Letters to the Editor

A Comment on the Call to Throw Away Your Fluorescence Induction Apparatus

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Recently, Trissl et al. (1993) suggested that some basic assumptions used in the interpretation of fluorescence induction curves are not supported by theoretical model calculations. Their results prompted Holzwarth (1993) to suggest that, although it may be premature to "throw away" fluorescence induction equipment, there is an urgent need to develop new analyses methods which will provide a firmer basis for the interpretation of fluorescence data. While the interpretation of fluorescence induction phenomena may well be complex, we believe that the conclusions drawn by these authors are exaggerated. Moreover, alternative (and better) fluorescence techniques for deriving the desired parameters exist, but are not widely used (and were overlooked by both Trissl et al. (1993) and Holzwarth (1993)). In this letter we: (*a*) briefly review the apparent problems posed by Trissl et al. in relating variable fluorescence to the quantum yield of photochemistry in PSII, (*b*) compare the analyses presented by Trissl et al. with Monte-Carlo simulations based on their "exciton-radical pair equilibrium model," and (*c*) comment on Holzwarth's call to revise the fluorescence induction technique and/or to develop new analysis methods.

We first consider the relationship between variable fluorescence (F_v), the maximum fluorescence yield (F_m), and the quantum yield of photochemistry in PSII (Φ_p). Trissl et al. correctly point out that if F_v/F_m is a quantitative measure of the quantum yield of photochemistry (the ratio of Q_a reduced to photons absorbed), then $[(F_v/F_m)/\Phi_p]$ should be constant. Using data from three published studies of fluorescence lifetimes (Schatz et al., 1988; Leibl et al., 1989; Roelofs et al., 1992), they calculated that the ratios of $(F_v/F_m)/\Phi_p$ for the three data sets are 0.653, 0.367, and 0.696, respectively. Inspection of the data in their Table 1, however, reveals a simple arithmetical mistake; the reported numbers are the products of F_v/F_m and Φ_p, not the ratios. The correct values are in fact 1.02, 1.02, and 0.88. These are relatively constant.

The three experimental data sets have approximately a 5-fold difference in the molecular time constants of excitation transfer, and twofold differences in both F_v and F_a (the complementary area over the fluorescence induction curve). We asked, therefore, if the variability between the three data sets affected their conclusions. One possibility is that, in all three studies, the lifetimes were measured on PSII preparations via detergents, and thus liable to artifact. Our experience has shown a more modest variability in vivo, which can be quantitively related to growth conditions (Falkowski et al., 1986b; Kolber et al., 1988; Greene et al., 1992; Falkowski et al., 1988).

We have measured changes in fluorescence lifetimes and their amplitudes during recovery from iron limitation in the chlorophyte alga, *Dunaliella tertiolecta* (Table 1). These lifetimes were measured without any background irradiance or inhibitors (i.e., the F_o state) and were calculated by global fitting of the experimental results to a three exponential decay; adding more components did not improve the fit. Over the course of 17 h after the addition of 250 nM $FeCl_3$ to the iron-starved culture, the amplitude of the 155-ps lifetime increased from 26 to 65%, while the amplitude of the 1240-ps lifetime decreased from 60 to 1.6%. The amplitudes of the lifetimes 17 h following the addition of iron are comparable to those observed in iron replete cells.

The short (i.e., 155 ps) and medium (i.e., 530 ps) lifetimes have been attributed to excitation trapping and charge stabilization, respectively, in functionally active PSII reaction centers, while the 1240-ps component corresponds to the excitation trapping by inactive, or closed reaction centers (Gentry et al., 1989; Haehnel et al., 1982). The fluorescence yield, F_o (all reaction centers open), can be approximated by:

$$F_o = k_f(\alpha_1\tau_1 + \alpha_2\tau_2 + \alpha_3\tau_3),$$

where k_f is the radiative rate constant and $< 1/\tau_i$. We make the simplistic assumption that, upon closure of the reaction center (F_m state), excitation normally reradiated by both the short and middle components when the traps are open, is reradiated at longer lifetimes (Holzwarth, 1986; Haehnel et al., 1982), and hence, $F_m \approx (k_f\tau_3)$. Based on these assumptions we calculated F_v/F_m from the measured rate constants (Table 1).

We simultaneously measured variable fluorescence on the same samples with single turnover saturating flashes using a pump-and-probe fluorescence technique (Mauzerall, 1972; Falkowski et al., 1986a,b; Kolber et al., 1988). Comparison of the F_v/F_m values, calculated from the measured rate constants, and those measured with single turnover flashes reveals a high degree of correspondence between the two methods; the former method gives values about 25% higher, because our simple approximation in the calculation of F_m

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TABLE 1 Results of fluorescence decay analyses on *D. tertiolecta* observed during the process of recovery from iron limitation

Time after addition of iron (h)	τ_1 = 155 ps	τ_2 = 530 ps	τ_3 = 1240 ps	F_o*	(F_v/F_m)*	$(F_v/F_m)^{\ddagger}$	(F_v/F_m)*/ $(F_v/F_m)^{\ddagger}$	$(\sigma_{PSII})^{\ddagger}$ A^2 λ 450 mm
0	α_1 = 0.26	α_2 = 0.14	α_3 = 0.60	0.85	0.31	0.25	1.22	309
2	α_1 = 0.46	α_2 = 0.29	α_3 = 0.25	0.53	0.57	0.40	1.40	270
7	α_1 = 0.61	α_2 = 0.29	α_3 = 0.10	0.38	0.70	0.53	1.31	190
17	α_1 = 0.65	α_2 = 0.34	α_3 = 0.016	0.30	0.74	0.63	1.17	139

Fluorescence measurements in the upper portion of the table were obtained with mode-locked, frequency doubled YAG laser operating in a single-pulse mode (30 ps). Fluorescence was measured at 685 nm with a microchannel plate photomultiplier and a scan converter digitizer; additional measurements at other wavelengths were obtained but are not shown here. Decay data were reduced by global fitting the experimental results to a three-exponential decays. The measured values of F_v/F_m were obtained using a pump-and-probe fluorescence method (Kolber et al., 1988).

* Calculated from fluorescence lifetimes.

‡ Measured with a pump-and-probe fluorometer as described by Kolber et al. (1988).

neglects other deactivation pathways. Moreover, in optically thin turbidostats (where cell growth is nutrient replete and limited solely by irradiance), there is no difference in F_v/F_m, as long as the cells are not grown at supersaturating irradiance levels (Kolber et al., 1988). The maximum values of F_v/F_m obtained are 0.65 under such conditions, and are remarkably similar in all species of microalgae we have examined (Kolber et al., 1988). We conclude that changes in variable fluorescence derived from single turnover flash measurements are consistent with picosecond lifetime measurements as well as with the theoretical relationship between F_v/F_m and the quantum yield of photochemistry in PSII.

Second, Trissl et al. (1993) calculated the complementary area (F_a) for simulated fluorescence induction curves using the three data sets. They normalized F_a to F_v and Φ_p by the expression $[(F_a/F_v) \cdot \Phi_p]$ and calculated the values as 0.976, 0.842, and 1.400. Based on the data they present in Table 1, the second number should be 0.971. In spite of this relatively minor arithmetical error, the conclusions presented by Trissl et al. appear to suggest that the complementary area over an induction curve is not proportional to the electron equivalents transferred to the acceptor side of PSII, as has been previously suggested (Malkin and Kok, 1966), and often assumed (e.g., Greene et al., 1992).

We reran Trissl et al.'s model using their set of ordinary differential equations and derived fluorescence curves with a Monte-Carlo simulation of the exciton-radical pair equilibrium. While our simulations of Trissl et al.'s model using their parameters led to virtually identical results as they reported, we ran a series of additional simulations (with 60,000 to 120,000 simulations runs per case) with a choice of τ_r (the time constant for relaxation of the radical pair to an inactive state) = 1 and 10 ns, as well as three cases in which we set the trapping and recombination constants equal. The results of our calculations reveal that, while $[(F_v/F_m)/\Phi_p]$ is somewhat sensitive to the time constants, in all cases $[(F_a/F_v) \cdot \Phi_p]$ remains remarkably constant. These results strongly suggest that the theoretical basis for relating the complementary area over a (properly measured) induction curve to the fraction of closed reaction centers and to the electrons transferred to the acceptor side can be supported, even within the context of the exciton-radical pair equilibrium model.

Finally, we considered the problem of fluorescence induction phenomena within the context of energy transfer between PSII reaction centers. The increase in fluorescence yield on sudden exposure to saturating light has long been observed to be sigmoidal. The early interpretation of this phenomenon as a measure of energy transfer between PSII reaction centers sharing a large array of antenna molecules (Paillotin, 1976), has been widely accepted, although this interpretation has been criticized (Ley and Mauzerall, 1986).

Most fluorescence induction curves are made on time scales that are much too long (10^{-4} s), and often are confounded by multiple turnovers on the acceptor side (this problem can even be observed in the presence of DCMU if the actinic light is weak). In the usual (slow) fluorescence induction experiment it is hard to deconvolute the probabilities of exciting open and closed reaction centers as the filling of traps progresses, particularly in multitrap units. One of the easiest ways of solving this problem is to provide the actinic source as single turnover flashes. Single-turnover, flash-induced saturation curves of fluorescence usually closely follow a cumulative one-hit Poissonian (the complement of an exponential) (Ley and Mauzerall, 1986; Mauzerall and Greenbaum, 1989; see also Falkowski et al., 1988), and have been used to derive the absolute optical cross section per active PSII reaction center for single or multitrap units (e.g., Table 1, last column). If the probabilities of trapping and escape from both open and closed reaction centers are equal, the saturation curve is still precisely Poissonian, irrespective of the value of this finite probability. If escape from closed traps is more probable than that from open traps, the curves are sharper (more linear) and are more shallow for the opposite case.

Single-turnover, flash-induced cross sections of variable fluorescence closely follow those for oxygen production (Falkowski et al., 1986a; Falkowski et al., 1988). Moreover, a plot of the ratio of open/closed reaction centers based on simultaneous measurements of changes in maximum variable fluorescence yields and oxygen evolved from single turnover flashes gives a slope of unity throughout almost all ranges of background irradiance (Falkowski et al., 1986a). These results suggest that: (*a*) the absorption cross section and quantum yield for photochemistry (as revealed by flash-induced oxygen evolution) can be quantitatively related to

fluorescence quenching both in the dark and under steady-state illumination, and (*b*) that changes in the maximum quantum yield of fluorescence can be quantitatively related to the fraction of open PS II reaction centers (Falkowski et al., 1988; Genty et al., 1989).

In conclusion, we agree with Trissl et al. and Holzwarth that the interpretation of the standard fluorescence induction curve is complex. However, their criticisms regarding fluorescence induction measurements in relation to both the quantum efficiency and the transfer of reducing equivalents to the acceptor side of PSII are unwarranted. We concur that the interpretation of the conventional fluorescence induction measurements within the context of the absorption cross section of PSII or the probability of transfer of excitation between reaction centers is poorly supported on theoretical grounds. We point out, however, that measurements of variable fluorescence induced by single turnover flashes can be conveniently used to derive the desired parameters, and that such measurements are consistent with both picosecond fluorescence lifetime analyses and with conceptual models of exciton trapping and charge separation in PSII.

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Response to Falkowski et al.

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The comments of Falkowski et al. in response to our article on theoretical fluorescence induction curves Trissl et al. 1993 require confirmation in some parts but also some criticism.

It is unfortunately true that our article contained some errors in the numerical values of F_v, F_m, F_a, and Φ_p. For parameter set No. 3 the rate constant for losses of the radical pair, k_r, was not quantified. An erratum has been printed (*Biophys. J.* 65:982–983, 1993). Despite this, all conclusions listed at the end of our article remain valid, in particular the statement of the absolute value of the complementary area depending on the rate constants selected for the exciton radical pair equilibrium model. Regrettably, this point is not discussed in the article of Falkowski et al.

Falkowski and his colleagues argue on whether the relations $(F_v/F_m)/\Phi_p$ and $(F_a/F_v) \cdot \Phi_p$ are constant or not. Recently, Dr. Jérôme Lavergne and I have succeeded in deriving analytical formulae for fluorescence induction curves derived from the exciton-radical pair equilibrium model. These equations allow effortless tests of any proposed relationship with high numerical accuracy, and they do not suffer from time-consuming computational calculations. With the availability of the analytical solution it is easy to prove that $(F_v/F_m)/\Phi_p = f(k_i)$, i.e., is a function of the rate constants, and that $(F_a/F_v) \cdot \Phi_p = 1$, i.e., is independent of the rate constants. The term $(F_a/F_v) \cdot \Phi_p$ expresses the number of electrons transferred. It thus turns out that the rela-

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