Simulation of Pulse-Amplitude-Modulated (PAM) fluorescence: Limitations of some PAM-parameters in studying environmental stress effects

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Abstract

Fluorescence parameters obtained during steady-state electron transport are frequently used to evaluate photosynthetic efficiency of plants. We studied the behaviour of those parameters as a function of irradiance-adapted fluorescence yields F_S and F'_M. Applied simulations showed that photochemical quenching evaluated by q_P is greatly influenced by the steady-state fluorescence level (F_S), and that its evolution is not complementary to non-photochemical quenching (q_N). On the other hand, the relative photochemical and non-photochemical quenching coefficients ($q_{P(rel)}$ and $q_{N(rel)}$) proposed by Buschmann (1995) represent better the balance between the energy dissipation pathways. However, these relative parameters are also non-linearly related when the F_S level is varied. We investigated the application of a new parameter, the relative unquenched fluorescence ($UQF_{(rel)}$) which takes into account the fraction of non-quenched fluorescence yield (F_S), which is related to closed photosystem 2 reaction centres not participating in electron transport. By using computer simulations and real *in vivo* measurements, we found that this new parameter is complementary to $q_{P(rel)}$ and $q_{N(rel)}$, which may facilitate the use of PAM fluorescence as diagnostic tool in environmental studies.

Additional key words: copper; *Chlamydomonas*; non-quenched fluorescence; parameter calculation; photochemical quenching.

Introduction

Pulse-Amplitude-Modulated (PAM) chlorophyll (Chl) *a* fluorescence measurements are widely used as a simple, rapid, and non-invasive method to assess the physiological state of higher plants and algae (Lichtenthaler and Rinderle 1988, Schreiber *et al*. 1994, Krause and Jahns 2003). Upon irradiation of a dark-adapted plant, the fluorescence signal rises quickly and then decreases to reach a steady-state level. This decrease, or quenching, of the fluorescence yield is due to both increased photochemistry and radiation-less deactivation processes. In the context of PAM fluorometry, equations were derived to distinguish and quantify two types of fluorescence quenching (Schreiber *et al.* 1986, Krause and Weis 1991, Roháček 2002): photochemical quenching (q_P), which is proportional to the photon energy captured by open photosystem 2 (PS2) reaction centres (RCs) and dissipated *via* photosynthetic electron transport, and non-photochemical quenching (q_N) which represents all the non-radiative processes of de-excitation (Table 1). During fluorescence induction of a dark-adapted leaf exposed to continuous irradiation, q_P is initially high but decreases as the electron carriers become reduced,while q_N increases in a complementary fashion as photo-protective mechanisms come into play (Roháček 2002, Krause and Jahns 2003). However, these two parameters can not be summed to a constant value, partly because they are normalized to

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Abbreviations: Chl, chlorophyll; F_0, minimum fluorescence yield in dark-adapted state; F'_0, minimum fluorescence yield in light-adapted state, F_M, maximum fluorescence yield in dark-adapted state; F'_M, maximum fluorescence yield in light-adapted state; F_S, steady-state fluorescence level in light-adapted state; LHC, light-harvesting complex; PAM, Pulse-Amplitude-Modulated; PS, photosystem; q_N and $q_{N(rel)}$, non-photochemical and relative non-photochemical quenching; q_P and $q_{P(rel)}$, photochemical and relative photochemical quenching; RCs, reaction centres; $UQF_{(rel)}$, relative unquenched fluorescence; Φ'_M, effective photosystem 2 quantum yield.
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different physiological states (Buschmann 1995, 1999), and partly because their derivations are based on different models of PS2 connectivity (Roháček 2002, Kramer *et al.* 2004). Another parameter determined by PAM fluorometry is the effective PS2 quantum yield (Φ'_M), which represents the plant's capacity to convert photon energy into chemical energy once steady-state electron transport has been achieved (Genty *et al.* 1989).

As shown in Table 1, fluorescence parameters are based on measurements obtained from both the dark-adapted (F_0 and F_M) and the light-adapted (F_0', F_S, F'_M, and Φ'_M) states. While the fluorescence levels obtained in the light-adapted steady-state (F_S and F'_M) are sensitive to any alteration of the overall PS2-PS1 electron transport and to the biochemical reactions linked to photosynthesis, the dark-adapted state fluorescence yields (F_0 and F_M) are less affected by these processes (Lazár 1999). Consequently, in the evaluation of the photochemical and non-photochemical energy dissipation processes, fluorescence yields at steady-state are more likely to have an impact. Since the steady-state fluorescence level (F_S) reflects the redox state of Q_A and F'_M represents fully reduced PS2 (Joliot and Joliot 1964, Lazár 1999), these fluorescence yields are important indicators in evaluation of energy dissipation processes.

Fluorescence quenching parameters are commonly employed to evaluate the photosynthetic efficiency of plants exposed to pollutants or other environmental stresses (Bolhàr-Nordenkampf *et al.* 1989, Schreiber *et al.* 1994, El Jay *et al.* 1997, Brack and Frank 1998, Naessens *et al.* 2000, Frankart *et al.* 2003, Juneau *et al.* 2003). However, some parameters seem to be more sensitive than others to environmental stresses. For example, Φ'_M and q_N were in several cases much more sensitive indicators of stress response than q_P, as seen in silicate-limited algal cells (Lippemeier *et al.* 1999) and in plants exposed to copper, mercury, and some herbicides (Juneau *et al.* 2001, 2002, Frankart *et al.* 2003). Furthermore, while F'_M could be strongly affected by low copper contents, reflecting effects on energy conversion processes in the photosynthetic apparatus, there was no effect on the calculated q_P value (Juneau *et al.* 2002). The insensitivity of q_P to some environmental factors suggests that this parameter is not always appropriate for assessing the overall photochemical activity of plants under stress. Although q_P is an approximate measure of the fraction of open PS2 RCs, it does not take into account the efficiency of these RCs (Genty *et al.* 1989).

Furthermore, when plants are exposed to some stress conditions (temperature or low irradiance, presence of pesticides or heavy metals), there is no relationship between the indicators of q_N and q_P energy dissipation processes in the light-adapted steady-state (Georgieva and Yordanov 1994, Juneau *et al.* 2001). The non-complementarity between these parameters may be due to the fact that these parameters do not refer to the same state of energy storage and dissipation *via* the photosynthetic apparatus (Buschmann 1995, 1999). As a solution to this problem, Buschmann proposed that two new parameters, called the relative photochemical and non-photochemical quenching coefficients ($q_{P(rel)}$ and $q_{N(rel)}$), should be used instead of q_P and q_N, in order that both parameters should be normalized to the total fluorescence quenched on going from the dark-adapted to the light-adapted state, *i.e.* having the same denominator, $F_M - F'_0$ (Table 1). The relative photochemical quenching coefficient has been criticized because it combines both photochemical and non-photochemical effects (Roháček 2002) and has not been widely used. However, q_P is also influenced by non-photochemical processes (Weis and Berry 1987), and as we show in our simulations below, $q_{P(rel)}$ is not subject to some of the distortions that affect q_P.

By doing simulations with physiologically relevant fluorescence yields, we attempted to determine how variations of F_S and F'_M may affect the calculated values of the PAM-fluorescence parameters. We found some unexpected deviancy in the behaviour of q_P under certain conditions, which could impact the use of this parameter for evaluation of environmental stress effects.

Table 1. Fluorescence parameters obtained at steady-state of electron transport (light-adapted state).

Parameter	Equation	Reference
Φ'_M	$(F'_M - F_S)/F'_M$	Genty *et al.* 1989
q_P	$(F'_M - F_S)/(F'_M - F'_0)$	Schreiber *et al.* 1986
q_L	$(F'_M - F_S)/(F'_M - F'_0)\ (F'_0/F_S)$	Kramer *et al.* 2004
q_N	$1 - [(F'_M - F'_0)/(F_M - F_0)]$	Schreiber *et al.* 1986
NPQ	$(F_M - F'_M)/F'_M$	Bilger and Björkman 1990
$q_{P(rel)}$	$(F'_M - F_S)/(F_M - F'_0)$	Buschmann 1995
$q_{N(rel)}$	$(F_M - F'_M)/(F_M - F'_0)$	Buschmann 1995
$UQF_{(rel)}$	$(F_S - F'_0)/(F_M - F'_0)$	This paper

Materials and methods

The different fluorescence parameters (Table 1) were simulated for realistic fluorescence yields that might be found when unicellular algae are exposed to different environmental conditions. The maximum dark-adapted fluorescence (F_M) was set to 1.000 and the background dark-adapted fluorescence (F_0) to 0.281, in order to obtain a value of 0.720 for $\Phi_M = (F_M - F_0)/F_M$, the maximum quantum yield in the dark-adapted state. We chose this value because of our current work on microalgae, rather than using the value of 0.840 which is more typical for higher plants (Björkman and Demmig 1987). However, we found that using different F_0 values did not affect the relative behaviour of the different fluorescence parameters (data not shown), *i.e.* the results are equally applicable to plants. F'_0 was set to 0.274 and both F_0 and F'_0 values were kept constant to simplify the analysis, since these fluorescence yields do not vary significantly unless plants are exposed to extreme environmental stress that causes structural alteration at the PS2 pigment level (Krause and Weis 1984). The fluorescence values obtained at steady-state electron transport (F'_M and F_S) were varied linearly from 0 to 1 using *Microsoft Excel*, with the limitations that $F_S > F'_0$, $F'_M > F_S$, and $F'_M < F_M$. Two simulations were done by varying either F'_M or F_S to obtain the difference between F'_M and F_S as a percentage of $F_M - F'_0$. Two other simulations were carried out by co-varying F'_M and F_S in the same or the opposite direction.

The green alga *Chlamydomonas reinhardtii* was grown in 0.625 AAP (Algal Assay Procedure) (USEPA 1971). Algae were taken in their exponential stage of growth and exposed to 10 mg m^{-3} $CuSO_4$ for 5 h. Fluorescence measurements were done using a PAM fluorometer (*FMS/2S*, *Hansatech*) as described in Juneau *et al.* (2002).

Results

The light-adapted steady state fluorescence parameters (Table 1) were evaluated using various realistic combinations of fluorescence yield values (F'_M and F_S). Modification of the difference between F'_M and F_S, and therefore in different levels of PS2 oxido-reduction states, can be obtained by: (*1*) variation of F'_M, (*2*) variation of F_S, and (*3*) variation of both F'_M and F_S. For all the following examples, F_0 and F'_0 values were set to 0.281 and 0.274, respectively. The variations in the difference between F'_M and F_S are presented as a percentage of $F_M - F'_0$, because it represents the maximum amplitude of fluorescence quenching in going from the dark- to the light-adapted state (Buschmann 1995).

(*1*) Variation of F'_M: The first example simulated the effect of high temperature or phosphate limitation where only F'_M varied (Juneau and Popovic 1999, Lippemeier *et al.* 2003). q_N decreased linearly with increasing difference between F'_M and F_S when only F'_M was increased (Fig. 1*A*). The different trend lines represent various degrees of PS2 reduction at steady state. When PS2 was almost fully oxidized ($F_S = 0.281 \approx F'_0$), there was more non-photochemical quenching than when the PS2 reduction state was high ($F_S >> F'_0$). For the same ($F'_M - F_S$) value, q_N was higher when $F_S = 0.281$ than when $F_S = 0.546$. However, under the same conditions q_P increased in a hyperbolic manner with the change of F'_M (Fig. 1*B*). Under low PS2 reduction state ($F_S = 0.281 \approx F'_0$), if the difference between F'_M and F_S was small (between 0 and 10 % of $F_M - F'_0$), there was a steep increase in q_P, but if the difference was larger there was almost no change in q_P. When the steady-state fluorescence level was high ($F_S = 0.546$), q_P increased slowly in a hyperbolic fashion as the F'_M value increased. As a result of this q_N and q_P behaviour, the sum of q_P and q_N was not constant (Fig. 2*A*), particularly when the F_S level was low. The effective PS2 photochemical yield value (Φ'_M) varied in a slightly hyperbolic manner (Fig. 1*C*), but it was much less influenced by the steady state fluorescence level than q_P.

Both the relative non-photochemical and photochemical coefficients ($q_{N(rel)}$ and $q_{P(rel)}$) varied in a linear way with F'_M (Fig. 1*D–E*), but only $q_{N(rel)}$ was influenced by the F_S level. Consequently, their sum was constant over the range of differences between F'_M and F_S (Fig. 2*B*). An alternative approach to estimating non-photochemical quenching was by using the parameter NPQ (see Table 1) which is not based on the F_S value. It decreased in a hyperbolic manner as F'_M increased and the amplitude of this decrease was much higher when PS2 was predominantly in an oxidised state ($F_S \approx F'_0$) (Fig. 1*F*).

(*2*) Variation of F_S: The difference between F'_M and F_S could increase due to a decrease of F_S without a change in F'_M, as it does when *Phaeodactylum tricornutum* is exposed to gradually increasing irradiance [10–300 μmol(photon) m^{-2} s^{-1}] (Flameling and Kromkamp 1998). Neither q_N (Fig. 3*A*) nor $q_{N(rel)}$ (data not shown) varied with the change of F_S level, since F_S is not involved in the calculation of either parameter. NPQ was also unaffected by the change of F_S, but its value was greatly modified by the F'_M level (data not shown).

On the other hand, q_P was influenced by both the set value of F'_M and the difference between F'_M and F_S (Fig. 3*B*). Contrary to the situation described in Fig. 1*B*, where the variation of F'_M produced non-linear changes in q_P values, here q_P varied linearly with the change of $F'_M - F_S$. Consequently, the sum of q_P and q_N was not constant and varies as q_P, since q_N did not change over the tested value range (Fig. 2*C*). The Φ'_M parameter

behaved similarly to q_P (data not shown), because F_S had the same impact on the calculation of Φ'_M and q_P (Table 1). $q_{P(rel)}$ followed the same trend as q_P with increasing $F'_M - F_S$ (Fig. 3*C*), but was not affected by F'_M since this fluorescence yield is found only in the numerator of the equation. Consequently, the sum of $q_{P(rel)}$ and $q_{N(rel)}$ will vary similarly to the sum of q_P and q_N, but without any influence of F'_M on the slope of the relationship (Fig. 2*D*).

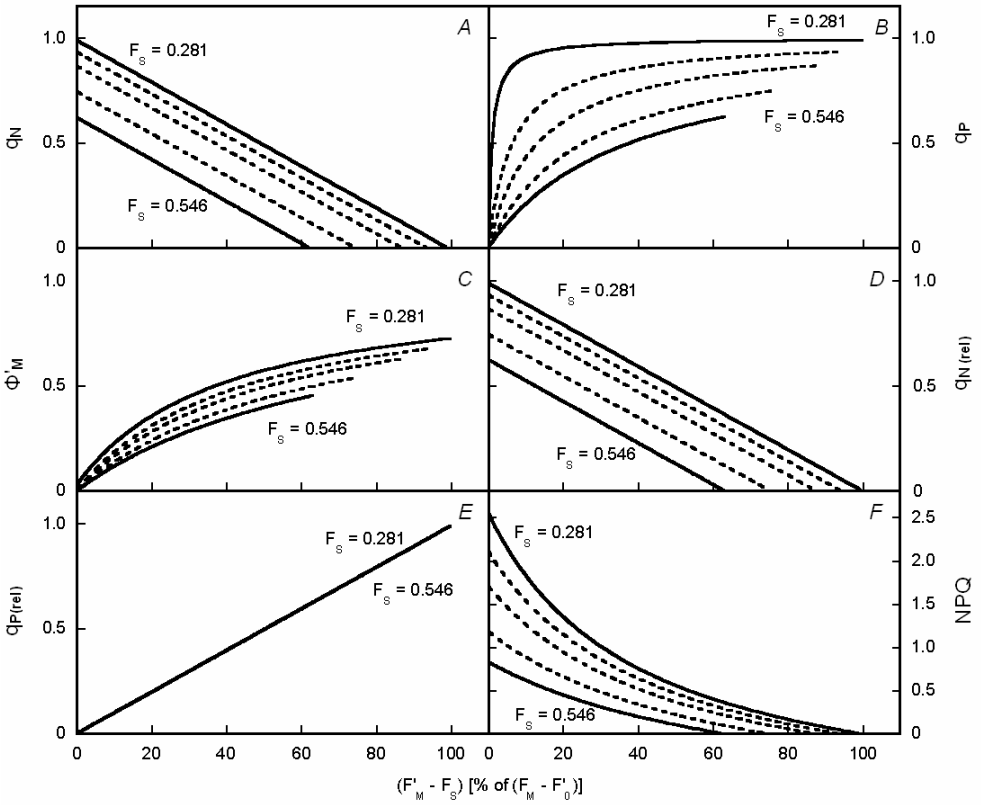

Fig. 1. Variation of the fluorescence parameters [relative values] as a function of the difference between F'_M and F_S, expressed as a percent of $F_M - F'_0$, when only F'_M is varied. F_0 = 0.281, F_M = 1.000, F'_0 = 0.274. The different curves in each figure represent the result when various F_S values are used for the simulation (*solid lines*, 0.281 and 0.546; *dotted lines*, intermediate values). F_0 = minimum fluorescence in dark-adapted state, F_M = maximal fluorescence in dark-adapted state, F_S = steady-state fluorescence level in light-adapted state, F'_M = maximum fluorescence in light-adapted state, and F'_0 = minimum fluorescence in light-adapted state.

(*3*) Variation of F_S and F'_M: When both F'_M and F_S increased from their initial values, the photochemical quenching measured by q_P varied markedly within a very narrow range of initial F_S values (Fig. 4*B*). The shape of the relationship passed from a positive to a negative hyperbola when the F_S value went from 0.426 to 0.462. When F_S was intermediate (0.439), there was no variation in q_P values throughout the entire range of the difference between F'_M and F_S. A less striking effect was observed for other values of F_S, but a positive hyperbola was obtained for all F_S values lower than 0.426, and a negative one was found when F_S was higher than 0.462 (data not shown). Therefore, when F'_M and F_S co-varied in the same direction, a small change in $(F'_M - F_S)$ might cause a large modification of q_P, as seen also in Fig. 1*B*. Contrary to q_P, $q_{P(rel)}$ behaviour was not influenced by the initial F_S value and was linear (Fig. 4*C*). The effective photochemical yield was slightly influenced by the initial level of F_S, and varied in a hyperbolic manner similarly to the case in Fig. 1*C* (data not shown).

Fig. 4*A* shows that q_N varied linearly with the difference between F'_M and F_S. The change of F_S value from 0.426 to 0.462 had little effect on this relationship. $q_{N(rel)}$ varied linearly like q_N; the NPQ coefficient varied in a hyperbolic fashion but was not greatly altered by the initial F_S value (data not shown). As a result of these effects, the sum of q_P and q_N varied almost linearly for the majority of the tested values except at low $(F'_M - F_S)$ values (<15 % of $F_M - F'_0$), where it followed the pattern of q_P variation for the different initial F_S levels (Fig. 2*E*). As expected, the sum of $q_{P(rel)}$ and $q_{N(rel)}$ stayed linear (Fig. 2*F*).

F'_M and F_S may also vary in the opposite direction as was found for the Chl-*b*-less barley mutant (Genty *et al.* 1989) and the green alga *Scenedesmus obliquus* (Heinze *et al.* 1996) under increasing irradiance. By using our

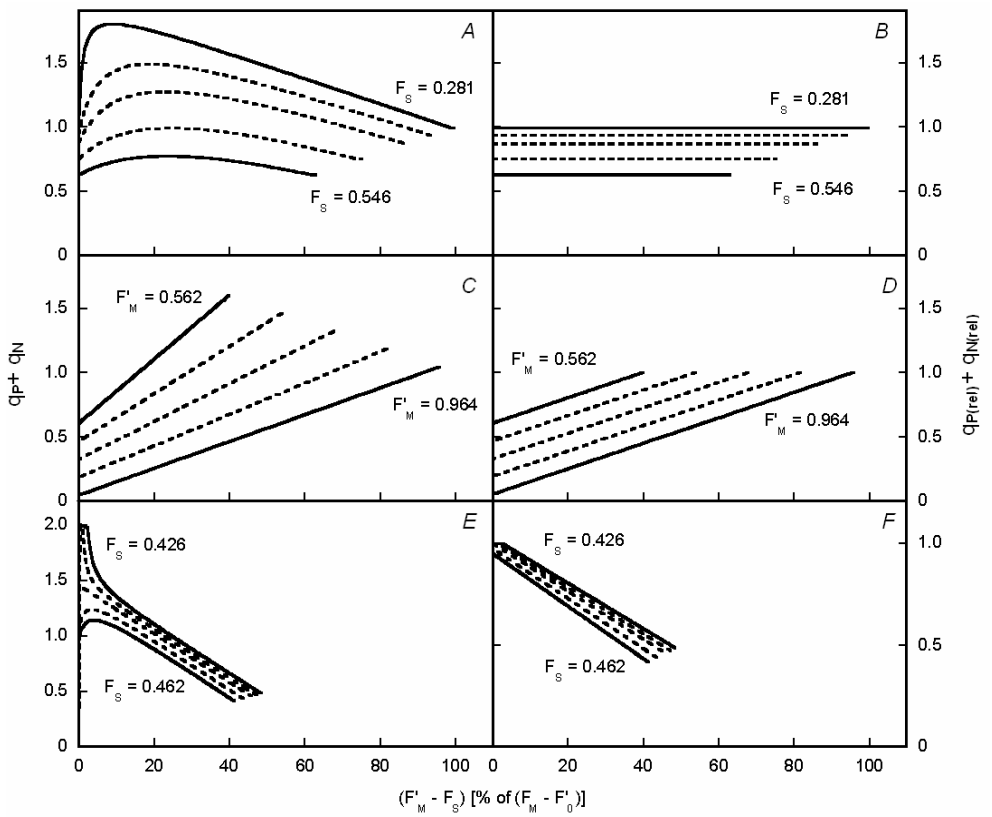

Fig. 2. Variation of the sum of q_P and q_N (*A, C, E*), and $q_{P(rel)}$ and $q_{N(rel)}$ (*B, D, F*) [relative values] as a function of $F'_M - F_S$, expressed as a percent of $F_M - F'_0$. The sums are for the simulations conducted in Figs. 1*A,B*, 3*C,D*, and 4*E,F*.

simulation approach under these conditions, we found that F_S had little effect on q_P curvilinear behaviour and that $q_{P(rel)}$ and $q_{N(rel)}$ were linearly related to the difference between F'_M and F_S (data not shown).

Discussion

By simulating fluorescence parameters for a wide range of physiologically relevant fluorescence yields, we found that the influence of F'_M and F_S on the behaviour of the calculated fluorescence parameters depended on their initial values and how $F'_M - F_S$ was altered (by varying only F'_M, only F_S, or both). Of the simulations done in this study, the ones in which $F'_M - F_S$ was altered by varying only F'_M (Fig. 1) or by varying F'_M and F_S in the same direction (Fig. 4), showed the strongest effect on q_P evaluation and significant deviations from linearity. This kind of inconsistency in the relationships of the currently used PAM-parameters could cause misinterpretation of data derived from experiments where plants are exposed to different environmental factors, as was shown for plants exposed to copper (Juneau *et al.* 2002) or high temperature (Georgieva and Yordanov 1994). Unfortunately, in many published papers the ranges of F'_M and F_S values are not given, so it is unclear what effect they might be having on the calculated parameters.

The lack of change seen in q_P values when the re-oxidation of PS2 was high ($F_S \approx F'_0$) (Fig. 1*B*), even when there was a significant decrease in F'_M, was found also when plants were exposed to high temperature (Juneau and Popovic 1999) or phosphate limitation (Lippemeier *et al.* 2003). In this situation (when F_S is low), q_P shows that there is no change in the fraction of open PS2 RCs. However, the efficiency of these open centres increases with the larger amplitude of $F'_M - F_S$, as indicated by the change of Φ'_M values (Fig. 1*C*). Therefore, the regulation of energy dissipation processes appears to be due to the simultaneous decrease of non-photochemical energy dissipation processes (Fig. 1*A,F*). On the other hand, when F_S is high, a significant increase in the fraction of open PS2 RCs seem to occur, while q_N and Φ'_M manifest similar trends in their changes as seen when F_S is low (*i.e.* decrease in non-photochemical processes and increase in PS2 RCs efficiency).

The question can be asked why, depending on the F_S level, the same increase in $F'_M - F_S$ would have different effect on q_P, known as an indicator of the fraction of open PS2 RCs? Also, this observation does not go along with the fact that the F_S level is linked to Q_A redox state (Lazár 1999). We note also a bizarre behaviour of q_P in Fig. 4*B*, where q_P increased, decreased, or stayed unchanged, all

within a narrow range of F_S values. Kramer *et al.* (2004) derived a new photochemical quenching coefficient (q_L) that takes into account the fact that PS2 units are connected (the "lake" model) rather than being independent (the "puddle" model), as was assumed in the derivation of the equations for q_P (Schreiber *et al.* 1986, Lavergne and Trissl 1995). We found that q_L behaves similarly to q_P in all the simulations done in our work (data not shown). Therefore, in the evaluation of the total quenching effect, it appears necessary to take into account the portion of PS2 RCs which does not participate in electron transport (dissipative RCs) (see discussion below).

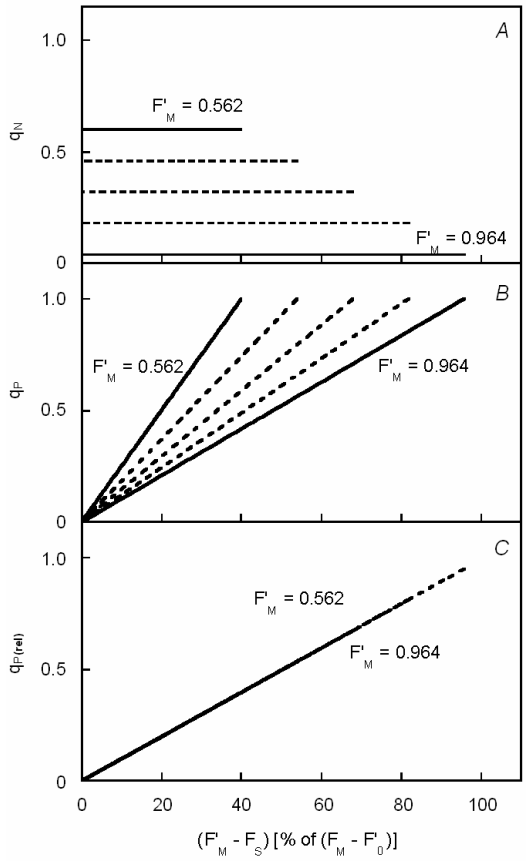

Fig. 3. Variation of the fluorescence parameters [relative values] as a function of $F'_M - F_S$, expressed as a percent of $F_M - F'_0$, when only F_S is varied. $F_0 = 0.281$, $F_M = 1.000$, $F'_0 = 0.274$. The different lines in each figure represent the effect of different F'_M values (from 0.562 to 0.964).

The simulations done in this work show that the most common fluorescence parameters used to evaluate photochemical and non-photochemical events (q_P and q_N) are greatly influenced by the fluorescence yields measured at steady-state of electron transport (F'_M and F_S) and that there is no complementarity between these two coefficients due to the fact that they are normalized to different physiological states; also that q_P represents only the fraction of open RCs and that q_P is not linked to the photochemical yield of these centres (Buschmann 1995, Kramer *et al.* 2004). For a better comparison of the photochemical and the non-photochemical energy dissipation processes, Buschmann (1995) proposed a new type of quenching coefficient, $q_{P(rel)}$ and $q_{N(rel)}$. These quenching coefficients were normalized to the same reference signal, *i.e.* they express the relative amounts of photochemical and non-photochemical quenching as a fraction of the total quenching on going from a dark-adapted to a light-adapted state. As shown in Fig. 2*B,C*, we confirmed earlier findings that the relative quenching coefficients are linearly related to the difference between F'_M and F_S when the effect of the saturating pulse (F'_M) given at steady-state of fluorescence was gradually increased, and therefore the sum of $q_{P(rel)}$ and $q_{N(rel)}$ is constant (Buschmann 1999). However, as it has been noticed for plants exposed to stress conditions, the difference between F'_M and F_S will change not only by a modification of F'_M, but also with a concomitant change of F_S, or by a modification of F_S only (Genty *et al.* 1989, Heinze *et al.* 1996, Flameling and Kromkamp 1998). Consequently, under these conditions, $q_{P(rel)} + q_{N(rel)}$ will not lead to a constant sum, as we demonstrated here (Fig. 2*D,F*).

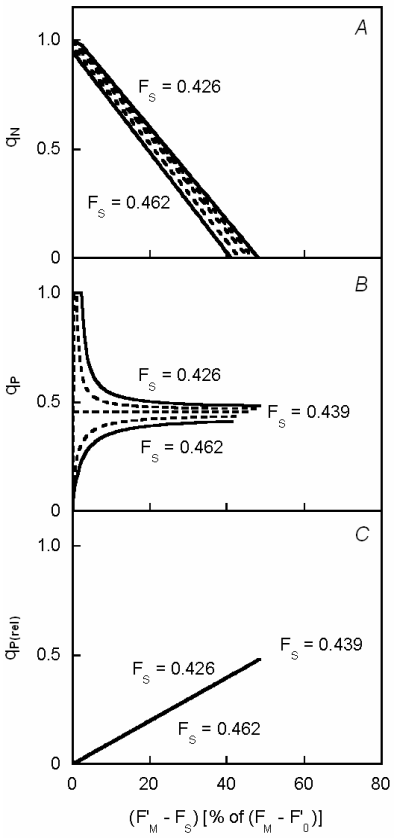

Fig. 4. Variation of the fluorescence parameters [relative values] as a function of $F'_M - F_S$, expressed as a percent of $F_M - F'_0$, when F_S and F'_M are varied simultaneously in the same direction. Initial values of $F'_M = 0.578$. $F_0 = 0.281$, $F_M = 1.000$, $F'_0 = 0.274$. The different curves in each figure represent the result when a narrow range of F_S values is used for the simulation (from 0.426 to 0.462).

As we found in our work, the non-constancy of the $q_{P(rel)} + q_{N(rel)}$ sum is caused by the variation of F_S, which is linked to the fraction of closed PS2 RCs, and therefore not participating in electron transport toward PS1 (Lazár 1999). The parameter $1 - q_P$ has been used to estimate the fraction of closed PS2 RCs (Demmig-Adams *et al.* 1996). However, our simulations showed that q_P does not have a consistent relationship with other parameters related to the fluorescence quenching effect. Consequently, we propose the relative unquenched fluorescence ($UQF_{(rel)}$) to deal explicitly with the contribution of F_S and to be normalized to $F_M - F'_0$, like $q_{P(rel)}$ and $q_{N(rel)}$:

$$UQF_{(rel)} = (F_S - F'_0)/(F_M - F'_0).$$

This new parameter is therefore a complement to the other relative quenching components ($q_{P(rel)}$ and $q_{N(rel)}$) occurring under continuous irradiation, and takes into account the fraction of non-quenched fluorescence yield related to the proportion of closed PS2 RCs present under continuous irradiation.

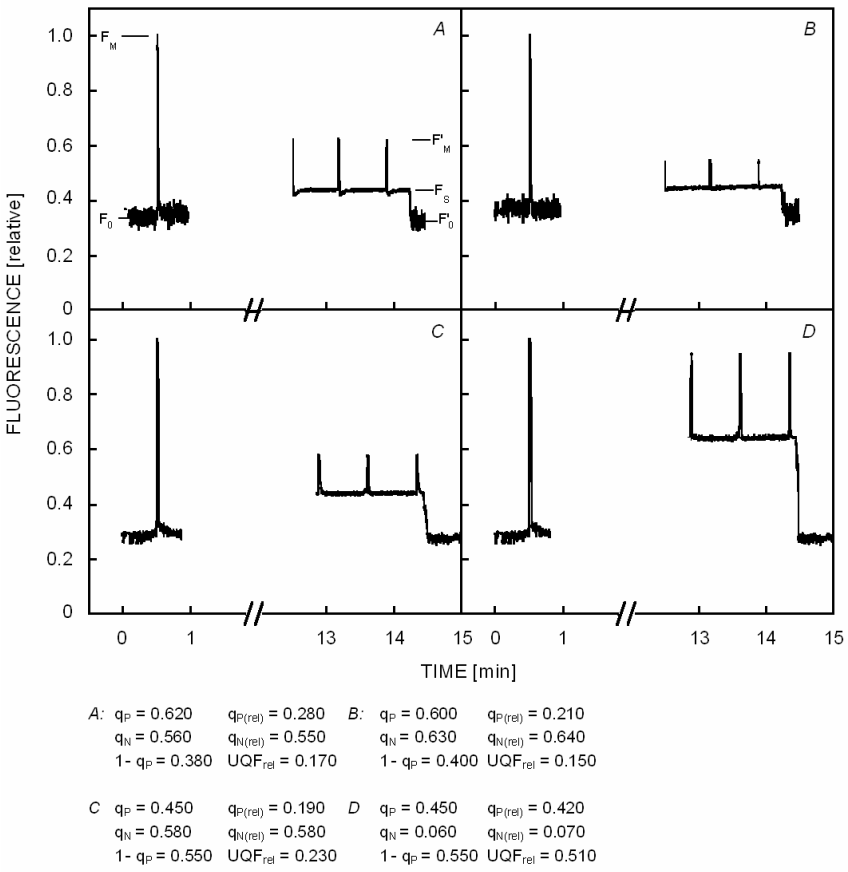

Fig. 5. Fluorescence kinetics showing the same q_P values, but different F_S and/or F'_M levels. *A* and *B*: the fluorescence induction kinetics of *Chlamydomonas reinhardtii* exposed to 10 mg m^{-3} copper for 5 h (*B*) compared to control cells (*A*). *C* and *D*: simulated fluorescence kinetics.

The following examples demonstrate the advantage of using the three relative fluorescence coefficients in the evaluation of plant stress response. When *C. reinhardtii* was exposed to 10 mg m^{-3} copper for 5 h (Fig. 5*B*), the F'_M value diminished by 25 % compared to the control (Fig. 5*A*), while the F_S value remained the same. The calculated values of q_P, q_N, and $1 - q_P$ showed only a slight change (3, 12, and 5 %, respectively), but $q_{P(rel)}$, $q_{N(rel)}$, and $UQF_{(rel)}$ showed larger variations (26, 17, and 10 %, respectively). This indicates that under these conditions, the photochemical part of the fluorescence quenching ($q_{P(rel)}$) is affected much more than one would expect based on the evaluation of q_P. Copper alters both the reducing and oxidising sides of PS2 (Barón *et al.* 1995) reducing the electron flow through PS2, and consequently the photochemical part of the quenching. At the same time, there is an increase in the non-photochemical processes, with almost identical values for q_N and q_{Nrel}.

Fig. 5*C,D* shows artificial fluorescence kinetics, which simulate the situation where both F_S and F'_M are affected, but q_P and $1 - q_P$ do not vary. Values of q_N, $q_{P(rel)}$, $q_{N(rel)}$, and $UQF_{(rel)}$ are clearly different, showing obvious differences in the photosynthetic electron transport activity and non-photochemical energy dissipation processes. In this example, the fluorescence kinetic of Fig. 5*D* shows that the acceptors in the electron transport

chain are more reduced than in Fig. 5*C* (F_S level is higher). However, if we consider only the comparison of q_P and $1 - q_P$ values, it would appear that the fluorescence quenching due to photochemical processes and the fraction of open PS2 RCs were not affected. As we might expect by the difference in the steady-state fluorescence levels observed between these two samples, $UQF_{(rel)}$ increased by more than 120 % in Fig. 5*D*, indicating that PS2 RCs are kept in a more reduced state.

The physiological meaning of the relative parameters discussed in our paper is somewhat similar to the one of the yields of dissipative processes for the energy absorbed by PS2 presented by Kramer *et al.* (2004). Indeed, they take into account the yield of energy dissipation, but these relative parameters are the only ones permitting easy analysis of the total quenching of the fluorescence signal on going from a dark-adapted to a light-adapted state. Our study shows that the values of the relative parameters seem to represent more adequately the proportion of the different quenching processes occurring in the light-adapted state. The concomitant use of the proposed parameters with the usual fluorescence parameters should permit a better evaluation of the effect of any environmental factor on the primary photosynthesis and energy dissipation processes in plants when PAM fluorometry is used as a diagnostic tool of the physiological state of plant.

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