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The peculiar NPQ regulation in the stramenopile *Phaeomonas* sp. challenges the xanthophyll cycle dogma

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ABSTRACT

In changing light conditions, photosynthetic organisms develop different strategies to maintain a fine balance between light harvesting, photochemistry, and photoprotection. One of the most widespread photoprotective mechanisms consists in the dissipation of excess light energy in the form of heat in the photosystem II antenna, which participates to the Non Photochemical Quenching (NPQ) of chlorophyll fluorescence. It is tightly related to the reversible epoxidation of xanthophyll pigments, catalyzed by the two enzymes, the violaxanthin deepoxidase and the zeaxanthin epoxidase. In *Phaeomonas* sp. (Pinguiophyte, Stramenopiles), we show that the regulation of the heat dissipation process is different from that of the green lineage: the NPQ is strictly proportional to the amount of the xanthophyll pigment zeaxanthin and the xanthophyll cycle enzymes are differently regulated. The violaxanthin deepoxidase is already active in the dark, because of a low luminal pH, and the zeaxanthin epoxidase shows a maximal activity under moderate light conditions, being almost inactive in the dark and under high light. This light-dependency mirrors the one of NPQ: *Phaeomonas* sp. displays a large NPQ in the dark as well as under high light, which recovers under moderate light. Our results pinpoint zeaxanthin epoxidase activity as the prime regulator of NPQ in *Phaeomonas* sp. and therefore challenge the deepoxidase-regulated xanthophyll cycle dogma.

Plain language summary

Phaeomonas sp., a poorly described photosynthetic microalga belonging to a sister group of diatoms, displays a large NPQ in the dark which recovers under moderate light. The linear relationship between NPQ and the amount of zeaxanthin provided a convenient experimental frame which allowed us to reveal that the modulation of the epoxidase activity is the main regulator of NPQ.

1. Introduction

When exposed to changing light irradiance in their environment, photosynthetic organisms usually develop different strategies to maintain a fine balance between light harvesting, photochemistry, and protection from excess photon absorption. High light can cause damages to the photosynthetic apparatus *via* the formation of reactive oxygen species (ROS) in photosystem II (PSII). High light can trigger several processes at the PSII level [1,2], including the high energy state quenching (qE), the photoinhibitory quenching (qI) and fluorescence decay caused by state transition (qT). Altogether, these phenomena contribute to the non-photochemical quenching (NPQ) of chlorophyll *a* fluorescence [1], but can be distinguished based on their different kinetic responses in the green lineage. The qE component is the fastest response to high light stress and prevents the over excitation of PSII by dissipating the excessive excitation energy as heat. This mechanism is always tightly coupled to the xanthophyll cycle (XC), which consists in the reversible de-epoxidation of some special carotenoids, the xanthophylls [3]. Increasing the photosynthetic rate leads to an enhanced concentration in de-epoxidized xanthophyll in the antenna of PSII, which in turn increases the rate of de-excitation of excited chlorophylls through heat dissipation. The decrease in the lifetime of the excited chlorophyll minimizes the probability of its interaction with O_2, and of the subsequent ROS production. In plants, the establishment of the qE mechanism can decrease the fluorescence lifetime from ~2 ns to ~0.3 ns, which reflects the dissipation, in the form of heat, of 80% of the excitonic energy [1]. Although qE has been widely studied in plants and green algae, the exact mechanism by which the excitonic energy is dissipated is still a matter of debate [4–9].

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The carotenoids and enzymes involved in the xanthophyll cycle (XC) can vary from one photosynthetic organism to another [2]. Violaxanthin de-epoxidase (VDE) catalyzes the two-step de-epoxidation of violaxanthin (Vx) into zeaxanthin (Zx), through the intermediate antheraxanthin (Ax), in land plants and green algae [3]. In most of the marine photosynthetic eukaryotes (diatoms, dinoflagellates), diadinoxanthin de-epoxidase catalyzes the one-step de-epoxidation of diadinoxanthin into diatoxanthin [2,10]. Zx epoxidase and diatoxanthin epoxidase catalyze the opposite reactions in Vx/Ax/Zx and diadinoxanthin/diatoxanthin cycles, respectively. De-epoxidases (commonly abbreviated VDE) are water soluble enzymes located in the lumen of thylakoids, require low pH for optimal activity and use ascorbate as a co-substrate, whereas epoxidases (commonly abbreviated ZEP) are stromal enzymes and use NADPH and O_2 as co-substrates. The luminal acidification coupled to the photosynthetic electron transfer is believed to be the main driver of the xanthophyll cycle by activating the de-epoxidase activity and therefore the conversion of the epoxidized pigments into their de-epoxidized counterparts. The pH-optimum of the VDE in flowering plants is around 5 [3,11,12]. In contrast, the epoxidation rate is usually implicitly regarded as being constant. Although it has been suggested that the down-regulation of ZEP activity allows a flexible adjustment of the retention time of Zx in response to daily light-stress periods experienced by the plant [13], the possible regulation of the epoxidase and the consequences on the xanthophyll cycle and NPQ has however not been properly investigated so far.

Not only the xanthophyll cycle, but also some peculiar PSII antenna subunits can be involved in the qE mechanism: PsbS, in terrestrial plants, mosses [14] and chlorophytes [15] or members of the LI818/LhcSR/Lhcx family, in mosses, chlorophytes, prasinophytes, dinophytes, haptophytes and diatoms [16,17]. Those subunits can modulate the quenching efficiency of the de-epoxidized xanthophyll, and the maximal NPQ capacity depends on the concentration of the protein [16,17]. PsbS [14], as well as some members of the LI818/LhcSR/Lhcx family [16,18], can probe the luminal pH thanks to acidic residues exposed to the lumen, which induces a conformational change in PSII antenna under high light, thereby facilitating the high energy quenching by the de-epoxidized xanthophyll. In chlorophytes, the role of PsbS is only transient during a low to high light transition [15]. Because of that, the quenching efficiency of the de-epoxidized xanthophyll is not constant, and the relationship between NPQ and the concentration of the de-epoxidized pigment is complex. At the contrary, in diatoms, the NPQ is proportional to the diatoxanthin content [19].

In *Phaeomonas* sp., a poorly described photosynthetic microalga belonging to pinguiophytes [20,21], a sister group of diatoms (bacillariophytes), we found a similar linear relationship where NPQ is strictly proportional to the amount of zeaxanthin. This linear relationship provided a convenient experimental frame which allowed us to show that the modulation of the zeaxanthin epoxidase activity is the main regulator of NPQ.

2. Materials and methods

2.1. Strain and growth conditions

Phaeomonas sp. (Roscoff Culture Collection, RCC503) was grown in F/2 medium [22], without silica, at 20 ± 1 °C in semi-continuous batch culture without shaking. The photoperiod was 12 h light/12 h darkness, and light irradiance was ~50 μmol photons $m^{-2} s^{-1}$. On a daily basis, cell concentration was determined using a Z2 Coulter Counter analyzer (Beckman Coulter, Indianapolis, USA). All the experiments were performed with cells in exponential phase, in the middle of the light phase (*i.e.* 6 ± 2 h after the onset of light). Before analysis, cells were concentrated by centrifugation and resuspended in their growth medium (supernatant).

2.2. Inhibitors

3-(3,4-Dichlorophenyl)-1,1-dimethyl-urea (DCMU), 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone (DBMIB), carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone (FCCP), and nigericin (Sigma-Aldrich, Munich, Germany) were dissolved in ethanol, whereas dithiothreitol (DTT) (Sigma-Aldrich, Munich, Germany) was dissolved in deionized water. We used 15 μM (final concentration) DCMU, 5 μM DBMIB (unless otherwise stated), 10 nM FCCP. For nigericin and DTT, we used concentrations which are saturating for the effect on NPQ, without affecting the photosynthetic rate were determined by measuring the dose response of NPQ and photosynthesis efficiency (ΦPSII, see below).

2.3. Absorption difference spectroscopy

Absorption difference signals were measured at different wavelengths with a Joliot-type spectrophotometer (*JTS-10*, *Bio-Logic*, *Grenoble*, *France*), equipped with a white probing LED and the appropriate interference filters (3 to 8 nm bandwidth).

To deconvolute the linear and quadratic contributions to the ECS signals, we used a low concentration of FCCP (10 nM) in order to ensure that there was no electric field (ΔΨ) in the dark. The building of the ΔΨ was then achieved with a short (duration ~10 ms) pulse of strong (4500 μmol photons $m^{-2} s^{-1}$) red illumination. Then, we followed the kinetics of the ΔI/I decay over the 500–600 nm range and we used the protocol described in Bailleul et al. [23] to deconvolute the ECS signals into linear and quadratic components. In brief, we considered that the ΔΨ followed a mono- exponential decay: $\Delta\Psi = \Delta\Psi_0 * \exp(-t/\tau)$, where t is time, $\Delta\Psi_0$ is the initial electric field generated by the light pulse, and τ is the electric field decay lifetime. Given that the linear and quadratic ECS are theoretically proportional to ΔΨ and $\Delta\Psi^2$, respectively, the ΔI/I spectro- temporal matrix is described as a sum of two exponentials: $y(\lambda,t) = A(\lambda) * \exp(-t/\tau) + B(\lambda) * \exp(-2.t/\tau) + C(\lambda)$. The kinetics of ECS relaxation (starting 50 ms after the end of the light pulse, to avoid redox signals from *c*-type cytochromes) at all wavelength were fitted by a global routine, using the Origin software, which considers the lifetime τ as a global (wavelength independent) variable, and the amplitudes of linear and quadratic components (A and B, respectively) as local (wavelength dependent) variables. The plots of the A and B amplitudes as functions of the wavelength provide the spectra of the linear and quadratic ECS components, respectively. The deconvolution was performed twice (on 2 independent biological samples) giving similar results.

For $\Delta\Psi_d$ measurements, we used the same light perturbation as above and followed the kinetics of ΔI/I at 542 nm and 576 nm, where pure linear ECS (ECS_{lin}) and quadratic ECS (ECS_{quad}) signals, respectively, are present (see Fig. 4B). It is to note that those two wavelengths were chosen to minimize the contribution of the cytochrome oxidized minus reduced absorption changes. The relationship between ECS_{quad} and ECS_{lin}, after being normalized to the ECS_{lin} increase upon a saturating laser flash (*i.e.*, 1 charge separation per photosystem), was fitted with the parabolic equation $ECS_{quad} + a * \Delta\Psi_d^2 = a * (ECS_{lin} + \Delta\Psi_d)^2$, where a is a constant. $\Delta\Psi_d$ then represents the electrical component of the PMF in the dark in *Phaeomonas* sp., expressed in charge separations by PS [23].

2.4. Fluorescence-based measurements

Chlorophyll fluorescence-based photosynthetic parameters were measured with a fluorescence imaging setup described in Johnson et al. [24]. The maximal quantum yield was measured as $(F_m - F_0)/F_m$, and the PSII quantum yield in the light was calculated as $\Phi_{PSII} = (F_m' - F)/F_m'$. F_0 and F_m are the minimal and maximal fluorescence intensity in dark-adapted cells, respectively whereas F and F_m' are the steady state and maximum fluorescence intensities in light acclimated cells, respectively [25]. Relative photosynthetic electron transfer rate ($rETR_{PSII}$)

was calculated as $I * \Phi_{PSII}$. The light saturation curves of $rETR_{PSII}$ were fitted with the exponential rise function $P = P_{max} * (1 - \exp(-E / E_k))$, where P_{max} is the maximal photosynthetic electron transport rate and E_k is the optimal light [26]. Because of the NPQ that develops in the dark (see Results), we calculated NPQ as $(F_m^* - F_m') / F_m'$, where F_m^* is the maximal fluorescence when cells are fully acclimated to low light irradiance (30–55 $\mu E\, m^{-2}\, s^{-1}$), corresponding to the maximal fluorescence yield and to the minimal de-epoxidation state of xanthophyll cycle pigments.

For calculations of the epoxidase rate, we used the kinetics of NPQ changes after DTT addition. Indeed, in the presence of DTT, only the ZEP participates to xanthophyll cycle with a kinetic constant kZEP:

$$\frac{d[Zx]}{dt} = -kZEP * [Zx]$$

$$\frac{d[Zx]}{[Zx]} = -kZEP * dt$$

Given the linear relationship between NPQ and Zx (see Fig. 3), this leads to:

$$\frac{dNPQ}{NPQ} = -kZEP * dt$$

$$NPQ\,(t) = NPQi * \exp(-kZEP * t)$$

Therefore we expect the NPQ (like Zx concentration) to decay mono-exponentially with time. Origin software was used to fit the NPQ kinetics with this function, where t is time, NPQi is the initial NPQ value at the offset of light (t = 0) and kZEP is the kinetic constant of the zeaxanthin epoxidase.

Light-induced changes in relative NADPH content (expressed in Volts) were performed at 25 °C using the NADPH/9-AA module of a DUAL-PAM (Walz, Effeltrich, Germany) in a square 1 × 1 cm opened cuvette with the following settings (see [27] for a critical discussion). The cell suspension (5E7 cell mL^{-1}) was stirred during measurements. The emitter unit (DUAL-ENADPH) and the photomultiplier detector unit (DUAL-DNADPH) were mounted at right angles. The measuring light intensity (365 nm) was set at 20 (on a scale of 1 to 20 in the DUAL-PAM software), the measuring frequency was set at 5000 Hz, and the coarse/fine sensitivity settings of the DUAL-DNADPH power supply were set to 2 and 10, respectively. Because actinic light at 620 nm induced some minor decrease of the fluorescence signal, values reported here correspond to the differences between light-induced changes in the presence and in the absence of DCMU (15 μM).

2.5. HPLC measurements

Pigments were extracted from whole cells in methanol. This was done by rapidly sampling 50 μL of the cell concentrate directly from the fluorescence setup (just after a saturating pulse), mixing it together with 950 μL of methanol (100%) in a 2 mL tube, and then put into liquid nitrogen. Before HPLC analysis, cellular debris were removed by centrifugation at 14,000 × *g* for 10 min. Forty microliters of pigment extract were subjected to reverse-phase HPLC (Prominence UFLC, Shimadzu, Kyoto, Japan) analysis using a set-up comprising a LC-20AT pump, a SIL-20AC auto-sampler, a DGU-20A5R degassing unit, a CTO-10AS VP Column Oven and a SPD-M20A online photodiode array detector. A Nova Pak C18 column (60 A°, 4 μm pore size, 150-mm length, 3.9 mm diameter) was used for separation. Pigments were eluted during 2 min with a gradient from 100% (v/v) solvent A (80% [v/v] methanol and 20% [v/v] 0.5 m ammonium acetate [pH 7]) to 100% (v/v) solvent B (90% [v/v] acetonitrile in water), then during 20 min with a gradient from 100% (v/v) solvent B to 31% (v/v) solvent B and 69% (v/v) solvent C (ethyl acetate) and during 3 min with a gradient from the latter solvent mixture to 100% (v/v) solvent A. The solvent flow rate was 1 mL min^{-1}. The relative concentrations of individual pigments were calculated by the area pics ratio of all identified pigments at 430 nm.

3. Results

3.1. Peculiar light-dependency of the xanthophyll cycle dependent NPQ in *Phaeomonas sp.*

In a previous publication on *Phaeomonas* sp. [28], a decrease of maximal fluorescence yield (F_m) in the dark and a concomitant increase in the concentration of zeaxanthin were observed, although not discussed. We first looked at the decrease of the maximal fluorescence yield upon a transition from low light to dark, and observed that it could decrease by 3 fold after 20 min in the dark (Fig. 1a). This correlated with significant changes in the pigment composition, ~25% of the violaxanthin (Vx) being converted into zeaxanthin (Zx) (Fig. 1g and Supplemental Fig. 1a). It is to note that when the kinetics of F_m change is followed in the dark, saturating pulses obviously disrupt the darkness. To rule out the possibility that the observed F_m decrease was not due to the saturating pulses themselves, we also measured the extent of the F_m decrease without pulses (Supplemental Fig. 6a). Even though the final extent of F_m decrease is slightly smaller in the absence of saturating pulses (2.5 fold decrease), the results confirm that the cells develop a large non-photochemical quenching (NPQ) of chlorophyll fluorescence in the dark (Fig. 1d). The extent of NPQ is usually calculated on the basis of the decrease in F_m, but the standard definition where the reference is the F_m in the dark cannot be used here, because F_m in the dark is changing with time in *Phaeomonas* sp. Instead we used the maximal fluorescence when cells are fully acclimated to low light irradiance as a reference (see Materials and methods), given that it corresponds to the maximal fluorescence yield in *Phaeomonas sp.* and is associated with minimal de-epoxidation state (see below). This NPQ in the dark reversed under low light conditions (Fig. 1e), leading to a relaxation of the F_m (Fig. 1b) and to a conversion of Zx back to Vx in approximately 15 min (Fig. 1h and Supplemental Fig. 1b). NPQ and xanthophyll de-epoxidation being usually responses to high light regime in other photosynthetic organisms, we also investigated the response of *Phaeomonas* sp. upon a transition from low light (maximal fluorescence yield) to high light. As expected we observed a decrease in F_m and a concomitant increase of NPQ and Zx (Fig. 1c, f, i). It is to note that the NPQ generated in high light also fully relaxes in low light in about 15 min (Supplemental Fig. 6e). In order to determine if NPQ changes depend on Zx amount in *Phaeomonas* sp., we tested the effect of dithiothreitol (DTT), an inhibitor of VDE. In the presence of DTT, the conversion of Vx into Zx in the dark and in high light and the generation of NPQ are prevented (Fig. 1d, g, f, i). In contrast, DTT did not significantly alter the dark to low-light transition, suggesting that the rate of de-epoxidation in low light is much lower than the rate of epoxidation (Fig. 1b, e, h). In all experiments reported above, the NPQ (Fig. 1d, e, f) follows the changes in the Zx concentration (Fig. 1g, h, i) and the total amount of XC pigments (i.e. the sum of Vx, Zx and their intermediate Ax) was constant (Supplemental Fig. 1).

We then investigated the light-dependency of the photosynthetic activity ($rETR_{PSII}$), the xanthophyll cycle (XC) and NPQ (Fig. 2). The $rETR_{PSII}$ showed a classical saturation curve, saturating at ~200 $\mu E\, m^{-2}\, s^{-1}$ (Fig. 2a). NPQ was null in the range of low and moderate light intensities where photosynthetic activity is limited by light, but it increased at higher light irradiances, reaching a maximal value under saturating light irradiance (at 250 and 450 $\mu E\, m^{-2}\, s^{-1}$, see Fig. 2b). These changes in NPQ correlated with changes in XC (Fig. 2c, d). The time (Fig. 1) and light (Fig. 2) dependencies of NPQ and pigment changes are very similar, suggesting a tight relationship between NPQ and Zx amount. To determine more accurately this relationship, we plotted one against the other and obtained a linear relationship (Fig. 3), a result which is in good agreement with previous finding in diatoms, the sister group of pinguiophytes [19]. Moreover, the observation that the linear relationship crosses the origin of the axes confirms that the use of F_m^* as a reference for unquenched cells is correct. Conversely, there is no obvious relationship between NPQ and

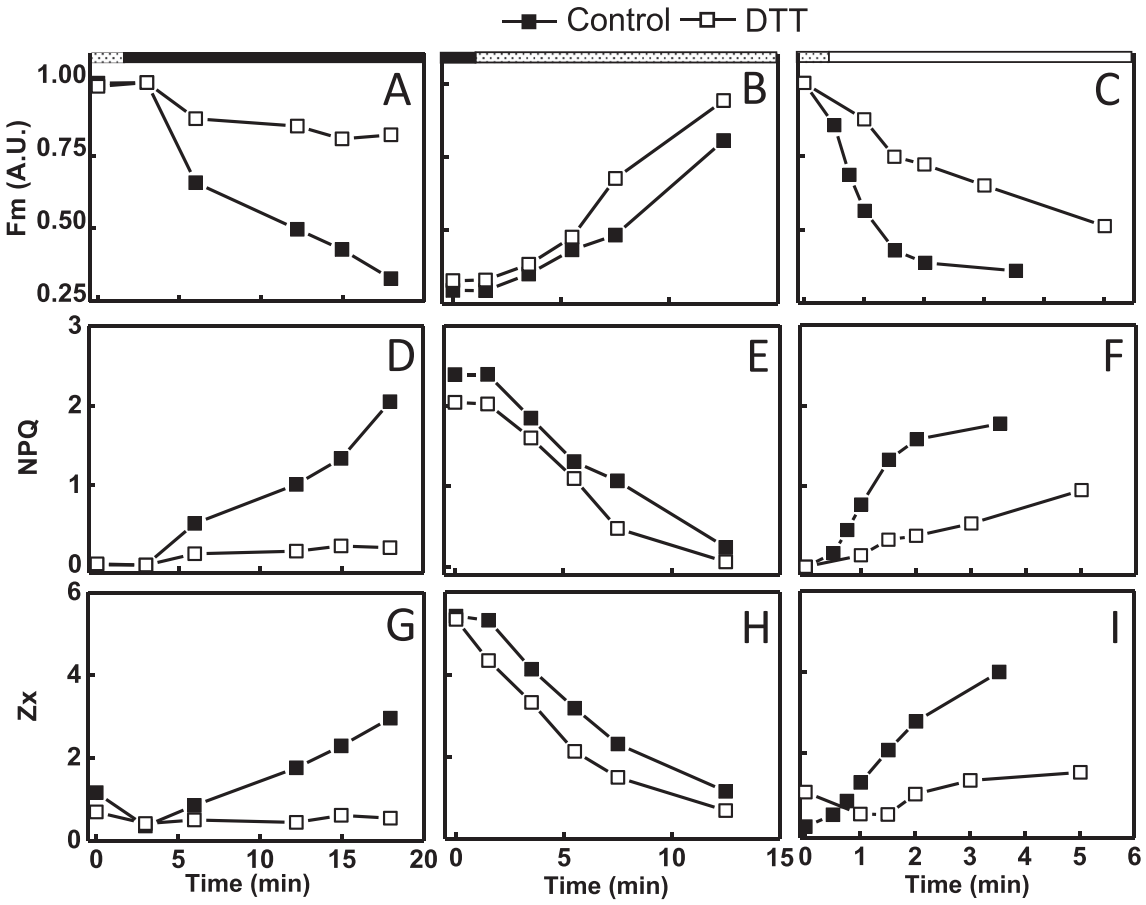

Fig. 1. Maximum chlorophyll fluorescence yield (F_m), non-photochemical quenching (NPQ) and zeaxanthin content in *Phaeomonas* sp. upon change of light intensity. Time-course of changes in the maximal fluorescence yield value (F_m) (a–c), NPQ (d–f) and Zx content (g–i) in the presence (open squares) or in the absence of 1 mM DTT (dark squares) upon transitions from low light to dark (left panels), dark to low light (30 μmol photons $m^{-2} s^{-1}$; central panels) and low light to high light (450 μmol photons $m^{-2} s^{-1}$; right panels). The rectangles above the panels indicate the light modes; open rectangles: high actinic light (450 μmol photons $m^{-2} s^{-1}$), dashed rectangles: low actinic light (30 μmol photons $m^{-2} s^{-1}$), dark rectangles: dark period. The amount of zeaxanthin is expressed as a percentage of the total pigment content (sum of carotenoids and chlorophylls, see Materials and methods). The data presented correspond to an illustrative representative experiment. See Supplemental Fig. 4 for averaged data with standard deviations corresponding to panels a–f.

Ax amount (Supplemental Fig. 3). Together, these results indicate that only Zx is involved in the fluorescence quenching.

3.2. Violaxanthin de-epoxidase is active in the dark in Phaeomonas sp., due to the presence of a dark trans-thylakoidal ΔpH

The peculiarity of the XC-dependent NPQ in *Phaeomonas* sp. comes from the unusual activity of its de-epoxidase in the dark (Figs. 1 and 2). VDE is believed to have no or little activity under neutral luminal pH, its activity being modulated by the osmotic component (ΔpH) of the proton motive force (PMF) [1,29]. In this respect, our results suggest that a significant PMF, including a ΔpH component is present in the absence of photosynthetic activity. The presence of a PMF in the dark has been already demonstrated in flowering plants [30] and in green algae [31], where it was shown to comprise both ΔpH and ΔΨ components [32]. More recently, a PMF in the dark was also demonstrated in diatoms by using the peculiar presence of two electrochromic probes with different dependency upon the electric component (ΔΨ) of the PMF [23].

In order to characterize the PMF in the dark in *Phaeomonas* sp., we first investigated the nature of the electrochromic shift (ECS) signal in *Phaeomonas* sp. using a similar protocol as in diatoms [23], *i.e.* by following the spectral dependency of the ECS relaxation. Fifty milliseconds after a saturating pulse, the absorption difference signals associated to *c*-type cytochromes redox state have fully relaxed. The remaining signal consists in the sole ECS, as demonstrated by the complete inhibition of the absorption difference signals upon addition of the uncoupler FCCP (data not shown). In the Fig. 4a, we show that the relaxation of the ECS depends on the wavelength, being faster at 576 nm compared to 542 nm, which suggests the presence of two different ECS components. These kinetics, very similar to the ones measured in diatoms [23], were fitted by the sum of a linear and a quadratic ECS components [33], which led to determine the spectra of those two components (Fig. 4b; see Materials and methods). Plotting the signal at 576 nm, which corresponds to a pure quadratic ECS, as a function of the signal at 542 nm, which corresponds to a pure linear ECS leads to the expected parabola (Fig. 4c, d). The relationships obtained in control conditions and in the presence of low concentration of the uncoupler FCCP were satisfyingly fitted by the same parabola, but the parabola in control conditions was shifted in regards of the origin of the axes (Fig. 4c, d). This indicates that a ΔΨ is present in the dark in control conditions, which is equivalent to 3 charge separations per photosystem and is partly suppressed in uncoupled conditions (Fig. 4d). Those results are in line with the ones obtained in diatoms [23] where two different ECS signals were found and used to reveal the presence of an electric field in the dark, which was also suppressed in the presence of the uncoupler FCCP. We then investigated whether a ΔpH was also present in the dark by using the H^+/K^+ exchanger nigericin, which

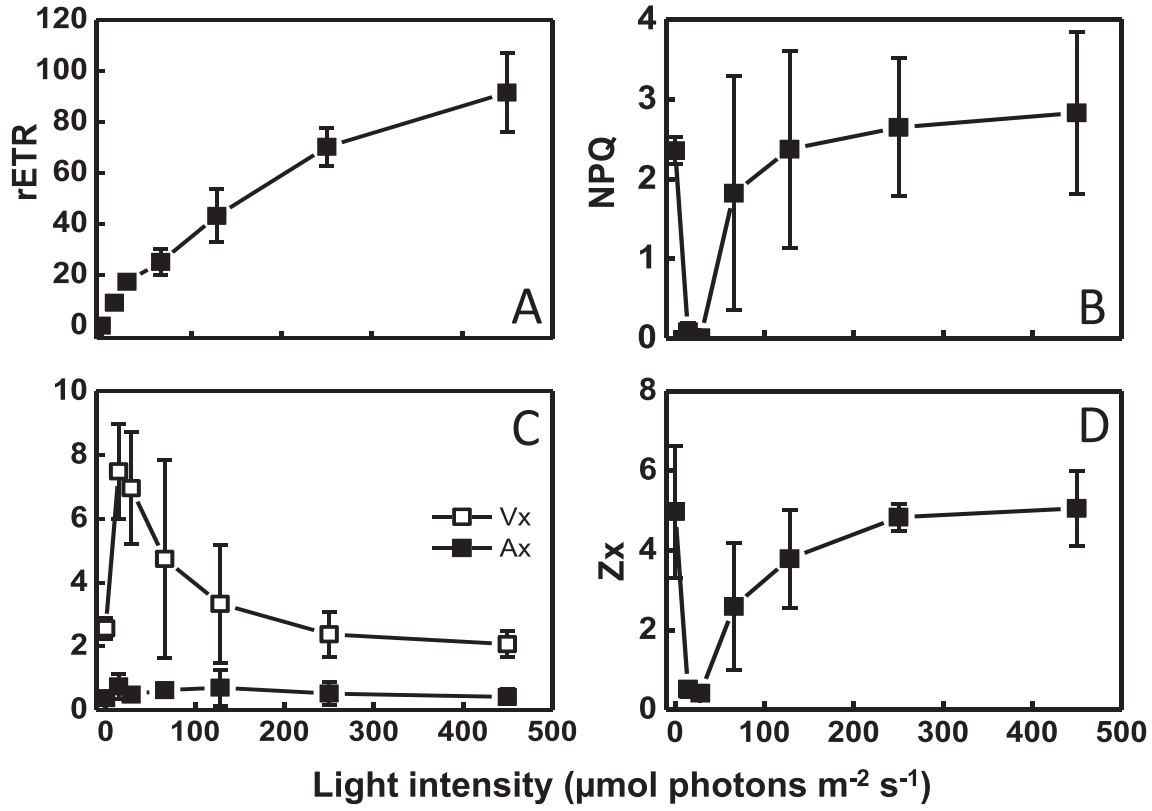

Fig. 2. Light-dependence of photosynthetic electron flow, NPQ and xanthophyll cycle pigments in *Phaeomonas* sp.
(a) Relative electrons transfer rate (µmol electrons $m^{-2} s^{-1}$), (b) NPQ, (c) antheraxanthin (Ax, black squares) and violaxanthin (Vx, open squares), and (d) zeaxanthin (Zx) in steady state as a function of the light intensity (µmol photons $m^{-2} s^{-1}$). The amount of xanthophyll pigments is expressed as a percentage of the total pigment content (sum of carotenoids and chlorophylls, see Materials and methods). The data presented correspond to the means of 3 biological and 9 technical replicates. In these experiments, NPQ, and thus Vx deepoxidation into Zx, were not always triggered at the same light intensity (see Supplemental Fig. 2 for individual datasets). This explains the extent of the standard deviation around 100 µmol of photons $m^{-2} s^{-1}$.

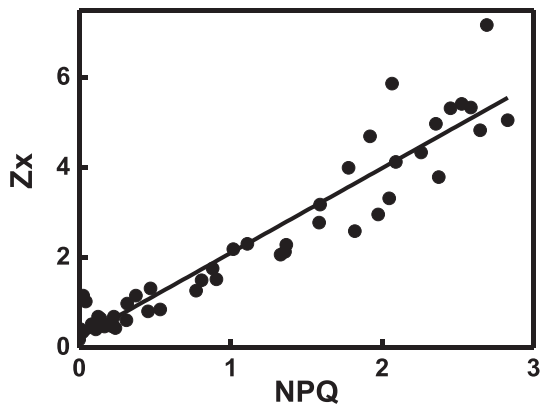

Fig. 3. Relationship between NPQ and Zeaxanthin content in *Phaeomonas* sp.
The amount of zeaxanthin (Zx, expressed as a percentage of the total pigment content) and the corresponding NPQ values were taken from Figs. 1 and 2. Linear regression was determined with Origin® software and best fit was for $Zx = 0.19 + 2.00 * NPQ$ ($R^2 = 0.91$).

suppresses the ΔpH component of PMF while maintaining the ΔΨ, and therefore insures a neutral luminal pH [34,35]. When nigericin is added to the cells before the dark period, the VDE activity in the dark is fully prevented as illustrated by the absence of NPQ in the dark (Supplemental Fig. 6a). This demonstrates that a ΔpH is present in the dark in *Phaeomonas* sp. In agreement with this, this inhibition of de-epoxidase by nigericin slightly accelerated NPQ relaxation (Fig. 5a). Thus, the ECS experiment and the effect of nigericin pinpoint the existence of a PMF in the dark which comprises both a ΔΨ and a ΔpH component. The latter allows the activation of the de-epoxidase, even in the absence of photosynthetic activity.

3.3. The zeaxanthin epoxidase activity is strongly regulated by the photosynthetic electron flow towards NADPH

The occurrence of a net de-epoxidation in the dark also indicated that the epoxidation rate is low. At the contrary, under low light irradiance, the NPQ fully relaxes in approximatively 15 min (Fig. 1e). This relaxation of NPQ, concomitant to the fast conversion of Zx back into Vx (Fig. 1h) suggests that the epoxidase is activated in the light compared to its dark status. Given that NADPH is the substrate for epoxidase, we investigated whether the light-driven electron transfer from H_2O to NADPH played a role in this light activation. For that, we investigated the consequences of photosynthetic inhibition on NPQ during a dark-to-light transition. We used the linear relationship between Zx and NPQ, discussed above, as an experimental advantage allowing us to probe ZEP activity through the measurement of the NPQ. As expected, inhibitors of the PSII (DCMU), or the cytochrome b_6f (DBMIB) did not suppress the generation of NPQ in the dark (Supplemental Fig. 6a). However, the relaxation of the NPQ during the dark to light transition was fully prevented in the presence of those inhibitors (Fig. 5a and Supplemental Fig. 6b), which indicates that the photosynthetic electron flow is necessary for the activity of ZEP.

Given that the epoxidation rate is dependent on photosynthetic flux, we then investigated its light-dependency. For this, we used the following protocol. First, we subjected the cells to a long period of darkness, which generated Zx accumulation and a strong NPQ. Second, we

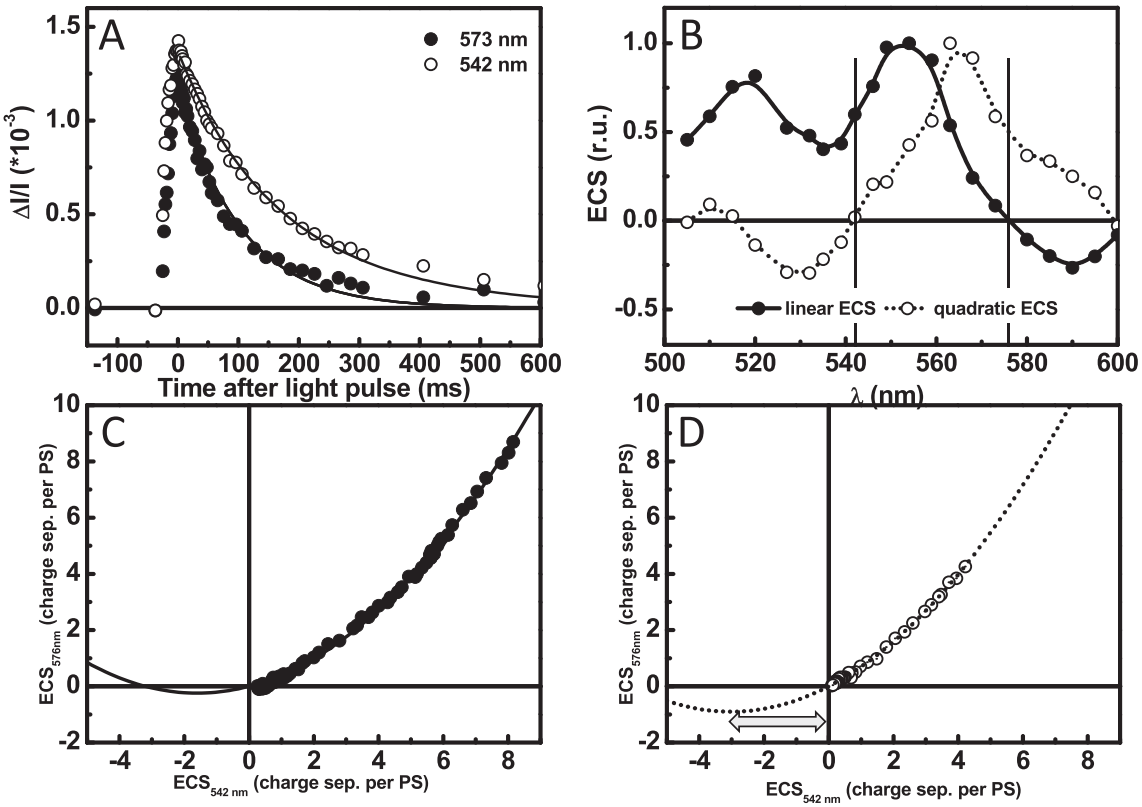

Fig. 4. Electrochromic Shift based measurements of the pmf in the dark.
(a) Kinetics of ΔI/I at 542 and 576 nm when a pulse of saturating light is applied to a dark-adapted *Phaeomonas* sample treated with low amount of uncoupler (see Materials and methods), (b) spectra of the linear (continuous) and quadratic (dashed) ECS spectra based on the deconvolution of the ΔI/I kinetics (see Materials and methods). Pure linear and quadratic wavelengths are shown with vertical bars. (c, d) Plot of the quadratic ECS *versus* the linear ECS during the decay of a pulse of saturating light, in the absence (c) or presence (d) of the uncoupler FCCP (10 nM). The horizontal arrow represents the extent of the electric component of the pmf already present in the dark, before the light perturbation.

added DTT to prevent further de-epoxidation. Then we measured the kinetics of the NPQ relaxation, reflecting the sole epoxidase activity, under different illumination (see Materials and methods). When the cells were kept in the dark, we did not observe any relaxation of the NPQ (Fig. 6a), which indicates that ZEP is almost completely inactive in the absence of photosynthetic activity. At the contrary, under low light, the NPQ relaxed rapidly (Fig. 6a), reflecting a fast ZEP activity. Unexpectedly, the relaxation of NPQ was slower under high light than under low light (Fig. 6a) despite a higher photosynthetic electron flow under high light (Fig. 2a). Altogether, these data indicate that the activity of ZEP presents an optimum under moderate light, being null in the dark, and slow under high light intensities (Fig. 6b). This light-dependency of ZEP activity mirrors the light-dependency of NPQ and Zx content (Fig. 2b, d) and pinpoints ZEP as the prime regulator of XC-dependent NPQ in *Phaeomonas* sp. instead of VDE as described in other organisms.

4. Discussion

It is generally assumed that NPQ comprises several contributions reflecting different phenomena: state transitions (qT), photo-inhibition (qI) or the high energy state quenching of fluorescence (qE) [1]. We observed that the NPQ, generated beforehand in the dark or in high light, fully relaxes under low light irradiance in a time-range (~15 min,

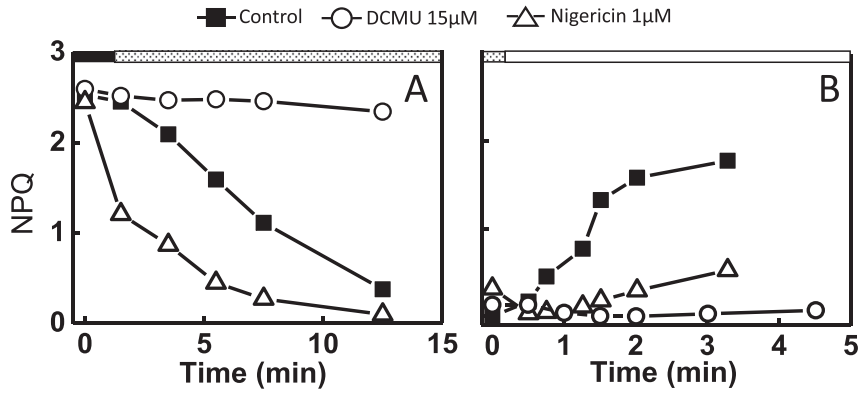

Fig. 5. Effect of PSII inhibitor and ΔpH uncoupler on NPQ changes upon change of light intensity in *Phaeomonas* sp.
Time-course of changes in NPQ upon transitions from (a) dark to low light (30 μmol photons $m^{-2} s^{-1}$) and (b) low light to high light (450 μmol photons $m^{-2} s^{-1}$) in the absence (dark squares) or in the absence of 1 μM nigericin (open triangles) and 15 μM DCMU (open circles). The rectangles above the panels indicate the light modes: open rectangles: high actinic light (450 μmol photons $m^{-2} s^{-1}$), dashed rectangles: low actinic light (30 μmol photons $m^{-2} s^{-1}$), dark rectangles: dark period. The data presented correspond to an illustrative representative experiment.

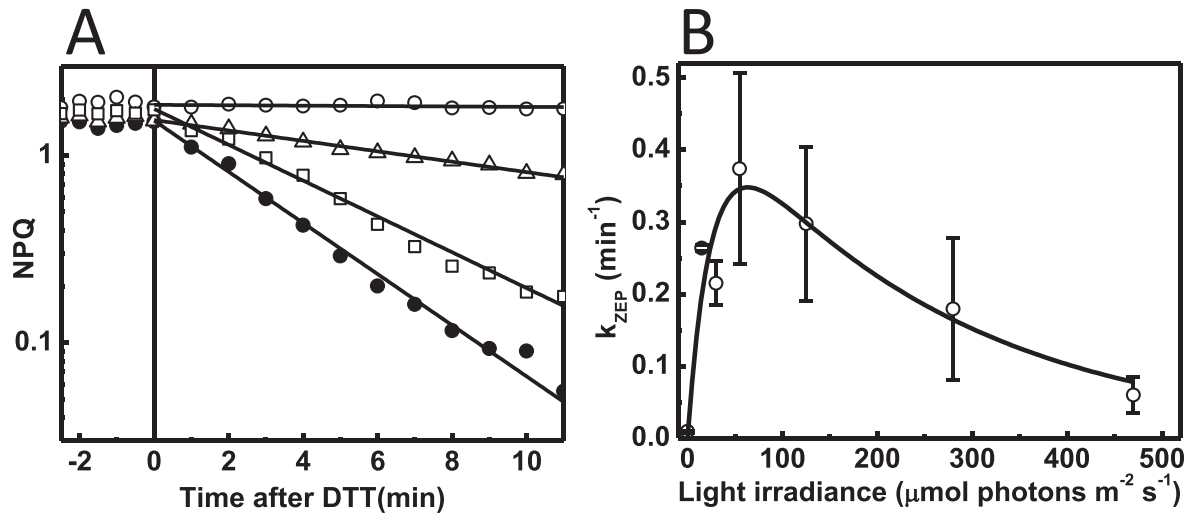

Fig. 6. Light dependency of the epoxidase activity. (a) Examples of kinetics of the mono-exponential decay of the NPQ after addition of DTT, under different light irradiances: dark (open circles), 30 μE m^{-2} s^{-1} (squares), 55 μE m^{-2} s^{-1} (close circles) and 470 μE m^{-2} s^{-1} (triangles). Lines represent the mono-exponential fitting of the NPQ decay. (b) Kinetic constant of the diatoxanthin epoxidation (k_{ZEP}) as a function of light irradiance. The kinetic constants are calculated through the mono-exponential fits of the NPQ decay like in panel A (see Materials and methods). Data represent the average ± S.D. from 6 independent experiments.

Fig. 1e and Supplemental Fig. 6e) that is not compatible with qI, which recovers more slowly [1]. State transitions (qT) and high energy state quenching can be distinguished based on their different modes of regulation. qT is regulated by the redox state of the plastoquinone (PQ) pool [36] whereas qE was originally defined in flowering plants as the NPQ component dependent on the synergetic action of a luminal pH acidification and the deepoxidation of xanthophyll pigments [37–39]. In the pinguiophyte *Phaeomonas* sp., we observed that the NPQ is suppressed (i) in the presence of a VDE inhibitor, which indicates that it is fully dependent on the xanthophyll cycle (ii) in the presence of the H^+/K^+ antiporter nigericin reflecting the role of ΔpH in the NPQ, and (iii) when photosynthesis is inhibited regardless of the redox state of the PQ pool: both in the presence of DCMU (PQ pool fully oxidized in the light) or DBMIB (PQ pool fully reduced in the light). Those data rule out the involvement of state transitions in the NPQ measured in *Phaeomonas* sp., like in the closely related diatoms (Owens, 1986), and indicate that the NPQ consists in a qE quenching. In plants, the qE mechanism is usually defined as the fastest (< 1 min) relaxing NPQ component [37–39], but another component was identified in *Arabidopsis thaliana* and called qZ [40], which displays qE features but different kinetics. It is formed within 10–30 min, independent of PsbS but strictly dependent on Zx. The relaxation of qZ strictly follows Zx epoxidation and therefore takes 10–30 min in *Arabidopsis thaliana* [40]. In *Phaeomonas* sp. the NPQ relaxes in ~15 min and is strictly proportional to Zx amount during its relaxation. The NPQ deciphered in this work displays identical properties as the qZ component, and we will therefore use that denomination from now on.

The linear relationship between NPQ and Zx (Fig. 3) which is expected by the Stern-Volmer theory for a homogenous quencher [41] is also informative regarding the mechanism of qZ. Such a linear relationship is found also in most diatoms [19,42]. It can be lost under prolonged high light exposure, but only because an extra pool of diatoxanthin, present in the lipid phase but not involved in the light-harvesting system, is being deepoxidized [43]. In the green lineage, there are two components of NPQ which are dependent on the xanthophyll cycle. The qE mechanism requires both the conversion of Vx into Zx and a conformational change of the light harvesting system catalyzed by the protonation of a PSII subunit which can be PsbS [14] or LhcSR3 [16]. Therefore, the kinetics of qE relaxation follows the PsbS/LhcSR deprotonation which is faster than the Zx epoxidation [16,44], and there is no linear correlation between qE and Zx during the relaxation phase [1]. Even though it has been proposed that Lhcx proteins in diatoms play a similar role as PsbS or LhcSR in the green lineage, sensing pH and inducing conformational changes in the PSII antenna [18,19], it is difficult to reconcile this with the linear relationship between diatoxanthin and NPQ in all conditions (generation or relaxation of NPQ, presence or absence of uncouplers). In diatoms, the linear relationship strongly suggests that the deepoxidation of diadinoxanthin into diatoxanthin is the only molecular event probably because Lhcx is not a pH sensor in this lineage and is not involved in conformational changes in PSII antenna [17 but see also 18]. Here also, the linear relationship between NPQ and Zx, both during the build-up and relaxation of the NPQ, rules out the involvement of another pH-sensing actor (*e.g.* PsbS, which is absent in the genomes of the closely related diatoms and brown algae). At the contrary, it strongly suggests that the deepoxidation of Vx into Zx is the only event involved in the fast changes in the PSII light-harvesting system leading to NPQ in *Phaeomonas* sp. This does not mean that the quenching efficiency does not depend on other factors. In *Phaeomonas* sp. like in diatoms, the concentration of another NPQ modulator (Lhcx in diatoms [17]) depends on the environmental conditions and can be regulated on longer time scale, modifying the characteristics of the PSII antenna (*e.g.* the number of diatoxanthin or Zx binding sites).

The behavior of the NPQ/qZ in *Phaeomonas* sp. and the light dependencies of ZEP and VDE enzymes draw a very different scheme from what is observed in flowering plants and green algae. In these organisms, it is generally accepted that VDE is inactive in the dark and becomes active as soon as the light-driven proton pumps acidifies the lumen [1,2]. Here, at the contrary, VDE is already active in the dark, due to the presence of a proton motive force which comprises not only an electric component, as the electrochromic shift experiments reveal, but also a ΔpH component. The presence of a proton motive force in the dark could be due to the hydrolysis of ATP generated by the respiratory process, like in plants [30], green algae [32] and diatoms [23] but could also be produced by an electrogenic chlororespiratory pathway, as previously proposed in diatoms [29,45,46]. There are two possibilities to explain the peculiar feature of an active VDE in *Phaeomonas sp.*: (i) the source of production of ATP in the dark is more efficient than in other photosynthetic organisms, and/or (ii) the pH dependency of the VDE is shifted towards more neutral pH. It is to note that it is the case in diatoms [29] leading to a slow but measurable deepoxidation of pigments during prolonged darkness periods [46].

The activity of the VDE in the dark does not mean that its rate is not regulated by light irradiance. At the contrary, the data presented here

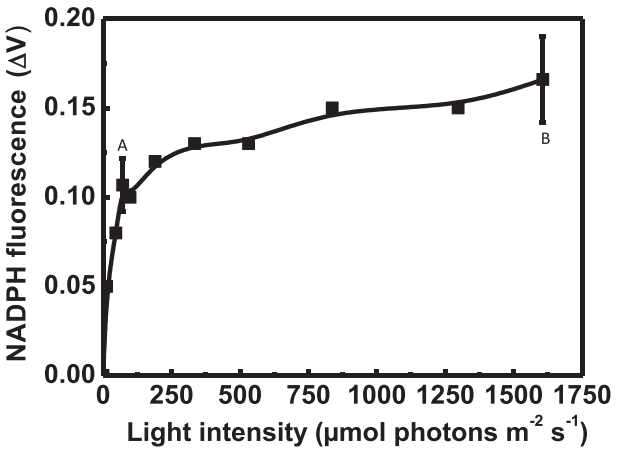

Fig. 7. NADPH fluorescence levels at different light intensities. Amplitude of NADPH fluorescence induced by a transition from dark to light (2 min illumination period with actinic light at 620 nm). Fluorescence in the dark was arbitrary set to 0. Values obtained at light intensities 72 (A) and 1602 (B) µmol photons $m^{-2} s^{-1}$ correspond to means of 2 biological and 5 technical replicates (p value < 0.02).

strongly suggest that VDE activity increases with light irradiance. Indeed, the generation of NPQ under high light is faster than in the dark. Moreover, the qZ and de-epoxidation state in steady-state high light (450 µmol photons $m^{-2} s^{-1}$) being similar to their dark values, one can roughly conclude that the ratio between VDE and ZEP activities is also similar in both conditions. Therefore, like for ZEP (Fig. 6b) whose activity increases significantly (by a factor 6), the rate of VDE must have similarly increased in high light compared to dark condition. However, the extent of the changes in ZEP activity out-competes the extent of the changes in VDE activity. We observed a > 10 fold increase of ZEP activity under low light irradiance compared to dark conditions (Fig. 6b). The high epoxidation rate under low light irradiance was fully inhibited in the presence of DCMU or DBMIB (Fig. 5a and Supplemental Fig. 4b, c), which inhibits the electron transfer from water to $NADP^+$. This most likely reflects that the increase of the concentration of the epoxidase substrate, NADPH, when photosynthesis occurs (Fig. 7) increases the epoxidase activity. A similar behavior was observed in diatoms to a smaller extent [47].

A surprising observation was that ZEP activity decreased under high light. In line with our interpretation of the dark to low light changes, this could have indicated that the availability of NADPH decreases under high light. The concentration of NADPH is actually even higher under high light (Fig. 7), so it seems more reasonable to imagine instead a direct modification of the Zx epoxidase in high light (*e.g.* by phosphorylation/dephosphorylation). This kind of mechanism is most likely involved in the light-induced down-regulation of ZEP in flowering plants. In *Arabidopsis thaliana*, Zx epoxidation is nearly completely inhibited when the PSII quantum efficiency if halved [13], and the reduction of NADPH to about 50% of wild-type in mutant defective in NAD kinase results in accumulation of high levels of Zx [48]. It was recently discovered that a M-type thioredoxin can affect the epoxidase activity in plants [49]. It is to note that a regulation of the activity of Zx epoxidase by the ΔpH itself has been proposed in diatoms [44] but such a ΔpH regulation of the epoxidase does not occur in *Phaeomonas* sp. (Supplemental Fig. 7).

The observation that the epoxidase activity changes by more than an order of magnitude depending on light irradiance contrasts with the generally implicit idea that only the de-epoxidase is regulated by the photosynthetic activity [1]. Even, the fact that the light dependencies of epoxidase activity and NPQ mirror each other strongly suggests that NPQ is mostly regulated through the regulation of the activity of the epoxidase, even though changes in the activity of the de- epoxidase also participate to XC regulation and qZ.

In most photosynthetic organisms, thermal dissipation of excess light energy (qE, qZ) usually takes place when the light absorption exceeds the photosynthetic electron transfer chain capacity [50]. The saturation of the electron transfer chain capacity happens in two light conditions: (i) under high light regime, when the electron flow to carbon fixation and alternative electron flows are saturated, and (ii) during dark-to-light transition, due to the deactivation of some enzymes of the Calvin-Benson-Bassham cycle in the dark, which leads to an electron bottleneck downhill PSI [50]. Because qE/qZ mechanisms usually need few minutes to operate, this leads to an initial situation where the excess photons are not taken in charge, resulting in an increase of the excited state lifetime in PSII and therefore in a high probability of ROS production [1]. Most photosynthetic organisms therefore respond to dark-to-light transitions by generating a transitory NPQ [17,51–53]. From this point of view, the fact that the PSII in *Phaeomonas* sp. is already quenched in the dark could represent an ecological advantage. The maximal quantum yield is diminished in the presence of a non-photochemical quenching (Supplemental Fig. 5) but it is obvious (and tautological) that the dissipating state of PSII cannot be a disadvantage in the dark or following a dark-to-light transition when the Calvin cycle is not yet activated. However, if such a dark qZ would be beneficial to photosynthetic organisms during the dark-to-light transition, this extreme event is likely not to be frequent in the marine environment. Understanding the ecological reason for developing a NPQ in the dark will need further work. Finally, the example of the NPQ in *Phaeomonas* sp. points out on the prime interest of exploring the diversity of the photosynthetic organisms to understand the constraints and regulations of the photosynthetic process in general.

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Author contribution

B.B., N.B. and P.C. planned and designed the research, B.B., B.I., N.B. and T.F. performed experiments, B.B., N.B. and P.C. analyzed data, B.B., N.B. and P.C. wrote the manuscript.

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