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Contribution to the discussion on the nonregulatory nonphotochemical quenching

LETTER TO THE EDITOR

Response to the Letter of Ulrich Schreiber in Photosynthetica (DOI 10.32615/ps.2024.023), as part of the discussion initiated by the paper of Győző Garab with the title: Revisiting the nonregulatory, constitutive nonphotochemical quenching of the absorbed light energy in oxygenic photosynthetic organisms

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This is a tardy reply, a short reflection upon the Letter of Ulrich Schreiber, whose contributions to chlorophyll fluorescence induction (ChlF) research and the design and construction of excellent chlorophyll fluorimeters can hardly be overestimated.

In general, Uli and I, probably both agree that numerous questions are still open and should be clarified on the origin and physical mechanism(s) of ChlF. Also, both of us think that this technique, with further technical developments and novel measuring protocols, will remain one of the key tools in photosynthesis research. At the same time, **we clearly disagree on a few, cardinal points** – as it is evident from the Letter by Ulrich Schreiber published in Photosynthetica.

Uli considers that "the Saturation Pulse (SP) method, with the help of which about 40 years ago the determination of F_v/F_m and the complementary PSII quantum yields Y(II), Y(NPQ), and Y(NO) became possible" provides correct data: "in normal photosynthetically active organisms the expressions derived for the fluorescence-based complementary PSII quantum yields Y(II), Y(NPQ), and Y(NO) are valid", except "when the OEC is damaged and the PSII acceptor pool cannot be filled up" and when PSI fluorescence to F_v, $F_v(I)$, is sizeable. For this latter case, it is proposed that Y(II) should be calculated using the intermediary level I_2 rather than the F_m level. This would evidently decrease the calculated value of Y(II) in dark-adapted healthy PSII – probably well below the quantum efficiency level of PSII photochemistry determined by other techniques. In my opinion, this would add to the (already long and cumulative) list of controversies of the Q_A model of ChlF. (For historical references and recent results and reviews, *see* my opening article and Garab *et al.* 2023.) However, I would like to stress that the problem with Y(NO) is more severe than just minor (10–20%) deviations from its expected (and/or independently determined) values.

To demonstrate that the fluorescence-based determination of Y(NO) might be seriously underestimated, I showed a PAM-based experiment on dark-adapted diuron-treated PSII core complexes (CCs); to generate F_m a train of single-turnover saturating flashes (STSFs) was used, instead of a SP. These data showed that the kinetic traces of ChlF were invariant on doubling the flash intensity (using two simultaneously fired STSFs). Consequently, with Y(NPQ) = 0 and F_v/F_m = 0.77 for all three cases, we obtain Y(II) = 0.77 and Y(NO) = 0.23 – using either one of the two flashes or the two together. Our data, using PSII CC and STSFs, and our approach in general, was heavily criticized by Uli. I will come back briefly to his overall critical remarks in separate paragraphs below. First, however, I would like to propose a similar, easy-to-perform experiment without the use of a train of single or double STSFs.

The proposed experiment may be performed on untreated or diuron-treated PSII CC, isolated thylakoids, algal cell suspension, or an intact leaf – using PAM fluorimetry and employing multiple turnover (MT) light pulses with controllable SP intensity. As explained by Klughammer *et al.* (2024): "In PAM fluorimetry (Schreiber *et al.* 1986) maximal fluorescence yield traditionally is determined by an MT-protocol, *i.e.*, using an about 300 ms long pulse of saturating light that leads to full reduction not only of Q_A, but of the secondary acceptor pools of PSII and PSI as well". By gradually increasing the intensity of the MT pulse, one may easily find the ~100% saturation level of the pulse for any given sample; and thus, the maximum of Y(II) (= F_v/F_m) and the minimum value of Y(NO) (= 1 – F_v/F_m) can be calculated. Then, upon gradually increasing the intensity of SP (>100%), one should find a gradual increase of Y(NO) at the expense of Y(II) – but, according to my experience, this is not observed in a broad range of SP intensity.

Very similar experiments can be performed using an 'OJIP device', which employs continuous (or quasi-continuous, high repetition rate fast LED pulse) excitation

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and fast chlorophyll fluorescence response detection. These devices resolve the intermediary, J and I (or I_1 and I_2), steps, between the minimum (O, F_o) and maximum (P, F_m) levels. In fact, a series of experiments of this type has been performed by Schansker *et al.* (2011), who recorded OJIP transients on untreated detached pea leaves and found that the P level was reached in less than about 200 ms and the F_v/F_m parameter did not change in a broad range of the photon flux density of the excitation, between 0.9 and 15 mmol(photon) m^{-2} s^{-1}. (The shape of the kinetic traces varied with the light intensity applied but the main 'OJIP characters' were retained; for similar experiments in the literature, *see* references cited in Schansker *et al.* 2011.)

Evidently, because under all these conditions no NPQ is discerned, the fluorescence-based values of Y(NO) cannot be correct.

The main problem with the discrepancy concerning the determination of Y(NO), in my opinion, is that a constant – *constitutive* – value, in the range of 0.2, does not warn the experimenter that important processes might be triggered by npq-silent dissipative processes (including light-induced and dissipation-assisted events). Evidently, by improving the time resolution of ChlF measurement (as by Laisk *et al.* 2024 and Klughammer *et al.* 2024) and using independent techniques (such as transient absorbance or photoacoustic spectroscopy techniques), the dependence of Y(NO) on the excess quanta can be made visible – also under physiologically relevant conditions. By these complementary tools even the partition of dissipation between different processes – such as triplet states, protein shock waves / local heat jumps, release of cations, and ROS production, events possibly involved in regulatory processes *in vivo* – might be revealed. Investigations of this type, and uncovering npq-silent processes triggered by excess excitation of PSII in intact systems under physiologically relevant conditions, may underline the physiological importance of Y(NO) – fully justifying the introduction of this parameter, albeit not in its original forms some 40 and 20 years ago.

Regarding Uli's remarks criticizing our experimental approach and rejecting our interpretation of ChlF (which, indeed, substantially deviates from the Q_A model), I must refer the reader of this Letter to key findings and references quoted in my opening article and in Garab *et al.* (2023). Here I briefly reflect on the main points of Uli's criticism.

(*1*) I do sharply disagree with the criticism regarding the use of PSII CC, because: (i) the referred complexes have been shown to display very high rates of oxygen evolution (Shen and Kamiya 2000); (ii) the samples display high F_v/F_m values, values in different batches typically range between 0.75 and 0.83; (iii) with PSII CC, ChlF measurements can be performed without any interference from PSI and $F_v(I)$; (iv) isolated dimers and monomers allow elucidating the origin of the sigmoidal rise of ChlF in the absence of energetic connectivity between PSII units; and (v) last but not least, atomic resolution structural data aid our understanding of structure-based steady-state fluorescence spectroscopy data – which, recorded between 4 K and 293 K (Andrizhiyevskaya *et al.* 2005) and 5 K and 180 K (Shibata *et al.* 2013), revealed the distinct origins of F685 and F695 from the CP43 and CP47 inner antenna complexes, respectively; and time-resolved fluorescence spectroscopic data on closed and light-adapted reaction centers – reflecting electron transfer and associated protein conformational relaxation processes (Szczepaniak *et al.* 2009, Sipka *et al.* 2021).

I would like to stress that I, of course, consider all laboratory and field measurements on intact organisms very important. However, for instance, in an intact leaf, besides uneven excitation due to leaf anatomy, many additional – physiologically important – factors might arise. These may include $F_v(I)$, spillover between the two photosystems, PSII heterogeneity, and state transitions, as well as structural and functional inhomogeneities in the leaf tissue. Identifying all these requires safe grounds concerning the origin of ChlF.

(*2*) As to the negative comments on cryogenic temperatures and ChlF measurements under physiologically non-relevant conditions, I must point out that (i) the seminal experiment by Kitajima and Butler (1975) – serving as the foundation of $Y(II) = F_v/F_m$ – were performed at 77 K; but (ii) the experiments of Joliot and Joliot (1979) – discovering the STSF-induced multistep fluorescence increments, after closing all active reaction centers by the first flash – were carried out on diuron-treated isolated thylakoid membranes at room temperature; and were extended to cryogenic temperatures only later (Magyar *et al.* 2018); (iii) the experiments by Magyar *et al.* (2018) – uncovering that these increments occurred only when sufficiently long waiting times were applied between consecutive flashes – were observed (and published) not only in PSII CC but also in thylakoid membranes and not only at cryogenic temperatures but also at 5 and 20°C; further, (iv) the experiments of Laisk and Oja (2020) – showing that the charge separated state of PSII in intact untreated leaves does not bring the system to F_m state – was performed at room temperature.

(*3*) Regarding Uli's arguments on the role of donor side effects, I have a weaker disagreement. We have emphasized that polarizable groups on the donor side of PSII are thought to play key roles in modulating F_v and that – *in the absence of diuron* – the S-state dependence (period-4 oscillation) of fluorescence must be taken into account (Sipka *et al.* 2022, and references therein). However, a DQ quenching *via* (the well-known strong quencher) $P680^+$ does not explain our data. Single, double, and repetitive laser flash-induced fast 819-nm absorbance transients were recorded on diuron-treated PSII CC; these experiments confirmed that only the first STSF is capable of generating stable charge separation and no correlation was found between the turnover of P680 and the waiting-time dependent multi-step fluorescence increments (Sipka *et al.* 2019). Evidently, with a $P680^+$ quenching mechanism, one should assume a gradual STSF-induced *depopulation* of $P680^+$ – parallel with the gradual fluorescence increments from the F_1 to the F_m level elicited by the same flashes (Magyar *et al.* 2018, Sipka *et al.* 2021).

I trust that our discussion on Y(NO) will further stimulate the use of chlorophyll fluorimeters with high

time resolution and the application of non-conventional protocols and complementary measuring techniques – which might eventually allow us to determine the true magnitude of Y(NO) and understand its nature and physiological roles in a large variety of organisms and under a broad range of environmental conditions.

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