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On the analysis of non-photochemical chlorophyll fluorescence quenching curves
I. Theoretical considerations

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ABSTRACT

Non-photochemical quenching (NPQ) protects photosynthetic organisms against photodamage by high light. One of the key measuring parameters for characterizing NPQ is the high-light induced decrease in chlorophyll fluorescence. The originally measured data are maximal fluorescence (Fm′) signals as a function of actinic illumination time (Fm′(t)). Usually these original data are converted into the so-called Stern–Volmer quenching function, $NPQ_{SV}(t)$, which is then analyzed and interpreted in terms of various NPQ mechanisms and kinetics. However, the interpretation of this analysis essentially depends on the assumption that NPQ follows indeed a Stern–Volmer relationship. Here, we question this commonly assumed relationship, which surprisingly has never been proven. We demonstrate by simulation of quenching data that particularly the conversion of time-dependent quenching curves like Fm′(t) into $NPQ_{SV}(t)$ is (mathematically) not "innocent" in terms of its effects. It distorts the kinetic quenching information contained in the originally measured function Fm′(t), leading to a severe (often sigmoidal) distortion of the time-dependence of quenching and has negative impact on the ability to uncover the underlying quenching mechanisms and their contribution to the quenching kinetics. We conclude that the commonly applied analysis of time-dependent NPQ in $NPQ_{SV}(t)$ space should be reconsidered. First, there exists no sound theoretical basis for this common practice. Second, there occurs no loss of information whatsoever when analyzing and interpreting the originally measured Fm′(t) data directly. Consequently, the analysis of Fm′(t) data has a much higher potential to provide correct mechanistic answers when trying to correlate quenching data with other biochemical information related to quenching.

1. Introduction

In order to protect their photosystems from irreversible damage, plants and other photosynthetic organisms are able to acclimate the photosynthetic apparatus to high light irradiation by various mechanisms in the short- and long-term. One important mechanism which is active predominantly in the short-term (s to min) is the dissipation of excess light energy as heat, known as "non-photochemical quenching" (NPQ). NPQ most likely consists of two or more specific mechanisms that are active in the antenna systems, converting electronic excitation energy into heat. While the detailed molecular mechanism(s) and the exact location of NPQ processes are still a matter of debate, there exists general agreement that for higher plants acidification of the lumen, the activation of a small protein (PsbS), and the activation of the xanthophyll cycle – i.e. the conversion of violaxanthin to zeaxanthin – act together to induce NPQ and thus provide the desired photoprotection effects [1–7].

The technical principles and procedures of measuring NPQ via the fluorescence emitted from the chlorophylls (Chls) of the photosystems are well established since several decades and commercial companies are now providing instrumentation that – with slight differences – all use the same principles for measuring NPQ [8]. A measurement usually starts – after appropriate dark adaptation of the photosynthetic tissue – with switching on a strong pulsed (pulse lengths typically between 200 ms and 1 s) light source that is intense enough to close all reaction centers (RCs) of PS II. The resulting fluorescence intensity (most instruments spectrally integrate over the entire emission wavelength range above 710–720 nm) provides the so-called Fm($t = 0$) value of fluorescence which characterizes the unquenched fluorescence level when all PS II RCs are closed. After a short waiting time an actinic (usually continuous) light source of adjustable intensity is switched on at time = 0 and the above-mentioned pulsed fluorescence measurement is repeated at certain time points t. The resulting data points provide the so-called Fm′(t) level of fluorescence, which represents the quenched fluorescence level at delay time t after the onset of the actinic illumination in a condition when all PS II RCs are closed (for reviews see Refs.

Abbreviations: NPQ, non-photochemical quenching; NPQ_{SV}, Stern–Volmer quenching parameter; PS I, photosystem I; PS II, photosystem II; qE, energy-dependent quenching; SI, supporting information

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[9,10]). It is important to note here that the Fm′(t) intensity represents the directly measurable experimental signal that characterizes quenching. When plotting Fm′(t) one usually observes a continuously decreasing function of t, starting from the unquenched Fm level of fluorescence down to the lowest level of fluorescence (for long delay time $t = \infty$, in most cases actually corresponding to the Fm′ value at the end of the actinic light phase) characteristic of the state with fully operative quenching mechanism(s) for a given actinic light intensity. The time course of the Fm′(t) function (albeit not the absolute intensity) can easily be compared between different laboratories and could thus also be used as the basis for further more detailed analysis of the rates, kinetic heterogeneity, and other kinetic characteristics of the NPQ quenching process(es). Interestingly, the measured Fm′(t) curves resulting from quenching induced by high actinic light intensity very often follow quite closely a bi-exponential function (see e.g. *Arabidopsis* data below). This observation might for example lead one to hypothesize that the observed time constants τ in this bi-exponential fluorescence quenching function might reflect the first order or pseudo-first order rates of physiological processes giving rise to quenching of the Chl fluorescence signal. Alternatively, one might hypothesize that the apparent bi-exponential Fm′ decay function does not reflect two independent (parallel) processes but rather a more complex connected process. In any case, it would seem natural at first glance to perform a direct analysis of the measured Fm′(t) quenching function and then try to correlate the observed rates etc. with other biochemical data known to be related to quenching. Yet, for reasons that will be discussed below, it is not common in the field to plot or to kinetically analyze the directly measured Fm′(t) function. Rather the measured Fm′(t) function is usually transformed into a different function, the $q_{SV}(t)$ or $NPQ_{SV}(t)$ function defined as

$$q_{SV}(t) = NPQ_{SV}(t) = \frac{Fm(t=0)}{Fm'(t)} - 1 \quad (1)$$

which is generally called the Stern–Volmer quenching parameter. The $NPQ_{SV}(t)$ function is then usually analyzed by kinetic analysis methods applying various different kinetic model functions. From the resulting kinetic parameters then often conclusions on the underlying quenching processes, e.g. their independence or connectedness, the relative contributions of different $NPQ_{SV}(t)$ components to the overall NPQ or their relationship to other parameters like e.g. the de-epoxidation state of the system, are drawn [9,10].

The conversion of the originally measured Fm′(t) function into the $NPQ_{SV}(t)$ function in mathematical terms involves the conversion of a function linear in $x(t)$ into a function of the form $A/x(t)$. It is the main purpose of the present work to show that such a conversion – while mathematically possible – is not "innocent" as far as the analysis and interpretation of the underlying physical and chemical processes of quenching are concerned. We will rather show that this conversion, in combination with the subsequent kinetic analysis and interpretation of the $NPQ_{SV}(t)$ curve, actually leads to a severe distortion of the kinetics as well as the relative contributions and time courses of the various possible contributing quenching processes. This distortion can be in type and magnitude so severe that conclusions drawn from the kinetic NPQ quenching analysis may become questionable, particularly when NPQ kinetics are directly compared with the kinetics of other processes such as xanthophyll conversion [11–13] and/or qE-related absorption changes at 535 nm [11,12,14]. In the first part of the paper we will focus on the analysis of simulated quenching curves whose properties are close to those typically observed experimentally. In the second part we will analyze experimentally measured quenching curves and demonstrate the distortion effects resulting from the use of the $NPQ_{SV}(t)$ curves. The present paper provides the theoretical basis and analysis of our claim. In a separate subsequent paper we will provide solutions to the problem, trying to properly extract the information on the underlying quenching mechanisms contained in the experimentally measured quenching curves.

2. Effects of conversion to $NPQ_{SV}(t)$ space

We will in the following analyze the effects on the kinetic quenching parameters resulting from a conversion of the original measured Fm′(t) data to the $NPQ_{SV}(t)$ space. In the first part we analyze and demonstrate those effects on simulated quenching curves which are assumed to be bi-exponential functions. In the second part we apply the same kind of analysis to various typical experimentally measured quenching curves deriving from dark-adapted leaves of Arabidopsis w.t. plants and various mutants affecting non-photochemical quenching. For this purpose we used the same NPQ quenching data that we have already published – applying the usual analysis in $NPQ_{SV}(t)$ space – from w.t. *Arabidopsis* plants and a range of *Arabidopsis* mutants affected in NPQ [13].

2.1. Simulated quenching curves

Experimental quenching curves in Fm′(t) space often follow relatively closely a bi-exponential function of the form

$$Fm'(t) = A_1 * \exp\left(-\frac{t}{\tau_1}\right) + A_2 * \exp\left(-\frac{t}{\tau_2}\right) + Fm'(t=\infty) \quad (2)$$

with amplitudes A_1 and A_2, lifetimes τ_1 and τ_2, respectively, and background Y_0 which is the value of Fm′($t = \infty$), i.e. when quenching no longer increases. It is important to follow quenching curves to that point, since otherwise the Fm′($t = \infty$) level and the long lifetime cannot be determined precisely in the fits. Fig. 1 shows an example of a bi-exponential Fm′(t) curve and the resulting curve after conversion into NPQ_{SV}-space. The figure reveals all the problems of such a conversion of the original Fm′(t) curve. The typical resulting sigmoidicity from this conversion is clearly visible to the naked eye and does not need any further analysis at this level. It follows from Fig. 1b and c that a bi-exponential fit of the $NPQ_{SV}(t)$ curve does not lead to a good fit quality since it cannot describe the sigmoidicity introduced into the curve by the conversion to NPQ_{SV} space nor does the curve at longer times follow a bi-exponential function. As is also visible both the resulting lifetimes as well as the amplitude ratios of the components differ largely from the values used as the input in the bi-exponential Fm′(t) function (Table 1).

We have simulated more such bi-exponential Fm′(t) quenching curves (for all simulation parameters see Table 1) for a range of decreasing amplitudes A_2 of the longer-lived lifetime component τ_2, leaving the lifetimes and the background value constant in order to study the effects of the conversion to NPQ_{SV} space in detail. This was done for two different overall assumed measurement time ranges (400 and 1000 s) that are characteristic of many experimental data published in the literature. The data were then transformed into the $NPQ_{SV}(t)$ space according to Eq. 1. The simulated Fm′(t) data were subsequently analyzed by a bi-exponential function (Eq. 2). Not surprisingly the result of the bi-exponential analysis exactly returns the input data used for the simulation (Table 1). The data transformed into $NPQ_{SV}(t)$ space were also analyzed using either a bi-exponential function with background (analogous to Eq. 2) or a Hill function as an example of a sigmoidal function. The Hill function has the form

$$NPQ_{SV}(t) = V_{max} * \frac{t^n}{k^n + t^n} \quad (3)$$

where V_{max} is the maximal rate, n the sigmoidicity parameter, and k the Hill constant. Let us start by inspecting the bi-exponential analysis data in NPQ_{SV}-space (Table 1). The first noticeable effect of the conversion into NPQ_{SV}-space is the severe distortion in the lifetimes. The original fast lifetime of 40 s is now changed to 74–160 s, depending on the amplitude A_2 of the slow phase, and its lifetime is also strongly dependent

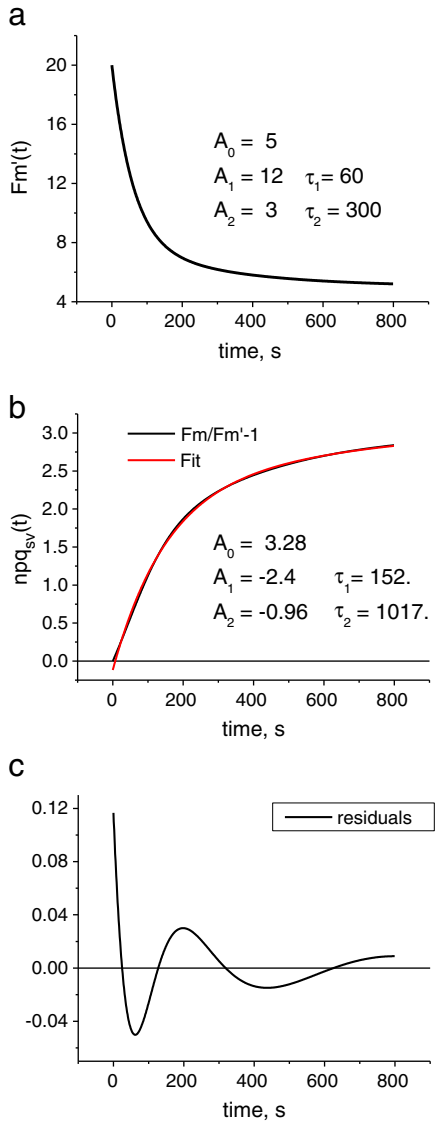

Fig. 1. Comparison of quenching analysis in the Fm′(t) and NPQ$_{SV}$ space. a) Bi-exponential model function for quenching in Fm′(t) space; b) bi-exponential fit (red) after conversion of the function to NPQ$_{SV}$ space (black); c) residuals between theoretical and fitted function in b). In a) and b) the fit parameters are shown in the inset.

on the assumed measuring time range. For smaller A_2 values actually no bi-exponential analysis is possible anymore (note: this is even the case for noiseless simulated data). This is most pronounced in the smaller time range (left half of Table 1) and somewhat less pronounced for the longer time range (right half of Table 1). Also the long lifetime (simulated value 200 s) is highly distorted, ranging from 270 s to 1434 s, i.e. scattered over a 6-fold range. The same hold for the ratio of the lifetimes. Likewise, the amplitude ratio A_1/A_2 of the amplitudes of the fast to the slow process are severely distorted in those cases where actually a bi-exponential function can be recovered, while in many cases the function cannot be separated anymore into the two constituting exponentials in NPQ$_{SV}$-space. In Supplementary Information (Fig. S1) several comparisons of the plots of simulated and theoretical functions are shown in NPQ$_{SV}$-space (all fits in Fm′(t) space are perfect as expected, fits not shown). From these plots it also follows that for a higher degree of bi-exponentiality the fits in NPQ$_{SV}$-space appear to be satisfactory but not perfect, becoming generally very poor for lower degrees of bi-exponentiality.

In summary, neither the form of the function, nor the lifetimes (rate constants), nor the amplitude ratio of the fast and the slow components are preserved by conversion into NPQ$_{SV}$-space. This is not surprising at all given the fact that actually a function linear in $x(t)$ is transformed into a function of the form A/$x(t)$ (or const/$x(t)$) when calculating the NPQ$_{SV}(t)$ function. Actually a function of the form const/$x(t)$ is close to a special form of a sigmoidal function, the function known also in biology as Hill function [15,16] (see e.g. Eq. 3). Indeed, it has been suggested occasionally in the analysis of NPQ data in NPQ$_{SV}$-space that the quenching seems to follow a sigmoidal function like e.g. a Hill function. For this reason we applied also a Hill function analysis to the same data sets in NPQ$_{SV}$-space. The results are given at the bottom in Table 1 and the plots in Fig. S2 (Supplementary Information SI). Actually, it appears that the formal fitting results in NPQ$_{SV}$-space are better when using such a Hill function despite the fact that this has only 3 free parameters, as compared to 5 free parameters for a bi-exponential with background. Also the severe dependence of the results on the assumed measuring time that was observed in the bi-exponential plots seems to be absent. Despite these formally better fits using a Hill function it must be kept in mind however that i) the original data clearly contained a bi-exponential function whose parameters cannot be recovered in NPQ$_{SV}$-space, and ii) that a sigmoidal Hill function would require an entirely different biochemical mechanism and/or reaction scheme for quenching than a mechanism that would be consistent with a bi-exponential function [15,16]. Thus the formally better fit in case of the Hill function fit should by no means be considered to reflect an improvement of the description but rather a fortuitous or accidental result. Clearly any insight into the molecular and biochemical basis of an (assumed) bi-exponential quenching function would simply be lost in this case. In some ways it is not surprising that original bi-exponential functions, in particular those with an amplitude ratio $A_1/A_2 > 5$ can be fitted well using a Hill function, or any other sigmoidal function for that case, since a mono-exponential decay function, when transformed into the reciprocal $1/\exp(-t/\tau)$ function actually is mathematically close to a standard sigmoidal function, the so-called logistic function which is defined as

$$P(t) = \frac{1}{1+e(-t/\tau)} \quad (4)$$

A small contribution of the second exponential typically adds to the apparent sigmoidicity and thus the fits of such originally bi-exponential functions - after conversion into the $1/x(t)$ space - with almost any sigmoidal function appears to be quite good, although there does not exist any fundamental justification for such a functional description.

2.2. Experimentally measured quenching curves

In the previous paragraph we simulated bi-exponential Fm′(t) quenching curves in order to study systematically the effects of the conversion and analysis in NPQ$_{SV}$ space upon change in the degree of bi-exponentiality. We now go on to analyze some characteristic data sets that derive from experimentally measured quenching and relaxation curves in Fm′(t) space. The data have been obtained from intact dark-adapted leaves of w.t. and several NPQ-related mutants of *Arabidopsis thaliana* carried out over a long measuring time (t_{max} = 60 min). The analysis of the same data in the traditional NPQ$_{SV}$-space has already been published [13] and discussed. For the present purpose the experimental Fm′(t) quenching curves were analyzed as bi-exponential functions with background on data that had been obtained from a long (60 min) actinic NPQ induction. In each case the bi-exponential function provides an excellent fit of the Fm′(t) data. The data typically contain a fast decay (time constant 10–30 s) and a long time constant that is at least an order of magnitude longer than the shorter one, with amplitude ratios ranging from 1 to 6 (see Table 2 for a collection of all data and Fig. S2 SI for the fit comparisons). Our focus here is to study what effects the conversion of these bi-exponential functions into NPQ$_{SV}$-space has, assuming different time resolutions of the measured data. This is an important point

Table 1
Analysis of simulated bi-exponential functions over time ranges of 400 s (left) and 1000 s (right) in Fm′(t) space and $NPQ_{SV}(t)$ space using a bi-exponential function and for comparison a sigmoidal Hill function. Note that in NPQ_{SV}-space the bi-exponential function is a rising function; therefore the amplitudes are expected to be negative in NPQ_{SV}-space, as compared to the positive amplitudes (decaying function) in the Fm′(t) function from which it is calculated.

Functions	Variable	$A_2 = 5$	$A_2 = 4$	$A_2 = 3$	$A_2 = 2$	$A_2 = 1$	$A_2 = 0$	Functions	Variable	$A_2 = 5$	$A_2 = 4$	$A_2 = 3$	$A_2 = 2$	$A_2 = 1$	$A_2 = 0$
$T_{max} = 400$ s								$T_{max} = 1000$ s							
Biexponential simulation and analysis parameters	Fm′(t)							Biexponential simulation and analysis parameters	Fm′(t)						
	Y_0	4	4	4	4	4	4		Y_0	4	4	4	4	4	4
	A_1	15	15	15	15	15	15		A_1	15	15	15	15	15	15
	τ_1 [s]	40	40	40	40	40	40		τ_1 [s]	40	40	40	40	40	40
	A_2	5	4	3	2	1	0		A_2	5	4	3	2	1	0
	τ_2 [s]	200	200	200	200	200	200		τ_2 [s]	200	200	200	200	200	200
	A_1/A_2	3	3.75	5	7.5	15	∞		A_1/A_2	3	3.75	5	7.5	15	∞
Biexponential analysis	$NPQ_{SV}(t)$							Biexponential analysis	$NPQ_{SV}(t)$						
	Y_0	4.8	4.61	4.66	4.77	4.93	5.18		Y_0	5.07	5.08	5.12	5.45	4.95	5.03
	A_1	−3.57	−4.7	−4.8	−4.94	−5.18	−5.52		A_1	−1.26	−2.61	−3.93	−4.74	−5.18	−5.48
	τ_1 [s]	159.59	156.25	133.52	113.54	96.	80.84		τ_1 [s]	97.43	113.61	116.82	109.64	97.4	74.28
	A_2	−1.35	–	–	–	–	–		A_2	−3.95	−2.60	−1.34	−0.89	–	–
	τ_2 [s]	333.89	–	–	–	–	–		τ_2 [s]	270.55	303.	422.19	1433.88	–	–
	A_1/A_2	2.64	–	–	–	–	–		A_1/A_2	0.32	1.0	2.93	5.33	–	–
Hill function analysis	$NPQ_{SV}(t)$							Hill function analysis	$NPQ_{SV}(t)$						
	V_{max}	5.46	5.29	5.18	5.11	5.16	5.3		V_{max}	5.56	5.41	5.26	5.14	5.05	5.06
	k	166.36	135.54	110.52	89.95	75.97	65.42		k	170.9	140.47	113.35	90.84	73.87	61.82
	n	1.25	1.31	1.39	1.5	1.67	1.9		n	1.26	1.29	1.36	1.49	1.76	2.15

which is often not considered carefully enough in experimental NPQ studies. The results of the bi-exponential NPQ_{SV}-analysis were calculated for three different time resolutions in the used data points (the results for two different time resolutions are shown in Fig. S2). At first glance the fit quality in NPQ_{SV}-space looks surprisingly good and it might lead to a first conclusion that the results of fitting in NPQ_{SV}-space could be meaningful. However, inspection of the detailed numbers in Table 2 shows that the same severe distortions due to the conversion to the NPQ_{SV}-space occur as were observed already in the previous set of data. The lifetimes are generally substantially longer than in the Fm′(t) data and the amplitude ratios are much smaller. However we see also the severe effects of choosing different time resolutions on the data points. Since the fast component in the Fm′(t) quenching data is for many photosynthetic tissues as low as 10 s, a rather high resolution of data points, at least at the beginning, is desirable. In practice, however, the application of such high time resolution might be critical, since a high frequency of saturating pulses can be expected to affect the effective actinic light intensity and by that to distort the measuring accuracy. Nevertheless it is important to evaluate the impact of different time resolutions on the kinetics analysis of fluorescence quenching. The first set shown in Table 2 uses a resolution of 1 s over the whole measuring range, a situation which is hardly fulfilled in any experimental data due to various reasons and was chosen here just to demonstrate the effects of the chosen time resolution. The next set uses a split time base with initially 3 s resolution, switching to 30 s resolution after 2 minutes. The third set uses a constant resolution of 20 s. The latter case

Table 2
The upper left matrix in the table provides the bi-exponential parameters derived from the Fm′(t) quenching data (same measurements as published in Ref. [13]) (note: the parameters used here are not the exact results from the analysis of the experimental data but the lifetimes and amplitudes were rounded). The bi-exponential functions were then simulated using various time resolutions per point and then transformed to the NPQ_{SV}-space and analyzed by a bi-exponential function with background (Y_0). The results are shown in the upper right, lower right and left matrices of the table for the different time resolutions.

Biexponential simulation parameters	Fm′(t)						Biexponential analysis	$NPQ_{SV}(t)$					
$T_{max} = 60$ min							$\Delta t = 1$ s						
	Y_0'	A_1	τ_1	A_2	τ_2	A_1/A_2		Y_0	A_1	τ_1	A_2	τ_2	A_1/A_2
WT	0.3	0.6	25	0.1	540	6		2.34	−1.62	37	−0.85	629	1.9
npq4	0.46	0.3	20	0.3	2000	1		1.47	−0.4	142	−1.09	3474	0.37
npq2	0.33	0.6	10	0.1	1200	6		2.15	−1.51	14.31	−0.76	1487	1.99
npq1	0.42	0.4	10	0.2	1350	2		1.48	−0.67	11.9	−0.85	1892	0.79
Biexponential analysis	$NPQ_{SV}(t)$						Biexponential analysis	$NPQ_{SV}(t)$					
$\Delta t = 3$ s $\Delta t = 30$ s							$\Delta t = 20$ s						
	Y_0	A_1	τ_1	A_2	τ_2	A_1/A_2		Y_0	A_1	τ_1	A_2	τ_2	A_1/A_2
WT	2.34	−1.65	41	−0.8	682	2.06		2.34	−1.54	40	−0.85	634	1.81
npq4	1.49	−0.39	153	−1.1	3597	0.35		1.47	−0.4	144	−1.09	3487	0.37
npq2	2.16	−1.49	15	−0.75	1582	1.99		2.15	−1.4	15	−0.76	1482	1.84
npq1	1.49	−0.66	13	−0.85	1961	0.78		1.48	−0.63	12	−0.85	1887	0.74

is close to typical experimental cases reported in the literature using e.g. PAM equipment or the like. We note however that even using only 20 s resolution all the parameters can still be extracted correctly in a bi-exponential fit (note: this is a simulation assuming no noise or other disturbances; actual experimental data sets will require a higher time resolution to compensate for such effects). But this is by far not the case for the fit in the NPQ_{SV}-space. Results are strongly dependent on the chosen time resolution. Thus in practice a split time base during the measurement is the most desirable situation, even when performing the analysis in the $Fm'(t)$ space.

3. Discussion

The $Fm'(t)$ data in non-photochemical quenching measurements by instruments like the PAM fluorimeter or similar represent the originally measured data. Without assuming or suggesting here any detailed quenching mechanism it is *a priori* an entirely reasonable hypothesis that these observed $Fm'(t)$ data and their kinetics, i.e. rate constants (lifetimes), amplitude ratios of fast and slow components, background level etc. should be correlated in some way with the kinetics and/or concentrations of the underlying physical and biochemical processes/compounds leading to quenching. One of the basic tasks in NPQ research that has to be solved in order to gain insight into the quenching mechanism(s) consists in finding and revealing these correlations. The analysis presented above shows however that the conversion of the measured Chl fluorescence quenching data from $Fm'(t)$ space to $NPQ_{SV}(t)$ space severely alters the time constants as well as the ratio of contribution of fast and slow processes. Consequently any interpretation of the kinetics and relative contribution of different quenching processes is essentially dependent on the applied analysis of the data. It is important to point out that the current understanding of different NPQ processes has been mainly derived from analyses of the relaxation characteristics of $NPQ_{SV}(t)$. The amplitudes of different relaxation components (which are thought to reflect different quenching components, such as qE, qT, qZ and qI) are usually taken as a measure for the relative contribution of different underlying quenching mechanisms to the overall NPQ. In comparison with the analysis of $Fm'(t)$ data, the analysis based on $NPQ_{SV}(t)$ data strongly overestimates the relative contribution of more rapidly relaxing NPQ components (usually qE) to the overall NPQ. It is thus clear that any reliable interpretation of fluorescence quenching data requires a careful evaluation of the underlying theories or models applied for the analyses.

Without going into details (all of this is well understood and published material) we note that actually the $Fm'(t)$ function can be directly related to the Chl excited state dynamics and the detailed primary molecular processes of photosynthetic charge separation, quenching, etc. occurring in the photosystems provided that ultrafast time-resolved data are available (see e.g. Refs. [8,17,18]) for derivations and the demonstration of multiple quenching components. Such data are indeed available for intact isolated photosystems (PSI as well as PSII) of many different photosynthetic organisms as well as for intact microalgae and intact chloroplasts or even intact leaves of higher plants [8,18–27]. One of the additional important negative consequences of the usually applied conversion of Chl fluorescence quenching data into the NPQ_{SV}-space thus also involves the loss of the direct correlation with ultrafast time-resolved fluorescence data.

It is worth recalling here how it actually became common practice to use $NPQ_{SV}(t)$ data for quenching analysis rather than the directly measured $Fm'(t)$ data. In early studies on non-photochemical quenching, changes in the variable fluorescence (and thus $Fm'(t)$ data) have been determined by applying different measuring principles to discriminate between photochemical and non-photochemical quenching processes. At that time, the non-photochemical quenching (mostly termed qN or q_{NP}) was defined as $(1 - Fv'/Fv)$ [28–30]. Also the first analyses of the different components of NPQ were derived from such $Fm'(t)$ data [29,31]. Based on the model by Kitajima and Butler [32], however, Demmig and co-workers introduced in 1989 the parameter k_D to quantify NPQ processes according to a Stern–Volmer type equation [2], and the now commonly applied NPQ_{SV} parameter $(Fm/Fm' - 1)$ was finally defined on the same basis by Bilger and Björkman [33]. The parameter NPQ_{SV} offered some practical advantages in comparison with the parameter qN or $(1 - Fv'/Fv)$: NPQ_{SV} can be determined without measuring F_0 or F_0', the value of NPQ_{SV} increases with increasing quenching, and NPQ_{SV} is not limited to the maximum value of 1. Furthermore, the parameters k_D and NPQ_{SV} were found to correlate linearly with the Zx concentration in contrast to the parameter $(1 - Fv'/Fv)$ [34–36].

Two caveats are in order here however which concern i) the actual lack of theoretical justification by Stern–Volmer theory, and ii) the later tacit extension of the pure NPQ_{SV} parameter to the time-dependent version $NPQ_{SV}(t)$ and its mechanistic interpretation.

As far as the first point is concerned the definition of NPQ_{SV} does correspond to the assumption of "collisional" (sometimes also called "diffusional") quenching (it is important to note here that "diffusional quenching" does not imply "excited state diffusion" but real Brownian diffusion of the quencher, and correspondingly of the quenched species, on the time scale of the excited state of the quenched species) in a homogeneous medium in three dimensions [37]. If these conditions were fulfilled in non-photochemical quenching then according to Stern–Volmer theory $(Fm/Fm' - 1)$ would be linear as a function of quencher concentration and the intercept of the line would be at 0 (i.e. at the origin). However even at the time of the introduction of the NPQ_{SV} treatment for non-photochemical quenching the above-mentioned authors did not assume that e.g. zeaxanthin as a quencher would actually be diffusing through the membrane on the time scale of the excited state lifetime of the antenna complexes. Diffusion in the photosynthetic membrane even for a small molecule like zeaxanthin would be much too slow to have any quenching effect. Furthermore, the membrane is not a three-dimensional system, which is another strict requirement for the applicability of this form of the Stern–Volmer equation (diffusion in two dimensions, like e.g. in a membrane, leads to much more complex formulae [37]). It was rather assumed that the quencher would be actually bound somehow to the antenna complexes. This situation would actually correspond more to the opposite extreme of quenching, i.e. "static quenching." Assumption of static quenching would however lead to a dependence (Fm/Fm') being linear in the quencher concentration, i.e. the intercept of the line will be at 1 [37]. This was however not found in these early studies for example for the dependence on the zeaxanthin concentration. These are but the two simplest cases. Any NPQ quenching situation deviating from these extremes would result in more complex special formulae and would have to be treated separately. It is in fact highly likely that a more complex situation than described by these two extremes is realized in non-photochemical quenching, given the fact that we are knowing already at least two quenching mechanisms and at least two quenching sites [8,13,17,18,38–43].

Whether actual Stern–Volmer quenching does indeed provide an adequate theoretical basis for describing non-photochemical quenching was thus never discussed nor proven in a stringent manner. Nevertheless the term came to life and persists till today, although Govindjee stated in his review article about non-photochemical quenching and actual Stern–Volmer quenching that "…In NPQ it is applied, however, in a quasi-solid-state system, in which only excitation energy moves, but not molecules. Thus, there is only a formal similarity between these two processes…". This statement should be interpreted in the sense that NPQ_{SV} as used in the present form represents merely a technical parameter that has a formal similarity to the real "collisional" Stern–Volmer quenching without any sound theoretical justification. At the same time the review of Baker and Oxborough gave some practical warnings regarding the use of the NPQ parameter [44].

The second caveat – which is even more severe for the present discussion – concerns the later extension of the static formula of NPQ_{SV} to the time-dependent form $NPQ_{SV}(t)$. Once the time-dependent Chl quenching measurements and commercial instrumentation for easy measurements had been introduced (for reviews see e.g. Refs. [9,10]) it

became common practice to use the same definition also for the time-development of the quenching (i.e. $NPQ_{SV}(t)$). The theoretical basis for this form, and its eventual mechanistic interpretation in terms of quenching processes, has never been discussed in the literature however. Rather it was tacitly assumed – without any further justification (simply because NPQ_{SV} was often plotted at various times after the onset of the actinic radiation) – that $NPQ_{SV}(t)$ would actually have a theoretical base in Stern–Volmer quenching. Such an assumption could actually be valid only in a situation where the quencher concentration would increase linearly with time on the seconds to minutes range after onset of the actinic radiation, a highly unlikely situation indeed. On top, all the other requirements for Stern–Volmer quenching discussed above would still have to be valid, which is not the case. There actually exists no general formula justified in Stern–Volmer quenching theory that would allow describing in a meaningful manner NPQ_{SV} as a function of time. In conclusion, neither the static nor the dynamic ($NPQ_{SV}(t)$) forms of the non-photochemical quenching analysis have any sound theoretical foundation in the physical phenomenon known as "Stern–Volmer quenching" which has been treated very extensively, in particular also regarding its applications to biological systems, in standard textbooks [37]. Given this situation we feel strongly that the existing connotation between the phenomenon of non-photochemical quenching on the one hand and the term "Stern–Volmer quenching" on the other hand should be discontinued altogether.

4. Conclusions

The $Fm'(t)$ data represent the directly measured Chl fluorescence quenching effects in photosynthetic organisms. Thus these data should be used when performing a kinetic analysis of quenching data and attempting to develop any quenching model or when correlating Chl fluorescence quenching data with kinetic data and/or concentrations of potential quenchers or quenching mechanisms. This should be done at least unless increasing knowledge about the underlying quenching mechanisms provides a better model for such analyses. The conversion of $Fm'(t)$ data into $NPQ_{SV}(t)$ was originally based on the assumption that fluorescence quenching represents a Stern–Volmer quenching process which in our hands is not warranted. Even if staying within the framework of Stern–Volmer quenching – which, given our present understanding is not justified – a relationship $NPQ_{SV}(t)$ vs. time is not supported by theory [37]. Performing kinetic analysis in the $NPQ_{SV}(t)$ space thus severely distorts the quenching kinetics and the contribution of different (slow/fast) components, entirely independent of the actual time function the quenching would follow. This is caused by the fact that a mathematical conversion from an $x(t)$ space to a $\mathrm{const}/x(t)$ space is involved in the calculation of $NPQ_{SV}(t)$ from $Fm'(t)$. While allowed mathematically this is not an innocent operation for a physical parameter that is assumed linear in $x(t)$. The resulting distortion is highly likely to reduce (or even completely wipe out) the possibility for a meaningful correlation with other NPQ related data. In fact any conclusions drawn on kinetics or mechanisms of NPQ quenching from the analysis of $NPQ_{SV}(t)$ data should be considered at least as being highly questionable.

The main purpose of this work is to point out the unfortunate and erroneous consequences of an inadequate analysis of time-dependent NPQ data and to provide some insightful examples by using simple analytical functions. It was not intended to propose at this point any new conclusions regarding possible NPQ quenching mechanisms nor do we propose here any actual kinetic form for the time-dependence of NPQ in real photosynthetic systems. It is clear however that important questions in NPQ research regarding e.g. the existence of multiple quenching mechanisms, multiple quenching sites etc. and questions whether these multiple mechanisms and sites operate independently of each other [13,17,18] or in a cooperative manner [5,12,45] need to be answered. In our view it will only be possible to find the correct answers to these questions if the analysis and interpretation of the quenching data are performed in a theoretically sound manner. We will thus in a subsequent experimental work present data and discuss in detail how Chl fluorescence quenching data can be analyzed properly and what kind of information can be drawn under such circumstances. In any case the present study strongly suggests that kinetic analysis and interpretation of $NPQ_{SV}(t)$ quenching data in the traditional manner should be discontinued since this may lead to severe misinterpretations of the underlying mechanisms. The analysis and interpretation should instead be applied to unconverted data directly in the $Fm'(t)$ space. No loss of information is associated with this form of analysis and everything (more reliable interpretations) is to be gained. One will in this case be able to properly test and compare various mechanistic models without the risk of arriving at the wrong conclusions. It is gratifying though that $NPQ(t)$ can be easily converted back to $Fm'(t)$ in all published experiments. Thus all the information reported in the literature can be still used after back-conversion and new analysis within this framework.

One final point of notice: The NPQ parameter in the definition used conventionally and discussed here (however without the link to the term "Stern–Volmer quenching") may still be used as a convenient pure "technical" parameter to exchange quantitative quenching data between laboratories, as long as it is not interpreted in a mechanistic manner. It may thus continue to be useful for two reasons: It is i) easily measurable with equipment that is available in most photosynthesis laboratories, and ii) it provides an absolute number that can be compared between laboratories to characterize plant behavior.

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Appendix A. Supplementary data

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