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BRIEF COMMUNICATION

Application of spectrally resolved fluorescence induction to study light-induced nonphotochemical quenching in algae

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Abstract

The light-induced nonphotochemical quenching (NPQ) can safely dissipate excess of absorbed light to heat. Here we describe an application of spectrally resolved fluorescence induction (SRFI) method for studying spectral variability of NPQ. The approach allows detection of spectrally-resolved nonphotochemical quenching (NPQ_λ) representing NPQ dependency on fluorescence emission wavelength in the whole spectral range of fluorescence emission. The experimental approach is briefly described and NPQ_λ is studied for the cryptophyte alga *Rhodomonas salina* and for green alga *Chlorella* sp. We confirm presence of NPQ_λ only in membrane-bound antennae (chlorophyll *a/c* antennae) and not in phycobiliproteins in lumen in cryptophyte and show that NPQ_λ is inhibited in the whole spectral range by NPQ inhibitors in *Chlorella* sp. We discuss variability in the quenching in the particular spectral ranges and applicability of the NPQ_λ parameter to study quenching locus *in vivo*.

Additional key words: fluorescence parameters; light-harvesting complex; photoprotection; photosynthesis; photosystem II.

Light-induced nonphotochemical quenching of chlorophyll (Chl) *a* fluorescence (NPQ) represents a regulated feedback mechanism allowing photoprotection of thylakoid membrane proteins during short periods (minutes) of excessive irradiation (*see e.g.,* Ruban *et al.* 2012, Derks *et al.* 2015). The process protects PSII and proceeds either in PSII reaction centrum (*see, e.g.*, Bruce *et al.* 1997, Komura *et al.* 2010, Krupnik *et al.* 2013) or in the associated antenna proteins (Belgio *et al.* 2014, Holzwarth *et al.* 2009, Xu *et al.* 2015). On a molecular basis, regulatory NPQ in antennae is connected with reversible switch of antenna proteins between light-harvesting and photoprotective mode (Krüger *et al.* 2011, 2013). The so-called energy dependent component (q_E) of NPQ is triggered by lumen acidification (Briantais *et al.* 1979, Gilmore and Yamamoto 1992, Belgio *et al.* 2013); however, there are some other factors (*e.g.,* role of ions) that need to be taken into account (Kaňa and Govindjee 2016). The pH sensitivity is controlled by several other allosteric regulators including xanthophylls (Niyogi *et al.* 1997, Kaňa *et al.* 2016) or PsbS protein in vascular plants (Li *et al.* 2000). Once the regulatory NPQ is induced, the quantum yield of Chl *a* fluorescence is lowered due to dissipation of excess excitation energy into heat, which can be detected as a change in sample temperature (Kaňa and Vass 2008).

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Abbreviations: CAC – chlorophyll *a*/*c* antennae complexes of cryptophyte; Chl – chlorophyll; DCMU – 3-(3,4-dichlorophenyl)-1,1-dimethylurea; F_t – fluorescence intensity at particular irradiance/time of measuring protocol; F_0 – minimal fluorescence intensity for open reaction center; F_M – maximal fluorescence intensity for closed reaction center measured with dark-adapted sample; F_M‘ – maximal fluorescence intensity for closed reaction center measured with light-adapted sample; NPQ – nonphotochemical quenching of chlorophyll *a* fluorescence; NPQ_λ – spectrally resolved nonphotochemical quenching of fluorescence; RC – reaction center; Rfd – fluorescence decrease ratio.
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However, direct measurement of the NPQ based on changes in the sample temperature is not well established [compare Kaňa and Vass (2008) with Kulasek *et al.* (2016)], therefore, the method of variable fluorescence is often used as a versatile and easy-to-use method to detect the extent of NPQ (*see*, *e.g.,* Bilger and Björkman 1990, Papageorgiou and Govindjee 2004, Demmig-Adams *et al.* 2014). In typical experimental approach, NPQ is calculated and measured based on a decrease in maximal Chl *a* fluorescence after illumination by actinic light (*i.e.,* as a decrease from dark level F_M to F_M‘ level on light) using Stern–Volmer formalism for collisional quenching [*see*, *e.g.*, Lakowicz (2006) or Holzwarth *et al.* (2013)]. An extent of the process of nonphotochemical quenching can be also defined in terms of quantum yields of nonphotochemical quenching, where regulatory and nonregulatory (basal) nonphotochemical quenching needs to be considered [for more details *see* Kramer *et al.* (2004) and review of Lazár (2015)]. Recently (Lazár 2015), the NPQ parameter has been found to be a ratio of quantum yield of the regulatory nonphotochemical quenching to quantum yield of nonregulatory (basal) nonphotochemical quenching.

In a standard (spectrally not resolved) experimental set-up, the decrease in the Chl *a* fluorescence is calculated as an integral value over the wide range of Chl *a* emission band (usually above 700 nm) and does not take into account changes at particular wavelengths. There are only few attempts that have tried to address fluorescence quenching spectrally. A spectrally resolved fluorescence spectroscopy at low temperature has allowed to attribute NPQ locus into light-harvesting antennae (Ruban and Horton 1994). Later, spectral resolution of NPQ at room temperature has allowed detection of different quenching components in *Arabidopsis* leaves (Lambrev *et al.* 2010) and role of red-shifted antennae in NPQ of *Chromera velia* alga (Kotabová *et al.* 2014) or absence of fluorescence quenching in phycoerythrins in cryptophyte (Kaňa *et al.* 2012b).

All these methods required application of a certain type of a spectrally resolved fluorescence induction (SRFI) method. The SRFI method can address regulation of light-harvesting in general. Initially, the method was used to study mechanism of state transitions and decoupling of phycobilisomes in the cyanobacteria *Synechococus* sp. PCC 7942 and *Synechocystis* sp. *PCC 6803* (Kaňa *et al.* 2009a, 2012a). Later, it has been adapted to more specific projects dealing with regulation of light harvesting in cyanobacteria *Synechocystis* sp. *6803* and its mutants (Kaňa *et al.* 2012a, Acuña *et al.* 2016, Bernat *et al.* 2017). Recently, it has been applied for several algal strains including *Chromera velia* (Kotabová *et al.* 2014) and cryptophyte algae (Kaňa *et al.* 2012b, Cheregi *et al.* 2015) to study NPQ locus and mechanism of state transitions. Here, the spectrally resolved nonphotochemical quenching (NPQ_λ) is characterized in more details for cryptophyte and green algae in terms of NPQ_λ kinetics and a role of inhibitors. The data reported here indicate that application of SFRI to detect NPQ_λ is a very promising technique for future research in photosynthesis as it allows detection of spectral changes occurring *in vivo* during natural energy equilibration/quenching processes.

The cryptophyte alga *Rhodomonas salina* (strain CCAP 978/27) and the green alga *Chlorella* sp. (Novotný *et al.* 2017) were grown in the cell suspension in artificial seawater medium with f/2 nutrient addition. The cultures were continually bubbled with air in a temperature-controlled bath (18°C) and illuminated by dimmable fluorescence tubes [30 μmol(photon) m^{-2} s^{-1}, day–night cycle of 12/12 h). Each sample was dark-adapted for 20 min before measurements. The measurements were carried out in the exponential growing phase (OD_{760nm} was in the range 0.2–0.3).

Nonmodulated technique of SRFI was measured with spectrometer *SM–9000* (*Photon Systems Instruments*, Brno, Czech Republic) with spectral bandwidth of 0.8 nm (in the range of 200–980 nm), the dark current of the instrument was automatically subtracted before measurements, based on the method described previously for cyanobacteria (Kaňa *et al.* 2012a) and algae (Kotabová *et al.* 2014). Samples were dark-adapted for 20 min before applying low-intensity measuring light [2 μmol(photon) m^{-2} s^{-1}] for the detection of fluorescence of open PSII reaction center measured with the dark-adapted sample – F_0. Maximal fluorescence intensities for the dark-adapted (F_M) and light-adapted sample (F_M’) have been measured at the end of 200-ms multiple turnover saturating actinic flashes [464 nm, 2,350 μmol (photon) m^{-2} s^{-1}]. The kinetic changes in spectra during actinic irradiation [464 nm, 1,100 μmol(photon) m^{-2} s^{-1}] were measured in the whole fluorescence spectrum with millisecond time-resolution scale (every 50 ms). The spectra of maximal fluorescence in the light $F_M'(\lambda)$ were detected after at different time of actinic irradiation (*see* description in figure legends). The spectrally resolved NPQ_λ was calculated based on the Stern–Volmer formalisms as $NPQ_\lambda = [F_M(\lambda) - F_M'(\lambda)]/[F_M'(\lambda)]$ for every wavelength. For comparison, the standard Chl *a* fluorescence induction has been detected simultaneously in the spectral range 690–710 nm (Fig. 1S, *supplement available online*) by *FL 100* spectrophotometer (*Photon System Instrument*, Brno, Czech republic) as described before (Kaňa *et al.* 2012a).

A measurement of NPQ_λ requires application of SRFI that has been described previously (Kaňa *et al.* 2012a). The SRFI method allows detection of the whole fluorescence spectrum in a wide spectral range (200–980 nm) with relatively high spectral resolution (wavelength accuracy of 0.8 nm, relative resolution reflecting FWHM $\Delta\lambda = 3$ nm, *see* Kaňa *et al.* 2012a), and with a fast detection rate (every 50 ms in our case). Evolution of the fluorescence spectra in time is plotted in a 3D graph (Fig. 1*A*) and as a 2D color-coded graph (Fig. 1*B*). There, the fluorescence emission spectra are represented as sections parallel to the wavelength axis (x-axis) at a given time of the measuring protocol (*see* top part of Fig. 1*B* labeled as *“Spectrum”*),

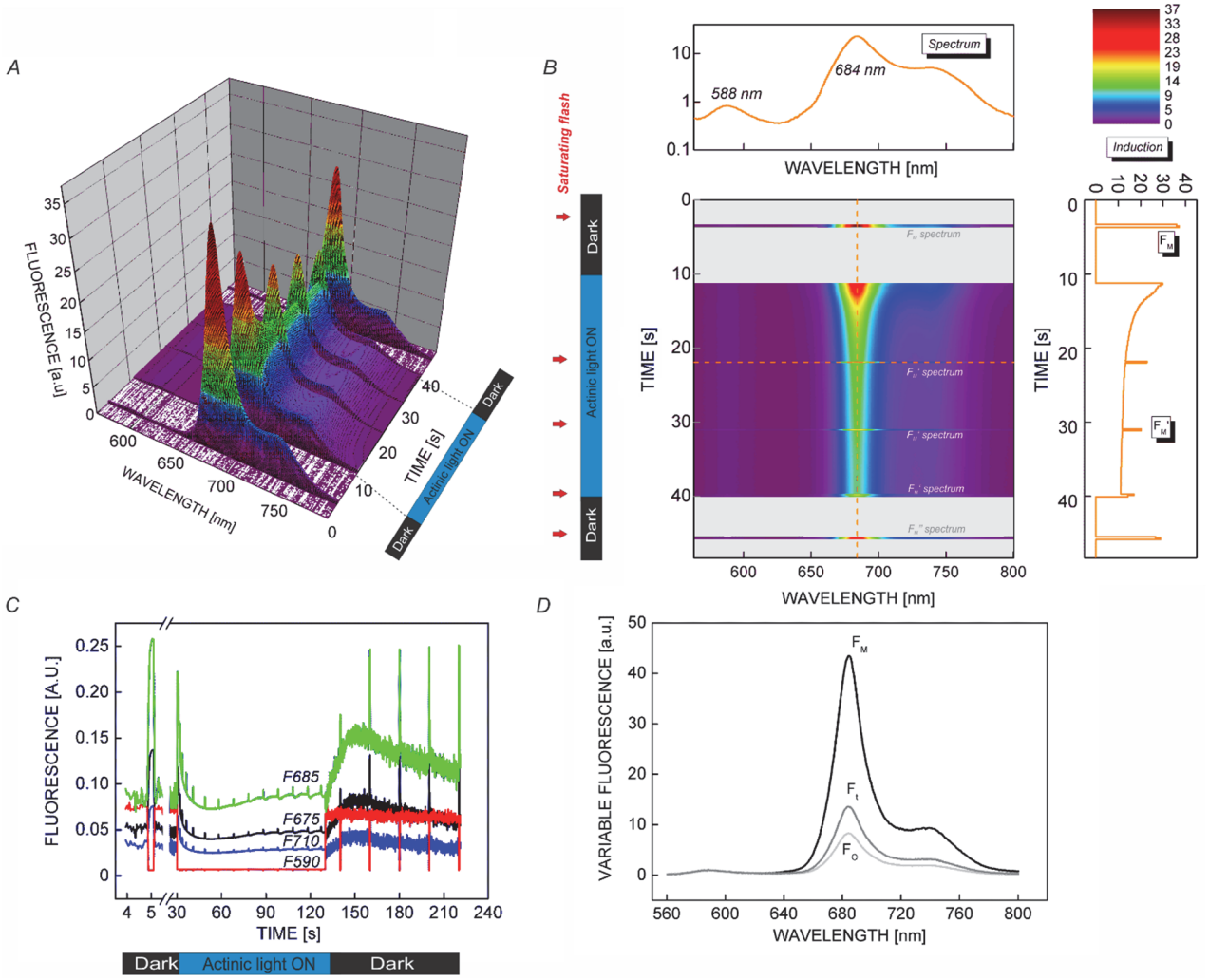

Fig. 1. The spectrally resolved fluorescence induction (SRFI) method applied to *Rhodomonas salina* cells. To explain the method, two different protocols were used; a short one (60 s, panels *A*,*B*) and the long one (220 s, panels *C*,*D*). Cells were dark-adapted for 20 min and blue light [464 nm; 1,100 µmol(photon) m^{-2} s^{-1}] was used to induce nonphotochemical quenching. The entire emission spectrum was measured every 50 ms. (*A*): 3D graph of fluorescence induction during a short protocol (60 s) with three saturating flashes applied during light period (F_M‘) and one before (*see* F_M) and after the light period. (*B*): Two dimensional (2D) replotting of SRFI data presented in panel *A*. The graph shows dependency of intensity of variable fluorescence F = f(λ,t), different intensity of fluorescence at particular time/wavelength is represented by artificial colors (*see* the scale). The timing of actinic light irradiation and application of saturating flashes are shown by *bars* and *red arrows*, respectively. Spectra are presented in the range between 560 and 800 nm, measured during the 60-s long protocol. The typical fluorescence spectrum at constant time (at t = 30 s of measurements) is presented (*see* insert *"Spectrum"* on the top) together with typical fluorescence induction at 685 nm (*see* inset "Induction"). (*C*): Examples of other fluorescence inductions measured with 220-s long protocol. The fluorescence induction at selected wavelength (F590, F685, F675, F710) are presented, the fluorescence intensity was corrected for different intensities of actinic light used for detection of F_O, F_M, and F_t. Data resembled simultaneous single wavelength range (690–710 nm) measurement by *FL 100* fluorimeter presented in Fig. 1S. (*D*): Fluorescence spectra measured at different stages of fluorescence induction, they were used for calculation of F_O, F_M (measured after 2 min on light during saturating flash), and Ft spectra (measured after 2 min in light) obtained from long protocol (220 s). The fluorescence intensity was corrected for different intensities of actinic light used for detection of F_O, F_M, and F_t.

a section parallel to the time axis (y-axis) shows fluorescence induction at a particular wavelength (*see* left part of Fig. 1*B* labeled as *"Induction"*). The approach allows us continual detection of the fluorescence induction at different wavelengths (Fig. 1*C*). The kinetic data (*i.e.*, fluorescence induction) are comparable with fluorescence measurements by other commercial spectrophotometers including pulse-modulated fluorimeters (Schreiber 2004) or other fluorimeters (*see*, *e.g.*, Kaftan *et al.* 1997 and data in Fig. 1S). The main advantage of the SRFI method is represented by simultaneous detection of fluorescence kinetics (kinetics of fluorescence quantum yield) in any selected wavelengths (*see* Fig. 1*C*, and previous applications in Kaňa *et al.* 2009a, 2012a; Kotabová *et al.* 2014).

As the method continuously detects spectra of all particular fluorescence parameters (F_t, F_0, F_M spectra in Fig. 1*D*), all fluorescence parameters defined for pulse-modulated fluorimeters (Rfd, F_V/F_M, Φ_{PSII}, q_P, q_N, Φ_{NPQ}, Φ_{NO}, *etc.*, *see, e.g.*, Lazar 2015, Schreiber 2004) can be detected spectrally by the SRFI method. Here, we have applied the SRFI method to study spectral variability in NPQ_λ (Fig. 2).

The spectra of maximal fluorescence for closed PSII reaction center in dark-adapted sample (F_M) and during actinic irradiation (F_M') were acquired during saturating flashes (Fig. 1*A*,*C*). NPQ_λ was calculated for all wavelengths (*see* Materials and methods) for two organisms, the cryptophyte alga *R. salina* and the green alga *Chlorella* sp. (Fig. 2). In *Chlorella* sp., we proved that NPQ_λ is inhibited in the whole spectral range after addition of DCMU (PSII inhibitor) or ammonium chloride that efficiently inhibits NPQ in various algae (Ruban *et al.* 2004, Kotabová *et al.* 2011, Kaňa *et al.* 2012b). The other model organism, which we used, cryptophyte alga *R. salina,* photosynthetic algae from red clade of photosynthesis evolved from a red alga ancestor (Zimorski *et al.* 2014). This alga is a very useful model organism for studying NPQ_λ as it contains two types of antenna systems (MacPherson and Hiller 2003) with different fluorescence emission: (*1*) phycobiliproteins (typical for red algae and cyanobacteria) located in thylakoid membrane lumen with fluorescence emission peak at 589 nm, which are tightly packed in the thylakoid lumen (Kaňa *et al.* 2009b); (*2*) membrane-embedded Chl antennae typical for green algae and higher plants, emitting in the 670–740 nm range, with maximum at 681 nm (Kaňa *et al.* 2012b). The Chl *a/c* antenna complexes (CAC) of cryptophyte consist of Lhcr and Lhcz proteins (Büchel 2015), and there is no xanthophyll cycle (no violaxanthin, no diadinoxanthin) (Funk *et al.* 2011, Kaňa *et al.* 2012b) that would affect the pH-dependent NPQ (Kaňa *et al.* 2012b). Our data show kinetics of NPQ_λ evolution on light, and confirm previous observations that there is no NPQ in phycobiliproteins (*see* F589 in Fig. 2*A*) and the fluorescence is quenched mostly in Chl-binding proteins (660–800 nm) in line with previous results proposing NPQ locus into CAC antennae (Kaňa *et al.* 2012b). We further suggest that the high NPQ_λ in this range could be attributed to some changes in CAC aggregation as antennae aggregation results in a shift of fluorescence emission maxima (*e.g.*, by 5 nm in LHCIIs of higher plants, *see* Johnson and Ruban 2009). The connection between the red shift of the F_{685} band and NPQ in antennae has been also proposed for intact leaves (Franck *et al.* 2005). However, a fluorescence quenching in the PSII inner antennae or in PSII RC cannot be excluded as a possible reason of NPQ_λ between F_{681} and F_{685}. It could include a quenching in the inner antennae, either by $P680^+$ (Bruce *et al.* 1997) or quenching in the inner PSII core antennae by Chl Z^+ (Komura *et al.* 2010, Miyake *et al.* 2011). Alternatively, some nonradiative dissipation directly in the reaction center has been also proposed as a mechanism of photoprotection (Cser and Vass 2007, Vass 2011). The importance of RC-type of quenching is species dependent, it has been proposed to be crucial in extremophylic red algae (Krupnik *et al.* 2013) and cyanobacteria (Ohad *et al.* 2010), in mesophilic red algae (Delphin *et al.* 1998), and in desiccated lichens (Komura *et al.* 2010, Miyake *et al.* 2011). On the other hand, it seems to be rather small in higher plants (Belgio *et al.* 2014). However, Fig. 2*A* indicates that emission from F_{685} is interconnected with F_{740}. It is visible in the similar changes in NPQ_λ at F_{685} and F_{740} at 4, 10, and 100 s. This seems to indicate energy equilibration and presence of one quenching mechanism across the whole emission band. In

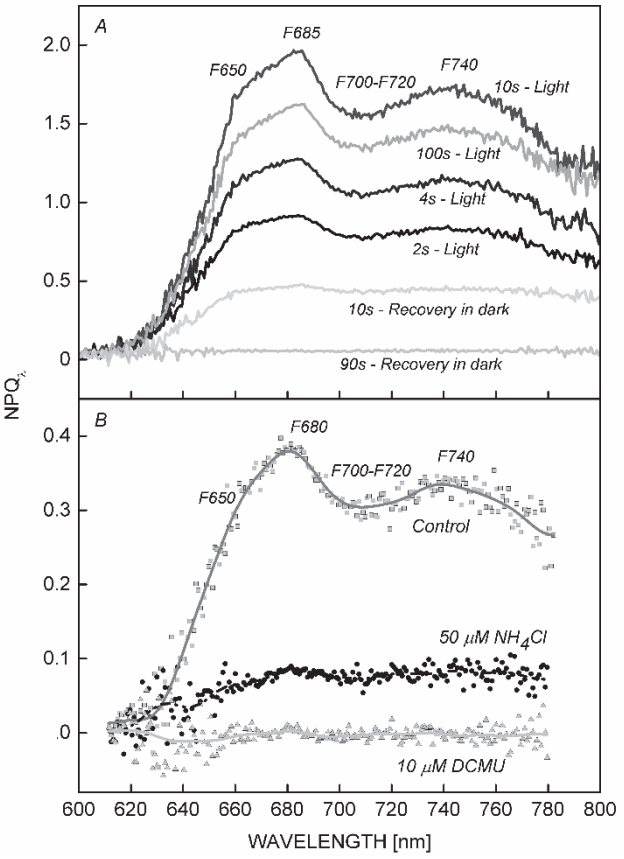

Fig. 2. Spectral variability of nonphotochemical quenching in *Rhodomonas salina* and *Chlorella* sp. NPQ was calculated based on Stern–Volmer equation from the SRFI data and defined the spectrally–resolved nonphotochemical quenching parameter NPQ_λ. (*A*): Induction of spectrally resolved NPQ on blue actinic light in cryptophyte algae *R. salina,* dark adapted cells were irradiated by blue actinic light [464 nm; 1,000 μmol(photon) m^{-2} s^{-1}] and fluorescence changes were observed during light (for 100 s) and dark period after irradiation. (*B*): Effect of inhibitors for spectrally resolved NPQ detected in green algae *Chlorella* sp.. Data represent control cells, cells with NH_4Cl (50 μM) and DCMU (5 μM) added before mesurements. The spectrally resolved NPQ was calculated after 100 s of blue light irradiation [464 nm, 300 μmol(photon) m^{-2} s^{-1}].

any case, to assess the importance of the quenching mechanism in the RC of PSII in cryptophyte, more experiments are required. Interestingly, we have shown also high NPQ_λ in the blue-shifted region (between F_{650}–F_{681}) similarly with NPQ_λ observed for *C. velia* (Kotabová *et al.* 2014). We tend to think that the blue-shifted NPQ_λ could be partially attributed to the quenching in the weakly coupled Chl pigments of light-harvesting antennae (Santabarbara and Jennings 2005). These pigments were described for LHCIIs and are characterized by blue-shifted emission maxima (650–675 nm, *see*, *e.g.*, Santabarbara and Jennings 2005).

We have also repeatedly confirmed a small drop in NPQ_λ between 700–720 nm (Kaňa *et al.* 2012b, Kotabová *et al.* 2014), which is also visible here in the data for cryptophyte and green algae (Fig. 2*A*,*B*). Recently, it has been shown that the relative fluorescence increase in the red fluorescence components around 720 nm (Lambrev *et al.* 2010) is a characteristic marker of NPQ conditions *in vivo* for higher plants; in fact, the actual quenching locus is the located around 682 nm (Lambrev *et al.* 2010, Miloslavina *et al.* 2008). The low importance of the fluorescence emission around 720 nm to actual NPQ has been confirmed also for Lhca antenna in red conformation because of their longest fluorescence lifetime (Passarini *et al.* 2010). There, F_{720} has been shown not to be involved in quenching, in contrasts with antennae with blue-shifted fluorescence emission (F_{681}), which exhibits short fluorescence lifetimes. Similarly, a relative increase in the fluorescence of F_{710} in comparison to F_{685} component has been also observed for dry lichens in the quenched state (Miyake *et al.* 2011). As there is usually increase in the fluorescence between 700–720 nm during stimulation of NPQ, the decrease of NPQ_λ between 700–720 nm could be caused by this fluorescence rise. An alternative explanation could be a higher involvement of fluorescence originating from PSI core (or their antennae) in this range (Franck *et al.* 2002, Rizzo *et al.* 2014, Giovagnetti *et al.* 2015), that usually does not show any NPQ even though it has kind of variable fluorescence as shown in theoretical models (Lazár 2013). Finally, the most red-shifted NPQ_λ (the F740 quenching, Fig. 2) can be attributed to aggregated forms of CAC antenna complexes in line with previous data (Komura *et al.* 2010, Miyake *et al.* 2011). However, these two possible quenching loci seem to be tightly interconnected as quenching at F_{685} and F_{740} behave similarly during fluorescence induction (Fig. 2*A*). In conclusion, we have shown applicability of SRFI method to measure spectrally resolved NPQ_λ in green algae and in cryptophyte.

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