Photoacclimation in *Dunaliella tertiolecta* reveals a unique NPQ pattern upon exposure to irradiance

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Received: 5 December 2010 / Accepted: 6 November 2011 / Published online: 20 November 2011
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**Abstract** Highly time-resolved photoacclimation patterns of the chlorophyte microalga *Dunaliella tertiolecta* during exposure to an off–on–off (block) light pattern of saturating photon flux, and to a regime of consecutive increasing light intensities are presented. Non-photochemical quenching (NPQ) mechanisms unexpectedly responded with an initial decrease during dark–light transitions. NPQ values started to rise after light exposure of approximately 4 min. State-transitions, measured as a change of PSII:PSI fluorescence emission at 77 K, did not contribute to early NPQ oscillations. Addition of the uncoupler CCCP, however, caused a rapid increase in fluorescence and showed the significance of qE for NPQ. Partitioning of the quantum efficiencies showed that constitutive NPQ was (a) higher than qE-driven NPQ and (b) responded to light treatment within seconds, suggesting an active role of constitutive NPQ in variable energy dissipation, although it is thought to contribute statically to NPQ. The PSII connectivity parameter \( p \) correlated well with \( F' \), \( F_m' \) and NPQ during the early phase of the dark–light transients in sub-saturating light, suggesting a plastic energy distribution pattern within energetically connected PSII centres. In consecutive increasing photon flux experiments, correlations were weaker during the second light increment. Changes in connectivity can present an early photoresponse that are reflected in fluorescence signals and NPQ and might be responsive to the short-term acclimation state, and/or to the actinic photon flux.

**Keywords** Connectivity · Constitutive NPQ · FRRF · Light acclimation · NPQ · qE · Photosynthesis · Chlorophyll a fluorescence

**Abbreviations**

CCCP Carbonyl cyanide 3-chlorophenylhydrazone

DCMU 3-(3,4-Dichlorophenyl)-1,1-dimethyl urea

NPQ Non-photochemical quenching

\( ((F_m - F_m')/F_m') \)

qE Energy-dependent fluorescence quenching

qI Photoinhibitory-induced fluorescence quenching

qP Photochemical fluorescence quenching

qT State-transition-caused fluorescence quenching

PF Photon flux

\( \sigma_{PSII} \) Functional absorption cross section of PSII in the dark

\( \sigma_{PSII}' \) Functional absorption cross section of PSII in actinic light

PSII Photosystem II

\( \Delta pH \) gradient *Trans*-thylakoid pH gradient

**Introduction**

During a dark–light transient, cells activate photosynthetic and, depending on the photon flux, photoprotective mechanisms. Activation of photosynthesis takes place in time scales from milliseconds, e.g. establishment of electrostatic forces that act on integral membrane structures to minutes...
for enzymatic reactivation of Calvin–Benson–Bassham cycle proteins (Portis 1992; Macintyre et al. 1997; Lazár 2006). RuBisCO reactivation in the light is complex and requires RuBisCO activase, ATP (Robinson and Portis 1988; Portis 2003), thioredoxin reduction and the existence of a trans-thylakoid pH gradient (ΔpH gradient) (Campbell and Ogren 1990). The degree of RuBisCO activation is dependent on the light intensity, light history, light exposure duration, the degree of inactivation reached before illumination, and may vary amongst species (Ernstsen et al. 1997; Hammond et al. 1998). However, full RuBisCO activation requires approximately 5 min in *D. tertiolecta* (Macintyre et al. 1997), a value that coincides with the up-regulation of photosynthetic O2 production in saturating photon flux (PF) (Campbell and Ogren 1990). During this timeframe increasing amounts of energy can be distributed towards carbon fixation and related photosynthetic processes. Especially at the beginning of the light phase the absorbed photon flux may exceed the energy conversion capacities (demand of photosynthetic processes) of the cell and require regulatory photoprotection (i.e. non-photocatalytic quenching, NPQ). Commonly NPQ is summarised to at least three processes (qE, qT and qI) of which only one process quenches absorbed photon energy, without contributing to photosynthesis, namely qE (e.g. Müller et al. 2001; Holt et al. 2004). The other two NPQ components, however, affect the fluorescence signal and can lower (quench) the fluorescence emission from the cell. During state-transitions (qT), absorbed photon energy can be re-distributed amongst PSII and PSI. Although this process can quench PSII fluorescence, it does not quench energy, and is, therefore, not a NPQ mechanism per se. State-transitions are effective in cyanobacteria and red algae, but might play a minor role in green algae and higher plants where dynamic changes in the energy distribution to either photosystem can be utilised to alter the production rate of ATP and NADPH (Campbell et al. 1998; Niyogi et al. 2001). qI is thought to be caused by photo-inhibition, i.e. damage of photosynthetic components, especially the D1 unit in PSII, but is more commonly used to describe a comprehensive suit of mechanisms with relaxation times between tens of minutes to hours, which includes NPQ mechanisms other than photo-inhibition and the repair thereof (Adams et al. 1995; Horton and Ruban 2005). The major component of NPQ in higher plants and chlorophyte algae is referred to as qE and relies on the build-up of a ΔpH gradient, which alone appears to activate qE and the conversion of violaxanthin to zeaxanthin, for expression of full NPQ, mediated by the enzyme violaxanthin de-epoxidase (Demming-Adams et al. 1990). The Psbs protein is a required subunit in PSII for full qE formation in higher plants (Li et al. 2000; Holt et al. 2004; Demming-Adams and Adams 2006), where qE correlates with violaxanthin de-epoxidation. Effective qE without xanthophyll cycle pigment conversion has been shown in green algae (Niyogi et al. 1997; Moya et al. 2001) and higher plants that lack zeaxanthin (Pascal et al. 2005; Ruban et al. 2007). qE activation kinetics are biphasic (Niyogi et al. 1997; Serôdio et al. 2005), with the rapid, and xanthophyll cycle independent phase reacting within seconds of light exposure (Li et al. 2009). For full qE activation both a suitable ΔpH gradient, which induces rapid qE, and violaxanthin de-epoxidation which requires some minutes (Niyogi 1999; Müller et al. 2001; Horton et al. 2008; Nilkens et al. 2010) is needed. Binding of H+ and zeaxanthin to PSI shifts the light harvesting complexes associated with PSII from an energy-transfer state to an energy-dissipation state due to a change in its conformation (Ruban et al. 2007). Additionally, PSI reaction core quenching has been previously suggested (Eisenstadt et al. 2008; Raszewski and Renger 2008). Here reactions in the PSI core cause fluorescence quenching and heat emission in a xanthophyll independent fashion detected in several algal species. Because this type of energy quenching has been shown in chlorophyte-like PSII (Niyogi et al. 1997; Niyogi et al. 2001; Holt et al. 2004) and algae that show structural differences in PSII, or a different photoprotective pigment suite (Olaiza et al. 1994; Delphin et al. 1996; Doerge et al. 2000; Sane et al. 2002), PSI reaction core quenching was suggested to be an efficient and probably universal energy dissipation system (Ivanov et al. 2008).

Activation of qE upon light exposure is dependent on the strength of the ΔpH gradient, which is controlled by a number of processes, such as the ATPase activation state and energy consumption by carbon fixation (Mills et al. 1980; Schreiber 1984). The higher the light intensity, the higher the ΔpH and therefore the higher the qE. When cells are exposed to saturating PF, significant photon absorption requires rapid energy dissipation, especially due to the slow activation kinetics of photosynthesis. An efficient, rapid, alternative quenching mechanism can provide an advantage to the cell as the formation of reactive and destructive oxygen species can be avoided. Higher plants and green algae respond to light exposure with up-regulation of both photosynthetic and NPQ mechanisms, although the kinetics and magnitude of the response depend on the species and light history (Niyogi et al. 1997; Moya et al. 2001).

The fast repetition rate (FRR) fluorescence technique uses a unique protocol to measure variable fluorescence. Instead of measuring fluorescence before and during a multiple turnover saturating light pulse, a sequence of rapidly fired sub-saturating flashlets is used to completely reduce the Q A pool. Because of the short duration of the flashlet sequence (about 280 μs), a fluorescence induction curve is measured within effectively a single PSII turnover event. From the kinetics of rise from *F* 0 to *F* m, the
functional absorption cross section $\sigma_{\text{PSII}}$ is calculated as well as the connectivity parameter $p$. The functional absorption cross section of PSII describes the efficiency of light utilisation of open PSII units and is equal to the product of the PSII efficiency and the optical cross section of PSII (Kolber and Falkowski 1993; Kolber et al. 1998).

From preliminary studies we obtained evidence that the marine chlorophyte \textit{D. tertiolecta} might possess some unique photoprotective features. Therefore, the current study presents observations on a unique, PF-dependent and rapid NPQ down-regulation upon light exposure in the marine chlorophyte \textit{D. tertiolecta}, in order to get a better understanding of the photoprotective mechanisms activated upon exposure to high irradiances.

**Materials and methods**

**Culture conditions**

Continuous cultures of \textit{Dunaliella tertiolecta} (Butcher 1959) (CSIRO strain CS-175) were grown in a flat-faced 1.6 l glass vessel (approximately 5 cm light path) under constant aeration, and irradiance (100 \(\mu\text{mol}\) photons m\(^{-2}\) s\(^{-1}\), 400 W Philips high pressure HPIT E40 lamp) at 18°C. Cells were kept in a stable physiological state by means of continuous dilution (flow rate 64 ml/h, giving a dilution rate of \(\sim 0.95\) day\(^{-1}\)) with fresh F/2 enriched seawater medium (pH 8.2) at a cell density of \(7.6 \pm 1 \times 10^5\) cells/ml and a pH of 8.7 ± 0.2 inside the culture vessel. A Coulter Counter (model ZM connected to a Coulter Multisizer, Beckman Coulter) was used to measure cell concentrations. Before measurement, cells were washed by gentle centrifugation and re-suspension of the pellet in fresh medium (pH 8.2) at a similar cell concentration as under growth conditions. Dark acclimation prior to measurement never exceeded 2 h.

**FRRF measurements**

Variable chlorophyll fluorescence was measured using a Fast Repetition Rate fluorometer (FRRF) (FastTracka-I, Chelsea Technology Group Ltd, UK). For a general description of a FRR fluorometer and FRRF theory see, e.g. Kolber and Falkowski (1993) and Kolber et al. (1998). A flashlet sequence (5 replicates, saturation flash length 1.1 \(\mu\text{s}\) and saturation flash period 2.8 \(\mu\text{s}\)) was applied every 13 s. Although the intensity of the individual flashlets is sub-saturating due to their short interval, the overall photon flux (\(\sim 30.000\) \(\mu\text{mol}\) photons m\(^{-2}\) s\(^{-1}\)) is highly saturating. Due to their extraordinarily high sensitivity, FRR fluorometers are mostly used in situ, especially in open ocean systems (Suggett et al. 2001, 2009; Moore et al. 2003). Although the FRRF was recalibrated by the manufacturer into the low sensitivity mode (0–150 \(\mu\text{g}\) chl a l\(^{-1}\)) the biomass (as in the growth conditions) was still too high, leading to saturation of the fluorescence signals. We, therefore, used neutral density filters (grey tinted polycarbonate films), shielding the photomultiplier light intake path of the apparatus to obtain suitable detection ranges (see Fig. 1 for a schematic drawing of the experimental setup). The data were fitted using the software provided by the

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manufacturer. Samples were kept in 50-ml culture vessels, under airtight conditions at constant stirring at room temperature (20–22°C). A cooling jacket was placed against the culture vessel and was facing the light source. A manually controlled halogen light source was used for application of PF of 50–470 μmol photons m⁻² s⁻¹ (FL 440 Walz GmbH, Germany). A FL 103 F short pass filter (<700 nm, Walz GmbH, Germany) was used block the near-infrared wave band. The PF was measured using a spherical (4π) quantum sensor. For differences between the multiple (e.g. PAM fluorometers) and single turnover protocols see Kromkamp and Forster (2003).

For calculations of variable fluorescence parameters, the standard nomenclature was used (refer to, e.g. Kolber and Falkowski 1993; Kromkamp and Forster 2003; Fujiki et al. 2007).

The functional absorption cross section (σₚₛₛᵢ) describes the maximal light utilisation efficiency for photochemistry in PSII, expressed in area per quantum (Å²). The same is true for σₚₛₛᵢ', but for a light acclimated state. Plastic PSII energy distribution can be distinguished between the lake model, where PSII centres are energetically connected, and the single unit model, where one PSII centre receives energy from its most adjacent light harvesting complex only. The connectivity parameter p is calculated from the kinetics of fluorescence increase during a flashlet sequence and describes the fraction of energetically connected PSII. Further details and algorithm are given in the literature (Kolber and Falkowski 1993; Kolber et al. 1998).

NPQ calculations were performed according to the Stern–Volmer equation with NPQ = (Fₘ − Fₘ')/Fₘ'. In the block light experiment, Fₘ' values were highest after the light treatment. Therefore, the maximal Fₘ, which was reached at the end of the dark phase following the block light treatment, was used for NPQ calculations (Fig. 2). For the purpose of this article, this block light treatment is referring to a dark to light transition, where the PF is constant during the light phase. Because Fₘ in the dark was lower than at low PF (Fig. 3), NPQ calculations were based on maximal fluorescence measured during the light experiments using consecutive increasing PF. This coincided with Fₘ' during lowest PF treatment (Fig. 3).

77 K fluorescence and measurements in the presence of CCCP

Cells were cultured in 500-ml conical glass flasks with a minimum of 200-ml head space at a constant PF of 100 μmol photons m⁻² s⁻¹ (Cool White light, Silvania fluorescent tubes) and a temperature of 18°C. Cells from the log-phase were harvested for the experiments. After washing in fresh F/2 pH 8.2 medium, cells were concentrated to a final density of 1 × 10⁷ cells/ml and dark incubated for 1 h prior to exposure to a saturating PF (660 μmol photons m⁻² s⁻¹; measured using a spherical (4π) light sensor). This was carried out in an open chamber (8-ml cylindrical Perspex Rod Oxygraph, Hansatech, UK) to allow gas exchange while the sample was stirred. Samples for low-temperature chlorophyll fluorescence emission spectra were taken by quickly pipetting 300 μl into Pasteur pipettes that had been sealed at the bottom, and plunged into liquid nitrogen. Sample handling took less...

Fig. 2 Representative fluorescence parameters measured by FRRF during a dark to light transition using a single irradiance intensity (‘block light treatment’) and darkness. a F', Fₘ' on the primary ordinate, and NPQ on the secondary Y-axis; b σₚₛₛᵢ (Sigma PSII) and maximal quantum yields as well as effective quantum yields during the irradiance treatment. The upward arrow indicates the start of the light period using a photon flux of 440 μmol photons m⁻² s⁻¹ (approx. 4 × growth light intensity) after dark incubation (1–2 h). The downward arrow indicates the end of the light treatment. An addition of 160 μM dissolved inorganic carbon aimed for detection of nutrient depletion (double arrowhead), which should not have occurred due to low cell densities in this experiment. Results were confirmed in two independent experiments.
than 3 s. All cells were kept in darkness at 77 K until fluorescence emission spectra were recorded using a spectrofluorometer (Hitachi 7500, Japan). Cells were excited with blue light of 435 nm wavelength (slit width 10 nm), while fluorescence spectra were recorded by the fluorometer (slit width 2.5 nm). For each sample, 3–5 spectra were recorded and the pipette rotated each time after a spectrum was taken, to reduce bio-optical interference with chlorophyll fluorescence. After baseline correction in OPUS (Bruker Optic GmbH, Germany), spectra were averaged for each replicate and de-convoluted (PeakFit, version 4.12, SeaSolve Software Inc.). Fits were forced for peak analysis at 685, 695, 702, 715, and 730 nm and fits were checked against residuals ($r^2 = 0.78$ ± 0.07 and 0.92 ± 0.04 for light and dark phases, respectively).

For experiments where the protonophore carbonyl cyanide 3-chlorophenylhydrazone (CCCP) (Sigma-Aldridge) was used, room temperature fluorescence signals were continuously recorded with a Diving Pam (Walz GmbH, Germany) using a smaller version of the Oxygraph chamber under similar PF and temperature. After cells were acclimated to the PF, CCCP was added to a final concentration of 200 µM. A saturation pulse train with a frequency of one saturation pulse min$^{-1}$ was applied, but intermitted after the actinic light was switched off to allow undisturbed $F_0$ (CCCP) determination.

**Results**

$F$, $F'_m$ and NPQ

Changes in $F'$ are influenced by PSII closure. Higher $F'$ values are caused by a higher degree of PSII closure. Upon the onset of high light (440 µmol photons m$^{-2}$ s$^{-1}$) $F'$ oscillated: very high $F'$ values were recorded within 1 min after light onset with almost the signal strength of $F_m$. $F'$ decreased thereafter for 4 min, followed by a rise until a maximum value was established approximately 5 min after the light was switched on (Fig. 2). $F'$ then decreased monotonically until the light was switched off. Only the addition of 160 µM dissolved inorganic carbon (as sodium bicarbonate, DIC, which we added to check on possible DIC limitation) caused a slight dip in $F'$, which, however, recovered quickly. When the light was turned off $F'$ decreased quickly due to opening of the PSII. After a few minutes $F'$ started to increase again, to reach a new steady state after 5 min. This increase is most likely related to a relaxation of NPQ, which was responsible for the slow but steady decrease in $F'$ after 3 min of exposure to high light.

When the cells were exposed to a low PF (50 µmol photons m$^{-2}$ s$^{-1}$, Fig. 3), $F'$ increased rapidly followed by a rapid and strong decrease, with an undershoot, until values showed a steady state at values just above $F_0$ as a result of PSII closure. At higher PF the undershoot disappeared and the final steady state value of $F'$ increased with increasing PF.

Upon a dark–light transient, it would be expected that maximal fluorescence signals would decrease as a result of elevated non-photochemical fluorescence quenching (Krause and Weis 1991; Campbell et al. 1998). In this study, however, $F'_m$ values increased compared to $F_m$ in the block light treatment (Fig. 2). The $F'_m$ increase (and therefore
NPQ down-regulation) was induced after approximately 1 min of actinic light onset, continued for ca 2.5 min, and was followed by a somewhat slower, but steady, decline until the signal was perturbed by addition of 160 μM DIC. $F_m'$ correlated strongly with $F'$ ($m = 1.39$; $r^2 = 0.91–0.96$). A strong correlation between $F'$ and $F_m'$ in FRRF measurements suggests a change in the absorption cross section of PSII during the transient, although the functional absorption cross section was found to be stable throughout the actinic light phase (Fig. 2b). The initial rise in $F_m'$ might be an indication of the dissipation of chlororespiration, but the following decrease in both $F'$ and $F_m'$ might be due to both induction of qE or a change in the absorption cross section of PSII due to a state-transition. We applied low-temperature chlorophyll fluorescence emission spectra to investigate the occurrence of state-transitions.

77 K emission spectra

Figure 4 shows a typical chlorophyll fluorescence emission spectrum in *D. tertiolecta*. Fluorescence emission peaks were not very distinct, with a small contribution at 695 nm ($F_{695}$) (PSII reaction centre). Emission at 715 nm ($F_{715}$) is regarded as a contribution from PSI, $F_{730}$ is considered as a vibration, while the origin of $F_{702}$ remains unclear. Emission spectra were normalised to the fluorescence yield at $F_{685}$ (light harvesting complexes of PSII). Murakami (1997) showed that the PSI/(PSII + PSI) ratio determined with biochemical techniques could be estimated accurately from the $F_{PSII}/(F_{PSII} + F_{PSI})$ ratio for different algal species. We used the $F_{685}/F_{715}$ ratio as a proxy for changes in the ratio of PSII to PSI.

**$F_{685}/F_{715}$ ratios**

$F_{685}/F_{715}$ ratio remained relatively constant at approximately 3.4 during the dark to light transient (Fig. 5). After 15 min a ~20% increase in the ratio PSII:PSI from 3.4 to 3.9 was observed. Upon the onset of dark exposure, values remained stable for approximately 1 min, declined thereafter, and established a quasi steady state for 20 min at a lower ratio of 2.9 indicating an increase in the absorption cross section of PSI. After 30 min of dark incubation, the PSII:PSI ratio increased again and reached an $F_{685}/F_{715}$ ratio close to values of that of far-red-light-treated samples.
(4.22 ± 0.34 vs. 3.83 ± 0.56 for far-red light, and 1 h dark-acclimated cells, respectively; Fig. 5). Our results suggest that state-transitions are limited to 25% of the PSII-antenna when the PQ pool is completely reduced by PSI-light (ratio changes from 4.2 to 3.4). Interestingly, PSII:PSI ratios were different after 1 h dark acclimation prior to light exposure (t = 0 in Fig. 5), and after the block light treatment. In the first case, cells were dark-acclimated after exposure to the growth PF, while the experimental light treatment was approximately three times as high.

CCCP

To further investigate the extent/occurrence of qE we added the protonophore uncoupler CCCP, which should collapse the ApH gradient and thus qE. After addition of CCCP the F’ signal increased within about 1 min to maximal levels (+50 ± 13% of F’(pre-CCCP)), with an exponential decline thereafter to values of 120 ± 13% greater than those of F’(pre-CCCP) (Fig. 6). This demonstrates the existence of a pH-driven qE process. However, after the initial rise in F’ as a result of the collapse of the pH gradient, F’ decreased again and a steady state was established within 10 min after CCCP addition, presumably due to a state-transition to the low fluorescent state. When actinic light was switched off, the F₀ signal increased (by +31 ± 12% of F’(pre-CCCP)). During the first 18 min no saturation pulses were given. But when they were applied (indicated by the double arrowhead) considerable oscillation in F’ was observed.

σPSII and NPQ

The functional absorption cross section of PSII (σPSII) decreased significantly, upon the onset of sub-saturating and saturation PF, within short time scales (Figs. 2, 3). While little acclimation was detected during the block irradiance treatment (Fig. 2), consecutive increases in energy pressure caused a stepwise decrease in σPSII to a minimum of 138 ± 6 Å² at the highest PF (Fig. 3). This decrease in σPSII is the result of NPQ processes, which facilitate in keeping the effective PSII efficiency relatively high (∆F/Fₘ₀ = 0.37 ± 0.08 at 470 μmol photons m⁻² s⁻¹, thus relatively open), therefore, limiting the opportunity for photodamage. Interestingly, the pattern in σPSII is not reflected by the pattern in NPQ (calculated as Stern–Volmer quenching: NPQ = (Fₘ₀ – Fₘ) / Fₘ₀). As σPSII remained constant during the illumination at 440 μmol photons m⁻² s⁻¹ NPQ increased, mirroring the changes in Fₘ₀ (Fig. 2).

Upon onset of darkness, σPSII recovered to a steady state in a fashion consistent with Michaelis–Menten kinetics within approximately 5 min. Recovery times coincided with the duration of NPQ acclimation (i.e., the time frame where NPQ has changed to a different quasi steady state). However, during this time NPQ first increased upon the onset of darkness, and then decreased to reach values similar to the values before the onset of the high light.

The pattern in NPQ and σPSII were more complex during the stepwise increase in irradiance. Whereas σPSII showed a stepwise decrease with increasing irradiance (best visible at the lower irradiance, Fig. 3), NPQ showed the expected oscillations mirroring changes in Fₘ₀. When NPQ reached steady states at each irradiance step, values were almost on the same level. Like the experiment with one high PF (Fig. 2), upon the onset of darkness NPQ first increased but then decreased to a value similar to the starting value.

In comparison to the pre-light treatment, σPSII was significantly reduced by 17% (data from Fig. 3; pre-light treatment 191 ± 11 Å², post-light treatment 159 ± 11 Å²), indicating a quasi steady state which remained for at least 10 min after light treatment.

To further investigate the relationship between NPQ and σPSII and to analyse the fraction of different quantum efficiencies, data from Fig. 2 were used for ΦNPQ, ΦD and NPQPSII calculations. Figure 7a clearly shows that NPQ

![Fig. 6](image-url) Continuous fluorescence at room temperature using a Diving-PAM. Data show one representative fluorescence trace during block light treatment of 660 μmol photons m⁻² s⁻¹ and darkness (downward arrow). Cells were poisoned with 200 μM CCCP (double arrowhead) after a light acclimated state was established. CCCP is a protonophore that dissipated the ApH gradient and relaxes energy-dependent quenching, but also prohibits photosynthesis. The open circle indicates the resumption of the saturation pulse train, which was interrupted prior to the light–dark transition. The oscillations might be caused by static interactions (see Vredenberg 2008).
and NPQ$_{\sigma_{\text{PSII}}}$ deviate from each other. NPQ$_{\sigma_{\text{PSII}}}$ does not show the early oscillation after light onset, and seems to decrease over the light phase, while NPQ increases. When plotted over $\sigma_{\text{PSII}}$, NPQ$_{\sigma_{\text{PSII}}}$ has high values in low $\sigma_{\text{PSII}}$ and low values in high $\sigma_{\text{PSII}}$ (Fig. 7b). The reverse is true for NPQ. The bottom panel of Fig. 7a shows that the quantum efficiency for fluorescence and photophysical decay ($\Phi_{\text{LD}}$) responds to the light treatment and decreases with exposure time. $\Phi_{\text{NPQ}}$ values are lower and respond in the opposite way to $\Phi_{\text{LD}}$. After an initial decrease values increase throughout the light phase. The sum of both parameters equals one, showing that the calculations of $\Phi_{\text{NPQ}}$ and $\Phi_{\text{LD}}$ are valid. Similar observations were made when consecutive increasing light was applied (Fig. 8). $\Phi_{\text{NPQ}}$ and $\Phi_{\text{LD}}$ respond in a converse fashion. Light exposure and increases in the PF elevated $\Phi_{\text{LD}}$, but decreased $\Phi_{\text{NPQ}}$. At high PF $\Phi_{\text{NPQ}}$ responses were limited while $\Phi_{\text{LD}}$ increased, suggesting that $\Phi_{\text{LD}}$ represents an active photoregulatory mechanism, even when $\Phi_{\text{NPQ}}$ appears to be at the end of its regulatory capacity. $\Phi_{\text{LD}}$ resembles the functional absorption cross section in the block light treatment (Fig. 7b), but not when the light is increased stepwise (Fig. 8).

Connectivity

The parameter $p$ describes the connectivity of PSII centres and migration of excitation energy from closed to open PSII. During the shift to HL ($440 \mu$mol photons m$^{-2}$ s$^{-1}$), $p$ remained relatively constant at a value of approximately 0.25, and increased within 3 min to 0.34 when the light was turned off (not shown). However, when the light was increased in smaller steps, a considerable fluctuation in connectivity was observed. Connectivity decreased during the first minute after the dark–light, and the next light increment transition (PF of 0–50 $\mu$mol photons m$^{-2}$ s$^{-1}$, and 50–200 $\mu$mol photons m$^{-2}$ s$^{-1}$, respectively, Fig. 9a). Thereafter values recovered in either a biphasic (dark–light) or in a linear fashion (low PF to higher PF treatment).

During the first 3.5 min of the first PF increment, $p$ correlated well with NPQ (Fig. 9b, d; $r^2 = 0.88 \pm 0.02$), while a weaker correlation coefficient was observed during the first minutes of the second light increment ($r^2 = 0.61 \pm 0.09$). NPQ showed an overshoot but stabilised at levels similar to dark values (Figs. 3, 8), whereas $p$ did not show this overshoot and stabilised at a value slightly lower than the one in the dark (Fig. 9a), suggesting a small decrease in connectivity. A further increase in irradiance to 200 $\mu$mol photons m$^{-2}$ s$^{-1}$ induced similar kinetics compared to the dark–light treatment albeit to a lower extent and $p$ stabilised at a value slightly below the value at the previous irradiance. Similar strong but negative relationships were found for the relationship between $p$ and $F'$ or $F''$, where the fluorescence decreased with an increase in connectivity (Fig. 9e, f; $r^2 = 0.89 \pm 0.05$ and 0.90 $\pm 0.05$ for $F'$ and $F''$, respectively). In the second light increment, correlation coefficients were weaker for $p$ versus $F'$ and $F''$ ($r^2 = 0.57 \pm 0.10$ and 0.59 $\pm 0.11$ for $F'$ and $F''$ in the first 3.5 min of 200 $\mu$mol photons m$^{-2}$ s$^{-1}$ irradiance treatment).
Discussion

When algal cells are exposed to saturating irradiances photoprotective mechanisms will be activated. Normally the first line of defence is the activation of the xanthophyll cycle, leading to the dissipation of (excess) energy as heat (qE) (Demmig-Adams and Adams 1993; Adams and Demmig-Adams 1995; Horton and Ruban 2005; Ljudmila et al. 2007; Papageorgiou et al. 2007). In D. tertiolecta, activation of the xanthophyll cycle takes place within minutes (Casper-Lindley and Björkman 1996). In the present work, we obtained clear evidence of the operation of qE when we added the uncoupler CCCP (Fig. 6). Addition of CCCP resulted in a sharp incline of the fluorescence signal as it collapsed the ΔpH gradient, dissipating qE. Nevertheless, the NPQ kinetics during the dark to light transient were not as expected. After a dark to light transition, electron transport activity is expected to cause an increase in the ΔpH gradient, which leads to an increase in qE. Activation of photosynthesis and PSII activity in D. tertiolecta operates according to expectations as can be seen from ΔF/F' and F' kinetics. Photosynthetic electron transport was, therefore, expected to elevate NPQ during the early phase of the dark to light transient, where a high photoprotective potential is required due to insufficient photosynthetic energy quenching. The initial rise of F'_m (NPQ down-regulation) is not in accordance to the expected decrease in both fluorescence parameters as a result of an increase in qE: one would expect a decrease. Casper-Lindley and Björkman (1998) showed for D. tertiolecta that exposure to saturating PF-induced de-epoxidation of violaxanthin, at very strong PF (1,200 μmol photons m⁻² s⁻¹), after a minimum of 5 min. The same authors also showed that after 45 min of high PF treatment only 60% of the violaxanthin pool was de-epoxidised, while maximal NPQ values were reached after approximately 15 min, indicating the effective potential of this species to quench excess absorbed quanta. This also demonstrates that in this species slow NPQ is not strictly connected to xanthophyll cycle de-epoxidation. Nevertheless, a sudden exposure to 440 μmol photons m⁻² s⁻¹ caused a decrease in NPQ during the first 4 min (Fig. 2) which might attribute to the disappearance of chlororespiration due to its influence on the ΔpH gradient. Chlororespiration can maintain a ΔpH gradient that is suitable to allow qE activation in the dark as this process uses the photosynthetic electron transport chain and result in a partly reduced PQ pool and H⁺ translocation over the thylakoid membrane in darkness (e.g. Peltier and Cournac 2002). Exposure to sub-saturating PF caused an even more rapid NPQ decrease, followed by an overshoot in NPQ, and steady values after approximately 7 min (Fig. 3). During following light increments the overshoot was not observed. However, in the following light increments the NPQ decrease occurred with similar kinetics to the dark–light transition, suggesting that down-regulation of NPQ in PF treatments is not primarily due to activation procedures of photosynthetic reactions. Exposure to 50 μmol photons m⁻² s⁻¹ (50% of growth light) for 10 min during the first light increment is expected to have resulted in significant activation of photosynthetic processes. Repetitive down-regulation of NPQ in increasing PF also rejects the hypothesis of an active NPQ in the dark due to

Fig. 8 Analysis of quenching yields subjected to a stepwise increase in irradiance (data Fig. 3). a Top panel NPQ calculated using the Stern–Volmer equation (($F_m - F'_m$)/$F'_m$), and as NPQ$_{psII}$ (($σ_{psII} - σ_{psII}^0$)/$σ_{psII}$). Bottom panel regulated NPQ ($Φ_{NPQ}$) and constitutive NPQ ($Φ_{psII}$). b Relationship between $σ_{psII}$ (bottom X-axis) and the two proxies for the NPQ (left Y-axis) or the quantum efficiency for constitutive NPQ (right Y-axis)
chlororespiration. Ten minutes of light exposure are sufficient for photosynthetic electron transport activation and down-regulation of chlororespiration under the applied PF regime. In case of chlororespiratory-induced active NPQ in the dark, the second light increment would not have induced a NPQ down-regulation.

A down-regulation of NPQ upon light exposure implies active NPQ mechanisms during growth PF conditions, and very slow de-activation kinetics, or NPQ activation in the dark. We checked whether the observed decrease in NPQ during the first 4 min of the high light exposure could be caused by a state II–state I transition, thus by transition from the high fluorescent to a low fluorescent state. The fact that we observed a decrease in the functional PSII cross section ($\sigma_{\text{PSII}}$) corroborates this, although the kinetics follow a completely different pattern (we come back to this later). Low-temperature fluorescence excitation scans were performed to check on the occurrence of state-transitions. Although the spectra shown in this study deviate from spectra found in higher plants and other algae (Harnischfeger 1977; Satoh et al. 2002), our results are in good comparison to other studies using D. tertiolecta (Gilmour et al. 1985; Vassiliev et al. 1995; Casper-Lindley and Björkman 1996). State-transitions operate on the time scale of minutes (Allen and Pfannschmidt 2000). Kinetics of the initial NPQ transient shown in Fig. 2 operate on the same time scale. However, when the PF is increased stepwise very rapid fluctuations are observed at the lowest two PFs, and these seem too fast to be explained by state-transitions, suggesting that the observed NPQ phenomenon is not caused by a state-transition. Low temperature fluorescence excitation scans of D. tertiolecta showed that during the first 10 min of exposure to high light the PSII:PSI ratio did not change, and then subsequently increased from 3.5 to $\sim 4$. This suggests an increase in the PSII absorption cross section during the second half of the light exposure. This shift was absent in NPQ and $\sigma_{\text{PSII}}$. When the cells were transferred from 660 µmol photons m$^{-2}$ s$^{-1}$ to darkness the PSII:PSI ratio first decreased, and then restored itself, which was not detected by room temperature fluorescence measurements using FRRF. If only qT would have caused the change in calculated NPQ, $F_m$ would decrease as a response to the light–dark transfer, whereas the opposite was observed. Therefore, it must be concluded that state-transitions did not show up in the fluorescence measurements in this study and state-transitions signals were overshadowed by other processes, probably qE.

Photoinhibition (qI) can also affect fluorescence signals. Recovery from qI requires repair of PSII reaction centres proteins, especially D1 (Ohad et al. 1994). This occurs on a time scale of hours. Hence, an effect of photoinhibition (qI) can be excluded based on the quick recovery of $F_i/F_m$ values in this study.

A decrease in NPQ should lead to an increase of the functional absorption cross section of PSII, as this is defined of that fraction of the optical cross section which is involved in photochemistry (Kolber and Falkowski 1993). As expected, upon exposure to HL (Fig. 2) an immediate decrease in the absorption cross section from 185 Å$^2$ to a more or less steady state value of approximately 140 Å$^2$ was noticed. Thereafter only a slight increase of $\sigma_{\text{PSII}}$ was measured, while NPQ continued to decrease. This trend in $\sigma_{\text{PSII}}$ is too weak to interpret it as a true signal. This shows that the behaviour in $\sigma_{\text{PSII}}$ does not match the behaviour in NPQ, whereas this might be expected as $\sigma_{\text{PSII}}$ is interpreted as that part of the optical absorption cross section involved in photochemistry (Ley and Mauzerall 1982). This suggests that $\sigma_{\text{PSII}}$ was mainly driven by processes other than NPQ. Activation of photosynthesis might affect $\sigma_{\text{PSII}}$ as more energy can be dedicated towards linear electron flow in the photosynthetic unit. In this case, electron transport rates (or the effective quantum yields) should elevate. Indeed, a small increase of $\Delta F/F_m$ was observed during the first 3 min of high light treatment (Fig. 2), indicating activation of photosynthetic electron transport through PSII. Application of lower light intensities, however, led to a brief decrease in $\Delta F/F_m$ (and electron transport rates) as well as in a decrease of the functional absorption cross section (Fig. 3), rejecting the theory of activation of photosynthesis being a major contributor to the development of $\sigma_{\text{PSII}}$. However, it seems likely that the effect of NPQ on $\sigma_{\text{PSII}}$ is counterbalanced by processes that contribute to the functional absorption cross section.

When the PF was increased stepwise, $\sigma_{\text{PSII}}$ initially decreased stepwise as might be expected due to increasing energy dissipation by NPQ mechanisms. Nevertheless, NPQ showed large oscillations, which are not visible in $\sigma_{\text{PSII}}$. To directly compare NPQ based on changes in $\sigma_{\text{PSII}}$ we made calculations similar to the Stern–Volmer approach by Suggett et al. (2006)

$$\text{NPQ}_{\sigma_{\text{PSII}}} = \frac{(\sigma_{\text{PSII}} - \sigma_{\text{PSII}}')/\sigma_{\text{PSII}}'}{\text{NPQ}_{\sigma_{\text{PSII}}}}$$

where $\sigma_{\text{PSII}}$ is the maximal functional absorption cross section measured in the dark, and $\sigma_{\text{PSII}}'$ is the functional absorption cross section measured during exposure with actinic irradiance. Figures 7 and 8 clearly show that the two proxies for NPQ (and NPQ$_{\sigma_{\text{PSII}}}$) show a different pattern. While NPQ$_{\sigma_{\text{PSII}}}$ decreases slightly as NPQ undergoes an oscillatory pattern in high PF, low light intensities induced patterns that resemble each other except of the rapid NPQ oscillation during the first minute. The discrepancy between NPQ and NPQ$_{\sigma_{\text{PSII}}}$ is therefore PF-dependent and might be associated with the extend of variable fluorescence produced by the flashlet sequence of the fluorometer. In high PF $\Delta F$ (i.e. the difference between
discrepancy between both proxies for NPQ was noticed for
light exposure. We do not know how to explain these
differences. It may be important to note that NPQ is based
on fluorescence induction curves of open PSII only (i.e. the
development of \( \Delta F \) during the flashlet sequence).

We noted a correlation between the connectivity
parameter \( p \) and changes in \( F \) and \( F_m' \) and NPQ. Connec-
tivity of PSII centres might increase the quantum efficiency
of PSII by use of excitons, which are transferred from a
closed to an open PSII. If connectivity would be absent, as
in the separate units model, an exciton hitting a closed PSII
would be lost. Zhu et al. (2005) demonstrated that an
increase in connectivity delayed the fluorescence induction
from O to J, without affecting the level of O. This suggests
that connectivity might not influence the level of \( F_0 \), \( F' \),
however, is affected by connectivity as show in this study.
We clearly show a strong correlation between connectivity
and variations in \( F' \) induced by exposure to (relatively low)
irradiances (Fig. 9e, f). One explanation might be that the
negative charges caused by reduced \( Q_B \) on the acceptor
side of PSII repel other PSII centres, hence causing a
positive relationship with NPQ (Fig. 9d). The decrease in
connectivity with increasing irradiances could not be
compared to other studies because this observation could
not be found in the literature. However, if connectivity
influences fast fluorescence induction as shown by Zhu
et al. (2005), \( \sigma_{PSII} \) and \( \text{NPQ}_{PSII} \) depend on energy distri-
bution amongst PSII centres. Because NPQ is calculated
from \( F_m \) and \( F_m' \), while \( 
\text{NPQ}_{PSII} \) is dependent on the fast
fluorescence induction, connectivity is likely to affect both
the parameters individually.

The sum of the quantum efficiencies for photochemistry,
heat dissipation and fluorescence should equal 1 (Schreiber
et al. 1995a, b). In this case, the quantum efficiency of heat
dissipation includes all processes contributing to NPQ, thus
including state-transitions, which is theoretically wrong
because state-transitions change the (optical) cross sections
of the photosystems without affecting loss of absorbed light
as heat. To better understand the apportioning of absorbed
light between the different processes we have calculated
the quantum efficiencies using the approach of Hendrick-
son et al. (2004). We favour this approach in our case
above the one by Kramer et al. (2004) because it does not
need knowledge of the minimal fluorescence in the light
activated state (\( F_0' \)). Hendrickson et al. (2004)
demonstrated that the results are very similar. The quantum
efficiency of photochemistry, \( \Phi_{PSII} \), equals the Genty
parameter \( \Delta F/F_m' \) (Genty et al. 1989). The quantum
efficiencies for heat dissipation and fluorescence are
expressed as the quantum efficiency for fluorescence \( \Phi_t \),
the quantum efficiency for photophysical decay or con-
stitutive NPQ (\( \Phi_D \)) and the quantum efficiency for regulated
NPQ (\( \Phi_{NPQ}, \text{i.e. } qE \)). \( \Phi_D \) is considered to be an
inherent energy dissipation process that is independent of
the (short-term changes in) photon flux, i.e. it summarises
that fraction of NPQ that is constantly lost as heat by
thermal radiation, non-regarding variances in photon flux.
\( \Phi_D \) should be constant. \( \Phi_t \) describes the same as \( \Phi_D \), but
for fluorescence. Hendrickson et al. (2004) summed the
\( \Phi_t \) and \( \Phi_D \) as \( \Phi_{LD} \):

\[
\Phi_{LD} = \Phi_t + \Phi_D = \frac{k_t + k_D}{k_l + k_D + k_P + k_N} \approx \frac{F'}{F_m} \tag{1}
\]

where \( k_l, k_D, k_P \) and \( k_N \) are the rate constants of fluo-
rescence, constitutional thermal dissipation, photochemical
and regulated-non photochemical quenching, respectively,
and \( F' \) (minimal fluorescence in the light). Because since
\( \Phi_l \) is small, \( \Phi_D \) is close to \( \Phi_{LD} \).

The quantum efficiency of NPQ that is regulated via the
ApH and the xanthophyll cycle (i.e. via \( qE \)) can be
expressed as:

\[
\Phi_{NPQ} = \frac{k_N}{k_l + k_D + k_P + k_N} \approx \frac{F'}{F_m^0} \frac{F'}{F_m} \tag{2}
\]

(Hendrickson et al. 2004). We used these equations to
calculate \( \Phi_{LD} \) and \( \Phi_{NPQ} \) using the data given in Fig. 2.
We can see that the photophysical decay fraction of NPQ is
larger than the \( qE \)-driven part of NPQ. It can be clearly
seen that kinetics of \( \Phi_{NPQ} \) resemble the kinetics in NPQ
(Figs. 7, 8), although the amplitude is less pronounced.
This is most likely because NPQ is not constrained
between 0 and 1 as is \( \Phi_{NPQ} \). What is also very interesting is
that \( \Phi_{LD} \) resembles the changes in the functional absorption
cross section. This can be more clearly seen when \( \Phi_{LD} \)
is plotted as a function of \( \sigma_{PSII} \). Here it can be seen that a
smaller functional cross section coincides with a larger
\( \Phi_{LD} \).

When the same procedure is followed for the stepwise
increase in irradiance as shown in Figs. 3, 8, partly different
results are obtained: as in the single high light exposure,
\( \Phi_{LD} > \Phi_{NPQ} \) and the kinetics of NPQ and \( \Phi_{NPQ} \) resemble
each other closely. However, the relationship between
\( \text{NPQ}_{PSII} \) and \( \Phi_{LD} \) is less clear and no relationship between
\( \sigma_{PSII} \) and \( \Phi_{LD} \) exists in the experiment where increasing PF
were applied. Clearly the relationship between NPQ and
changes in the functional cross section and the corre-
sponding quantum efficiencies need further study.
The name constitutional NPQ (photophysical decay) suggests that this does not vary significantly with different irradiances. This is indeed observed in a number of higher plant studies (Ahn et al. 2009; Guadagno et al. 2010). These latter studies also expanded the analysis of the portioning of quantum efficiencies to a better description of the importance of qE, qI and qT in energy apportioning amongst PSII centres compared to the higher irradiance in the second light step (open symbols, $r^2 \leq 0.61$). For readability reasons $F'$ has been normalised to 0.4 and not 1 in (e). Data show mean and SD ($n = 3$)

When cells are exposed to high PF, but have not been further, it seems possible that similar responses operate independently from other NPQ mechanisms. Numbers in the legend refer to the photon flux [closed symbols (50 μE) = 50 μmol photons m$^{-2}$ s$^{-1}$; open symbols (200 μE) = 200 μmol photons m$^{-2}$ s$^{-1}$]. Please note that data from the first and second light increment are plotted on the same timeline for improved comparability. A positive correlation between NPQ and $p$, while correlations were negative for $F'$ (e) and $F_m'$ (f). $F'$ and $F_m'$ in (e, f) have also been normalised to values prior to light treatment. Changes on the Y-axis therefore depict the relative change of $F'$ and $F_m'$, which explains why $F'$ values can be higher $F_m'$. Correlation coefficients were stronger ($r^2 \geq 0.88$) in cells exposed to the first light increment (closed symbols) compared to the higher irradiance in the second light step (open symbols, $r^2 \leq 0.61$). For readability reasons $F'$ has been normalised to 0.4 and not 1 in (e). Data show mean and SD ($n = 3$)

**Fig. 9** Connectivity $p$ (a), NPQ calculated using the Stern–Volmer equation ($F_{m} - F_{m}'/F_{m}'$) (b) and $F'$, $F_m'$ in (c) during the first minutes of the dark–light transition and the following higher irradiance treatment. Data were extracted from Fig. 3 (i.e. the experiment, where cells were exposed to consecutive increasing photon fluxes) and rearranged for better comparison. Filled symbols show the first light treatment, open symbols the following irradiance step. Numbers in the text refer to the photon flux [closed symbols (50 μE) = 50 μmol photons m$^{-2}$ s$^{-1}$; open symbols (200 μE) = 200 μmol photons m$^{-2}$ s$^{-1}$]. Please note that data from the first and second light increment are plotted on the same timeline for improved comparability. A positive correlation between NPQ and $p$, while correlations were negative for $F'$ (e) and $F_m'$ in (f). $F'$ and $F_m'$ in (e, f) have also been normalised to values prior to light treatment. Changes on the Y-axis therefore depict the relative change of $F'$ and $F_m'$, which explains why $F'$ values can be higher $F_m'$. Correlation coefficients were stronger ($r^2 \geq 0.88$) in cells exposed to the first light increment (closed symbols) compared to the higher irradiance in the second light step (open symbols, $r^2 \leq 0.61$). For readability reasons $F'$ has been normalised to 0.4 and not 1 in (e). Data show mean and SD ($n = 3$)

that they occur between measurements conducted by the measurement protocol (13 s).

The rapid, and xanthophyll cycle independent, fraction of $q_E$ can act as an efficient photoprotective mechanism in algae and might be attributed to PSII reaction centre quenching, whether this is due to charge recombination, direct P680$^+$ quenching, spill-over or conformational changes in the PSII core subunits (Olaiza et al. 1994; Doege et al. 2000; Eisenstadt et al. 2008; Ivanov et al. 2008; Raszewski and Renger 2008). As constitutive thermal dissipation ($\Phi_{D}$) originates in the PSII core (Ivanov et al. 2008), it can be concluded that *D. tertiolecta* is capable of rapidly changing PSII reaction core properties to avoid photodamage. However, changes of the connectivity parameter $p$ show that both, constitutive NPQ and dynamic energy distribution amongst PSII centres contribute to the rapid and efficient photoprotection strategy of *D. tertiolecta*.

**Acknowledgments** The authors like to thank three anonymous reviewers who helped to improve the quality of the manuscript. SI was funded by Monash Graduate Scholarship and Monash International Postgraduate Research Scholarship. Experiments at JB’s laboratory were funded by the Australian Research Council. This is NIOO publication 5100.

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