their fluorescence induction instruments given the ambiguities associated with that technique (Holzwarth 1993). The concern was whether or not the physiological inferences that were being drawn from fluorescence induction measurements were strongly supported by the biophysical bases of the measurement technique being used. An analogous question with respect to modern *in situ* variable fluorescence methods is: with a given *in situ* measurement of $F(t)$, is this transient signal understood well enough so that photophysiological parameters of PSII can be derived from it with confidence? If the answer is no, then the question becomes: what are the limits on the physiological, metabolic, and ecological inferences that can be drawn from *in situ* measurements of phytoplankton variable fluorescence? The factors discussed in this overview represent only a few of the issues now known to affect variable fluorescence use *in situ*, primarily with respect solely to phytoplankton assemblages. Continuing to review the accuracy of these variable fluorescence approaches in phytoplankton, and identifying similar challenges when using these approaches with corals, seagrasses, and benthic algae, will remain an important part of using variable fluorescence techniques *in situ*.

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