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.

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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.

[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

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

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

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

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

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

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