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Antagonist effect between violaxanthin and de-epoxidated pigments in nonphotochemical quenching induction in the qE deficient brown alga *Macrocystis pyrifera*

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ABSTRACT

Nonphotochemical quenching (NPQ) of Photosystem II fluorescence is one of the most important photoprotection responses of phototropic organisms. NPQ in *Macrocystis pyrifera* is unique since the fast induction of this response, the energy dependent quenching (qE), is not present in this alga. In contrast to higher plants, NPQ in this organism is much more strongly related to xanthophyll cycle (XC) pigment interconversion. Characterization of how NPQ is controlled when qE is not present is important as this might represent an ancient response to light stress. Here, we describe the influence of the XC pigment pool (ΣXC) size on NPQ induction in *M. pyrifera*. The sum of violaxanthin (Vx) plus antheraxanthin and zeaxanthin (Zx) represents the ΣXC. This pool was three-fold larger in blades collected at the surface of the water column (19 mol mol^{-1} Chl $a \times 100$) than in blades collected at 6 m depth. Maximum NPQ was not different in samples with a ΣXC higher than 12 mol mol^{-1} Chl $a \times 100$; however, NPQ induction was faster in blades with a large ΣXC. The increase in the NPQ induction rate was associated with a faster Vx to Zx conversion. Further, we found that NPQ depends on the de-epoxidation state of the ΣXC, not on the absolute concentration of Zx and antheraxanthin. Thus, there was an antagonist effect between Vx and de-epoxidated xanthophylls for NPQ. These results indicate that in the absence of qE, a large ΣXC is needed in *M. pyrifera* to respond faster to light stress conditions.

1. Introduction

The nonphotochemical quenching (NPQ) of Photosystem II (PSII) chlorophyll (Chl) *a* emission is a proxy to measure the thermal dissipation in the photosynthetic apparatus of plants and algae [1]. Dissipation of excess energy as heat is one of the most important photoprotection mechanisms of phototropic organisms. This process confers strong fitness to plants grown under field conditions [2] and provides resistance to environmental stress [3]. NPQ is a complex and finely regulated process that in higher plants consists of four different components: (1) qE, energy or ΔpH-dependent quenching; (2) qT, state transition quenching; (3) qI, photoinhibitory quenching and, (4) qZ, a zeaxanthin (Zx) dependent quenching [4]. The carotenoid Zx plays an important role in the qE, qZ and qI components of NPQ, either as a direct quencher or as a modulator of these processes in the photosynthetic apparatus of higher plants [5,6]. Zx is formed under saturating light conditions. A pH lower than 6 in the thylakoid lumen activates the violaxanthin de-epoxidase enzyme (VDE) that converts violaxanthin (Vx) into antheraxanthin (Ax) and then into Zx. The back conversion reaction takes place in darkness and is mediated by the Zx epoxidase enzyme [7]. The Vx to Zx conversion in high light and the back reaction in darkness are known as the xanthophyll cycle (XC) [7]. The XC is present in higher plants, mosses, lichens, green algae and brown algae [6,8,9], while in diatoms, xanthophytes, haptophytes and dinoflagellates a homologous cycle exists in which diadinoxanthin is interconverted into diatoxanthin, known as diadinoxanthin cycle [6,10].

Of the different components involved in photoprotection, qE is the fastest component of NPQ since it is induced in high light and

Abbreviations: Ax, antheraxanthin; Chl, chlorophyll; Dd, diadinoxanthin; DPS, de-epoxidation state of the xanthophyll cycle pigment pool; Dt, diatoxanthin; ΔpH, proton gradient across the thylakoid membrane; FCP, fucoxanthin-chlorophyll protein; HL, high light; HPLC, high performance liquid chromatography; LHC, light harvesting complex; LL, low light; MGDG, monogalactosyldiacylglycerol; NPQ, nonphotochemical quenching of PSII florescence; PSII, photosystem II; qE, energy-dependent quenching; qI, photoinhibitory quenching; qZ, zeaxanthin-dependent quenching; qT, state transition quenching; Vx, violaxanthin; VDE, violaxanthin de-epoxidase; XC, xanthophyll cycle; ΣXC, xanthophyll cycle pigment pool; Zx, zeaxanthin; ZE, zeaxanthin epoxidase

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disappears in darkness in seconds to minutes [1]. This component is activated by a drop in the pH of the thylakoid lumen (formation of a transthylakoid proton gradient, ΔpH) that is sensed by the PsbS protein in higher plants [11] or LHCSR proteins in green algae [12]. Protonation of PsbS induces conformational changes of PSII antenna complexes that promote thermal dissipation in this system [11]. Zx modulates the PsbS-dependent quenching since the presence of this pigment enhances the formation of qE and retards its relaxation [13]. Also, lutein accelerates qE formation in a similar way as Zx [14] in some tropical plants that have a lutein cycle (formation of lutein from lutein epoxide) involved in photoprotection [15]. In contrast to qE, qZ is a slowly developing (10 to 30 min after light exposure) and slowly relaxing (10 to 60 min in darkness) component of NPQ that depends on the formation of Zx and is indirectly dependent on ΔpH since a low pH is necessary to activate the VDE [4,5]. qI is the longest lasting component of all the NPQ components [16]; it has been associated with PSII damage and its relaxation requires the repair of this system [17]. Zx also participates in qI since any sustained down regulation or inactivation of PSII is accompanied by the retention of this pigment [3]. The relative contribution of each component to NPQ development depends on the intensity and the duration of the exposure of the organism to light [5]. In higher plants, qE is the most important response to short term (min) exposure to saturating light since it allows a flexible and rapid switching between a light harvesting and energy dissipation function of the light harvesting antenna system [2].

The brown alga *Macrocystis pyrifera* has a slow induction of NPQ upon exposure to high light, and the fast NPQ relaxation phase that takes place in the first few minutes after light to dark transition is absent in this alga [18]. In contrast to higher plants, preillumination of this alga does not accelerate NPQ induction [18]. NPQ follows the accumulation of Zx upon illumination in any pre-acclimation condition, and the disruption of the ΔpH in light does not collapse NPQ in *M. pyrifera* [19]. Thus, qE is not present here and NPQ is strongly associated with XC activity [18,19]. In other Chl *c* containing organisms such as diatoms, NPQ is also closely related to XC pigment interconversion [6,20]. However, diatoms show a ΔpH related NPQ formed immediately upon illumination [21]. The lack of qE has been reported only for *M. pyrifera* [18] but several other brown algae show similar characteristics of NPQ control [22,23].

There are important evolutionary implications of the differential control of NPQ between higher plants and brown algae [18]. Probably the xanthophyll cycle appeared before the separation of the green and red algae (brown algae) lineages and light harvesting evolutionary events must have influenced the mechanisms of photoprotection related to this cycle [18]. It is not known how NPQ is controlled when it is independent of the ΔpH, but strongly related to the XC. *M. pyrifera* is a good model to investigate the response to light stress of an organism in which NPQ is independent of the ΔpH (absence of qE that has a fast response). In this work, we characterize the effect of the xanthophyll cycle pigment pool (ΣXC) on the rate and the extent of NPQ induction and its relaxation in *M. pyrifera*. The size of the ΣXC in this alga varies significantly in blades exposed to different light conditions [24]. An increase in the concentration of XC pigments is characteristic of sun-acclimated organisms [25–27]. In higher plants a high concentration of XC pigments slows the formation and relaxation of NPQ [28]. A 40% decrease in the rate of NPQ induction was detected in *Arabidopsis* mutants that overexpress XC pigments [28]. Similar reduction in NPQ induction rate has been reported for organisms grown in intermittent light that possess a large ΣXC [29]. In an organism that lacks the fast ΔpH-dependent NPQ component, a large ΣXC might represent a physiological constraint if this pool negatively affects the rate of NPQ induction. Here we have investigated how the size of the ΣXC affects the development of NPQ in *M. pyrifera*. We show that a large ΣXC is associated with an increase in the rate but not in the amplitude of NPQ induction. We also found that this process is related to the depoxidation state of the xanthophyll cycle and not to the absolute concentration of the photoprotective pigments zeaxanthin and antheraxanthin.

2. Materials and methods

2.1. Plant material

Macrocystis pyrifera (L) C. Agardh samples were collected by scuba diving from Campo Kennedy kelp forest (31° 41.96 N; 116° 40.90 W), near Ensenada Baja California, México in November of 2009. Three blades were collected from the surface, and then at depths of 1, 2, 3 and 6 m in the water column. Blades were collected from a single organism early in the morning, tagged, kept in darkness and transported in coolers with seawater to the laboratory. Tissue discs (1.2 cm diameter) from each blade were cut with a cork borer 10 cm above the pneumatocyst, along the central axis of each blade. The discs of the three blades from the same depth were pooled and maintained in 250 mL Erlenmeyer flasks with 200 mL of filtered seawater at 17 ± 0.5 °C. The discs were maintained under illumination (75 μmol photons m^{-2} s^{-1}; cool-white fluorescent bulbs) for 4 h, followed by at least 8 h of dark incubation. This light to dark treatment favored epoxidation of xanthophyll cycle pigments. After this treatment, the discs showed a maximum Photosystem II (PSII) quantum efficiency higher than 0.7, which is characteristic of non-stressed photosynthetic tissue of *M. pyrifera* [24]; this efficiency was estimated from the F_v/F_m ratio, where F_v is the variable chlorophyll (Chl) fluorescence and F_m is the maximal Chl fluorescence, see e.g. [1].

At the sampling site, irradiance in the 400 to 700 nm range was measured in the water column with a quantum scalar PAR (Photosynthetic Active Radiation) irradiance sensor QSPL-2101 (Biospherical Instruments, San Diego CA, USA). Three profiles of the irradiance changes with the depth were measured during the sampling period. Irradiance data were fitted using the Marquardt–Levenberg algorithm of Sigma Plot software (Jandel SSP Scientific SPSS, Chicago, IL, USA) to the Lambert–Beer model for the reduction of irradiance in the water column: $E_z = E_0\ e^{-k_d\ z}$, where E_z is the irradiance at depth z, E_0 is the irradiance at surface and the light attenuation coefficient is represented by k_d [30].

2.2. Experimental setup

To investigate the effect of the xanthophyll cycle pool size (ΣXC) and light intensity on the de-epoxidation and NPQ formation in *M. pyrifera*, the algal discs, collected from different depths, were exposed to five different light intensities. PSII florescence and pigment concentration were measured in samples exposed for 10 min to 45, 90, 550, 950, and 1550 μmol photons m^{-2} s^{-1}. Light was provided by 50-Watt halogen dichroic lamps (Techno Lite) and the intensity was adjusted by using homemade neutral density filters (plastic sieves) placed between the lamp and the sample. During the light period, algal discs were placed in homemade acrylic chambers with continuous flow of seawater maintained at 17 ± 0.5 °C and supplemented with 2 mM $NaHCO_3$ to avoid any possible CO_2 limitation. The light intensity was measured in situ with a cosine-type quantum sensor of a pulse amplitude modulated fluorometer (Diving-PAM; Heinz Walz, Effeltrich, Germany).

To evaluate NPQ relaxation and XC epoxidation, an independent experiment was performed. Surface samples were collected in June of 2010 from a different organism than the one in which induction experiments were performed. The samples were acclimated as mentioned above (maintained at 75 μmol photons m^{-2} s^{-1} during 4 h, followed by 8 h of dark incubation) and then exposed to 1000 μmol photons m^{-2} s^{-1} for 60 min. Further samples were taken at 4, 6, 10, 20, 50 min of light exposure and maintained in darkness for up to 60 min. NPQ and the concentrations of the XC pigments concentration were monitored during light and dark treatments in each subsample. According to

Nilkens et al. [4], NPQ and DPS relaxation data were evaluated using the equation $y = A_1 \exp(-t/\tau) + y_0$, where, A represents the amplitude and τ the lifetime of the first kinetic component, and y_0 represents the amplitude of the second component.

2.3. Photosystem II chlorophyll a fluorescence measurements

Photosystem II chlorophyll *a* emission was measured with a Diving-PAM (Walz, Germany); it was monitored by placing the PAM fiber optic at a 60° angle with respect to the light exposed side of the discs and this optical geometry was maintained during the duration of the experiments. NPQ was monitored in tissue discs illuminated for 10 min at five different light intensities (see Section 2.2). To measure the maximum florescence emission of PSII (F_m or F_m'; without or with non-photochemical quenching, respectively, i.e., in dark-adapted, or light-exposed samples) saturating flashes (halogen dichroic lamp of the Diving-PAM; 0.8 s; 3500 μmol photons m^{-2} s^{-1}) were applied every 30 s. NPQ was calculated as $(F_m - F_m')/F_m'$.

2.4. Pigment analysis

During the NPQ induction and relaxation experiments, samples for pigment analysis were taken at different time points during the illumination period and after the light was switched off. A tissue disc was collected from an individual chamber and immediately frozen and stored in liquid nitrogen (77 K). Pigments were extracted and analyzed as described in [24]. Pigments were extracted in 3 mL 100% cold acetone by grinding the discs that had been earlier frozen with liquid nitrogen. The extracts were maintained overnight at 4 °C and centrifuged at 13,000 × *g* (4 °C) for 5 min, and stored at −20 °C before analysis by HPLC [24]. The HPLC was done using a Shimadzu AV-10 system (Kyoto, Japan), equipped with a Zorbax Eclipse XDB C8 (Agilent Technologies, Englewood, CO) reverse-phase column (4.6 mm × 150 mm, 3.5 μm sizeparticles). The system was calibrated with external standards (DHI Inc., Hoersholm, Denmark).

The de-epoxidation state (DPS) of the xanthophyll cycle pigment pool was calculated as [Zx] + 0.5[Ax]/ΣXC [31]; here, Ax and Zx stand for antheraxanthin and zeaxanthin, respectively, and ΣXC is the sum of Vx, Ax and Zx concentrations. The DPS represents the photoprotective state of the XC, as the carotenoids Ax and Zx are involved in the dissipation of energy as heat that confers photoprotection to the photosynthetic apparatus [32].

3. Results

3.1. Concentration of xanthophyll cycle pigments in blades from different depths

The concentration of the XC pigments in the blades of *M. pyrifera* decreased with the depth of collection of the samples (Table 1). The de-epoxidation state (DPS = [Zx] + 0.5[Ax]/ΣXC) of the XC pigments was lower than 2% in all samples (Table 1). The DPS was associated mainly with the presence of Ax. The concentration of this pigment was five times higher in surface blades than in 6 m depth samples (Table 1). Zx was detected only in samples collected at the surface and 1 m depth (Table 1). Surface blades had a Vx concentration of ~19 mol mol^{-1} Chl *a* × 100; and a ΣXC concentration of ~20 mol mol^{-1} Chl *a* × 100 (Table 1). Vx decreased with depth and was three-fold lower in blades collected at 6 m. Further, there was a 25% reduction of ΣXC in 1 m depth blades; its size was more than three times lower in blades collected at 6 m depth than in surface blades (Table 1). Fig. 1 shows that there is an exponential reduction of the ΣXC with depth. The change in the size of the XC pigment content follows the reduction of light intensity with depth (Fig. 1). A 30% reduction of the intensity reaching the surface was detected at 1 m depth and at 6 m the reduction increased to 88% (Fig. 1). These results show that light plays an important role in the regulation of the size of the ΣXC.

Table 1
Concentration of xanthophyll cycle pigments in dark-adapted blades of Macrocystis pyrifera, collected from different depths in the water column in the ocean. The concentrations, as mol mol^{-1} Chl a × 100, of violaxanthin (Vx), antheraxanthin (Ax), zeaxanthin (Zx), the size of the XC pigment pool (ΣXC) and the de-epoxidation state of this pool (DPS) are presented.

Depth	Vx	Ax	Zx	ΣXC*	Chl *c*:Chl *a*	DPS
0	19.1 ± 0.9	0.5 ± 0.3	0.1 ± 0.1	19.7 ± 1	0.13 ± 0.01	1.87 ± 0.7
1	14.3 ± 0.7	0.3 ± 0.01	0.1 ± 0.1	14.7 ± 0.3	0.13 ± 0.01	1.7 ± 0.7
2	12.2 ± 0.6	0.3 ± 0.1	n.d	12.5 ± 0.3	0.13 ± 0.01	1.2 ± 0.4
3	9.7 ± 0.8	0.2 ± 0.0	n.d	9.9 ± 0.3	0.13 ± 0.01	1.03 ± 0.8
6	6.0 ± 0.5	0.1 ± 0.1	n.d	6.1 ± 0.2	0.13 ± 0.03	1.07 ± 0.3

* The ΣXC is the sum of [Vx] + [Ax] + [Zx]. The DPS is expressed as ([Zx] + 0.5[Ax]/ΣXC) × 100. Results represent the average of 5 independent measurements ± SD. n.d., not detected.

3.2. NPQ and XC pigment de-epoxidation in samples from different depths

Tissues collected from different depths were exposed to five different light intensities and NPQ induction was monitored for 10 min. A short illumination period of 10 min was chosen for the analysis of XC pigment de-epoxidation effect on NPQ induction in order to reduce the effect of PSII damage, especially in samples exposed to high light intensities. Two light intensities (45 and 90 μmol photons m^{-2} s^{-1}) were below the light-saturation parameter (E_k) reported for blades grown above 9 m (102 μmol photons m^{-2} s^{-1}; [24]) and three other light intensities (550, 950 and 1550 μmol photons m^{-2} s^{-1}) were higher than E_k. Samples collected from all depths showed an increase in NPQ upon exposure to light. Transient NPQ formation was observed in surface blades when exposed to 45 and 90 μmol photons m^{-2} s^{-1}. NPQ in the samples increased in the first 4 min after light exposure and decreased after this illumination period (Fig. 2). This transient NPQ induction was also observed in 1 m and 2 m depth samples. The lowest NPQ was measured in blades collected from 6 m depth (Fig. 2). NPQ at 10 min was higher in samples from all depths exposed to higher

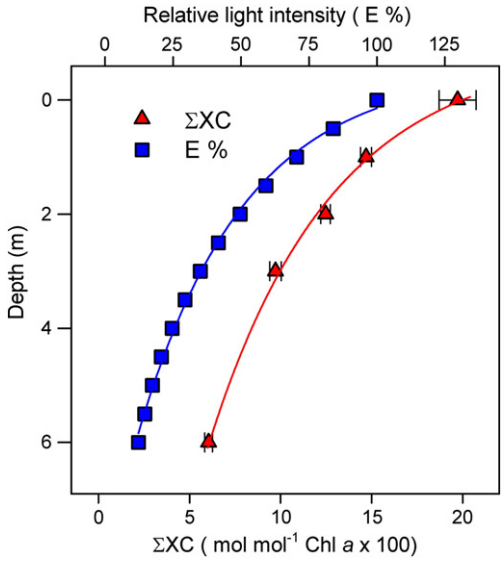

Fig. 1. Depth variation of the xanthophyll cycle pigment pool (ΣXC) in *Macrocystis pyrifera* blades and irradiance (E) in the water column. Irradiance is normalized to the intensity reaching the surface. Light data was fitted to the Lambert–Beer model of reduction of irradiance in the water column with an extinction coefficient (k_d) of 0.4 m^{-1} (continuous blue line). Also, ΣXC variation was modeled with an exponential model of reduction of this pool with depth (continuous red line).

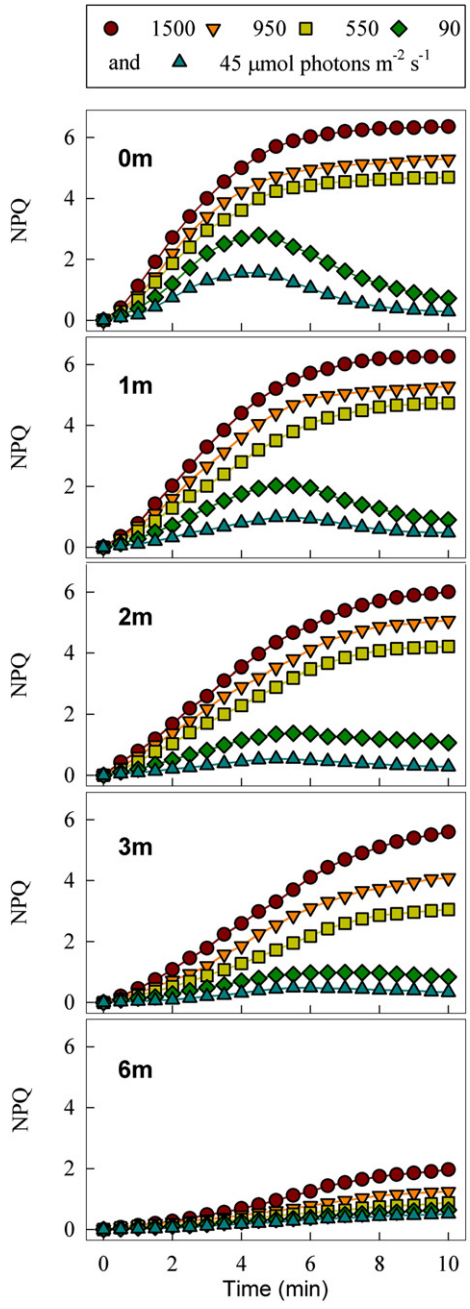

Fig. 2. Time course of nonphotochemical quenching (NPQ) in *Macrocystis pyrifera* blades collected from different depths. Triplicate samples of each depth were exposed to five different irradiances. The data represent the average of the three replicates.

light intensities (Fig. 2). Maximum NPQ after 10 min of light exposure was similar in samples collected from 3 m to the surface; however, the induction of this process was faster in surface than in deeper blades (Fig. 2). Half of the NPQ measured at 10 min (approximately 3 units of NPQ) was detected at ~2, 2.5, 3.5 and 4.5 min in samples collected at surface, 1, 2, and 3 m, respectively.

In *M. pyrifera*, NPQ is not dependent on the formation of the transthylakoid proton gradient (ΔpH), but it is closely associated with XC activity [18,19]. Therefore, the change in the concentration of XC pigments was measured under all light treatments to relate NPQ induction to XC de-epoxidation. The change in the concentration of XC pigments in samples collected at different depths and exposed to five irradiances is shown in Fig. 3. Data of the change of XC pigments was normalized to Chl a concentration. There were no significant changes in Chl or carotenoid content on leaf area basis during the experimentation period. In surface samples, Vx concentration decreased from 18.7 to 12.5 mol mol^{-1} Chl $a\times100$ in 10 min of exposure to 1550 μmol photons m^{-2} s^{-1} (Fig. 3). Vx de-epoxidation decreased with the collection depth of the samples and with light intensity (Fig. 3). A transient decrease in Vx was observed in surface and 1 m depth samples exposed to 45 and 90 μmol photons m^{-2} s^{-1} (Fig. 3). This transient change was also observed in Ax and Zx. The concentration of these pigments increased in samples from surface and 1 m depths in the first minutes after exposure to 45 and 90 μmol photons m^{-2} s^{-1} and decreased towards the end of the exposure period. Ax showed a net accumulation upon exposure to light and reached a maximum concentration after 5 min in all samples exposed to 550 μmol photons m^{-2} s^{-1} and higher irradiance (Fig. 3). A fast accumulation of Ax was observed under all the light conditions for surface blades, and there was a decrease in the rate of accumulation of this pigment in samples collected at depth (Fig 3). In contrast to Ax, the increase in Zx concentration was slower in the first minutes of light exposure (Fig. 3). The time lag for Zx appearance increased with the depth of collection of the samples. In 3 m blades Zx accumulation was detected after 2 min of light exposure. Therefore, there was a faster accumulation of de-epoxidated pigment in samples acclimated to higher irradiances.

The change in the concentration of XC pigments was reflected in DPS variation during the light exposure period. DPS increased in samples from all depths upon exposure to light due to the accumulation of Ax and Zx. Maximum DPS was detected after 10 min and was higher in samples from all depths exposed to irradiances above 550 μmol photons m^{-2} s^{-1} (Fig. 3). DPS at 10 min of light exposure was similar (~26) in samples collected from 3 m to the surface; however, similar to NPQ induction, the rise of this parameter in time was faster in surface than in deeper blades (Fig. 3).

3.3. NPQ and XC pigment de-epoxidation in relation to ΣXC size

The effect of the ΣXC size on NPQ induction and DPS was analyzed. Maximum NPQ formation was related to the ΣXC size of the blades (Fig. 4A). When exposed to 550, 950 and 1550 μmol photons m^{-2} s^{-1}, the samples with a ΣXC of ~6 mol mol^{-1} Chl $a\times100$ (6 m depth) showed significantly lower (Two-way ANOVA, $p<0.05$; Tukey a posteriori test) NPQ than samples with a larger xanthophyll cycle pigment pool (Fig. 3A). NPQ levels in blades collected from 0 m, 1 m, 2 m and 3 m depths (ΣXC larger than 6 mol mol^{-1} Chl $a\times100$) were similar after 10 min of exposure to different light treatments (Two-way ANOVA, $p<0.05$; Fig. 4A). The NPQ was ~6 in these samples after 10 min of exposure to 1550 μmol photons m^{-2} s^{-1} and a 20% and 30% reduction was observed in tissues exposed to 990 and 550 μmol photons m^{-2} s^{-1}, respectively (Fig. 4A).

The de-epoxidation state of the XC pigments showed a similar relation with the size of the ΣXC as NPQ (Fig. 4B). The lowest DPS after 10 min of light exposure was detected in samples with the smallest ΣXC (6 m depth samples; Fig. 4B). DPS after 10 min of light exposure was not different in samples with a ΣXC size higher than ~12 mol mol^{-1} Chl $a\times100$ (Fig. 4B). The NPQ formed within 10 min of light exposure was similar in 3, 2, and 1 m, and surface samples. However, maximum DPS was different at each light intensity. Maximum DPS after 10 min of light exposure was approximately 27% in samples exposed to 1500 μmol photons m^{-2} s^{-1}, and decreased in samples exposed to 950 and 550 μmol photons m^{-2} s^{-1} (Fig. 4B).

3.4. Relation between NPQ induction and XC pigment de-epoxidation

The size of the ΣXC and the irradiance level influenced DPS and NPQ induction in *M. pyrifera*. The formation of de-epoxidated pigments and NPQ induction followed a similar trend between different

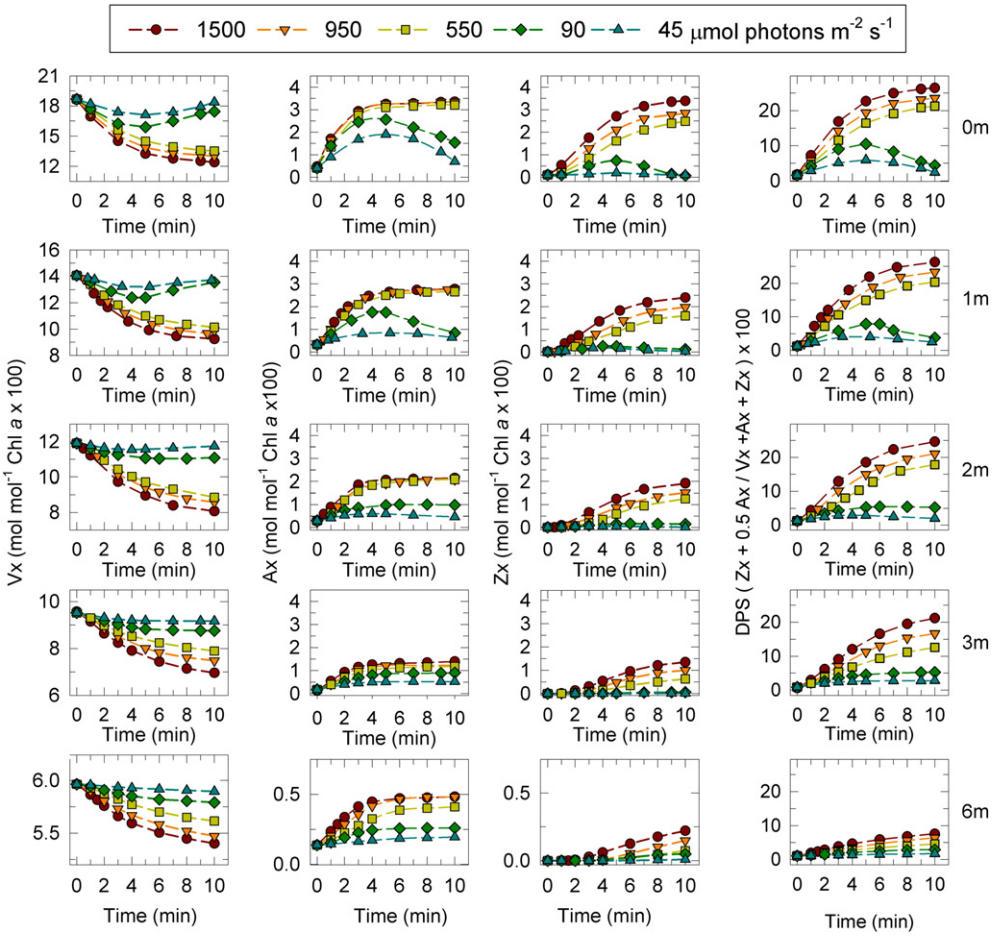

Fig. 3. Time course of the change of violaxanthin (Vx), antheraxanthin (Ax) and zeaxanthin (Zx) concentration in *Macrocystis pyrifera* blades collected from different depths. The change of the de-epoxidation state of the XC pigment pool (DPS) is also presented. Triplicate samples of each depth were exposed to five different irradiances. The data represent the average of the three replicates. Note the change in the scale of the Y axis in graphs of each collection depth.

light treatments and in samples from different depths (cf. Figs. 2 and 3, Fig. S1 in the supplementary material). The linear regression analysis between NPQ and the amount of Zx formed in the different samples and light treatments yielded an r^2 of 0.87 (Fig. 5A). The unexplained variance of this linear relation could be associated with the lack of accumulation of Zx at the beginning of the light treatments (cf. Fig. 3). It is known that Ax also contributes to NPQ mechanism [31,33]. This pigment was rapidly formed upon light exposure (cf. Fig. 3). The relation of the concentration of Ax plus Zx and NPQ is shown in Fig. 5B. An interesting pattern appeared from this result;

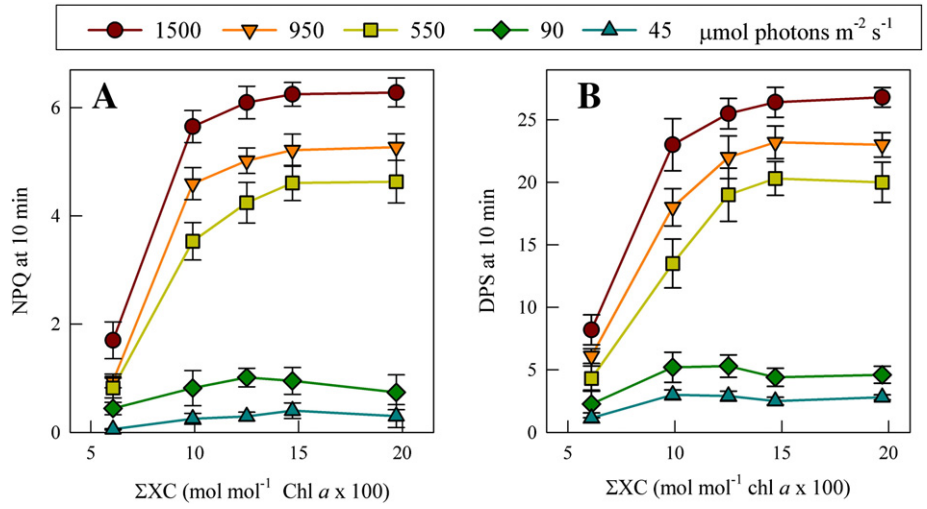

Fig. 4. Relation of the xanthophyll cycle pool size (ΣXC) and the non-photochemical quenching (NPQ; Panel A). In Panel B, the relation of the ΣXC with the de-epoxidation state of the XC pigment pool (DPS) is presented. The data represent the average of the three replicates ± standard deviation of each variable measured at 10 min of light exposure in *Macrocystis pyrifera* blades collected from different depths.

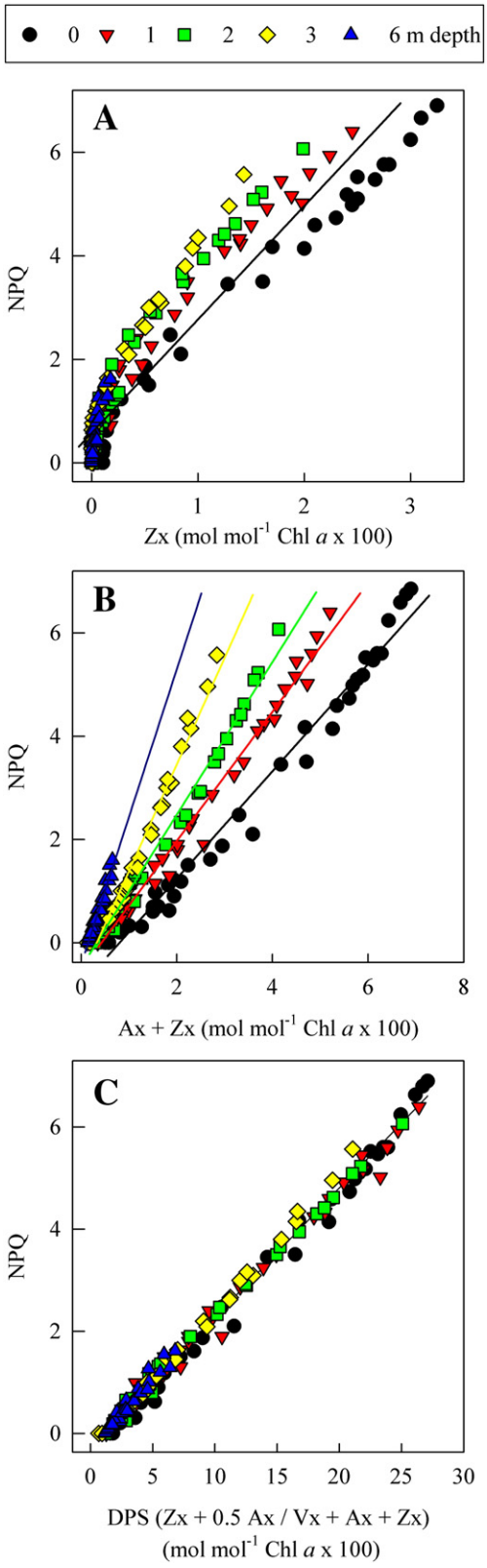

Fig. 5. Relation of nonphotochemical quenching (NPQ) of Photosystem II fluorescence, and the concentration of zeaxanthin (Panel A), antheraxanthin plus zeaxanthin (Panel B), and de-epoxidation state of the XC pool (Panel C) in *Macrocystis pyrifera* blades collected from different depths and exposed to different irradiances. The colors of the symbols and regression lines represent data from samples collected at different depths.

NPQ was linearly related to Ax plus Zx concentration with an r^2 higher than 0.96 when the relation is constructed with data obtained from each individual collection depth (Fig. 5B). The steepest slope of this relation was detected in the tissue collected at 6 m depth that showed the smallest ΣXC (Fig. 5B; cf. Fig. 5C). The slope of the relation decreased from 6 m to surface samples (Fig. 5B). This indicates that a higher concentration of Ax plus Zx is needed to induce the same NPQ in samples with a larger ΣXC than in tissue with lower concentration of XC pigments. However, when the relative amount of de-epoxidated pigments is considered instead of the absolute concentration of these pigments, comparable NPQ appears to be induced in samples with a different ΣXC size exposed to the different light intensities. The linear relationship between NPQ and DPS has an r^2 of 0.99 considering all the data of the different light treatments, exposure time and collection depths (Fig. 5C). Therefore, NPQ formation in *M. pyrifera* depends on the relative increase of de-epoxidated pigments and not on the absolute concentration of these pigments. This indicates that there is an antagonist effect for NPQ induction between de-epoxidated pigments and Vx.

3.5. Relation between NPQ relaxation and epoxidation of XC pigments

The antagonist effect between de-epoxidated and epoxidated pigments was also investigated in samples maintained in darkness after different preillumination periods. NPQ relaxation in darkness is slow and follows Zea epoxidation [19]. However, the reappearance of Vx in darkness and its effect on NPQ relaxation have not yet been investigated.

NPQ relaxation in samples collected at surface and exposed to different periods to high light (4, 6, 10, 20, and 50 min) and the change of XC pigment concentration are presented in Figs. 6 and 7, respectively. The blades collected for this experiment showed a smaller ΣXC than surface blades used in the NPQ induction experiment. Vx concentration was ~14 mol mol^{-1} Chl $a \times 100$ (Fig. 7). Also, maximum DPS was ~40% after the exposure of the sample to 1000 μmol photon m^{-2} s^{-1} for 60 min (Fig. 7). NPQ relaxation (Fig. 6) and Zx epoxidation (Fig. 7) after high light exposure followed a monophasic exponential decay. Two NPQ relaxation phases were detected in *M. pyrifera*. The maximum amplitude (A) of the main NPQ relaxation phase was fully developed after 10 min of preillumination and represented more that 80% of the NPQ induced in light (Table 2). Lifetime (τ_1) of this phase was between 2 to 6 min (Table 2). The second component (y_0 in Table 2; see also [4]) was detected in all treatments and its contribution to NPQ increased with the length of the preillumination period (Fig. 6; Table 2). Relaxation of NPQ was incomplete even in samples preilluminated for 4 min (Fig. 6). In samples preilluminated for 50 min, the slow relaxation phase represented more than 20% of the total NPQ; further, this phase did not relax within the time range of the experiment (Fig. 6).

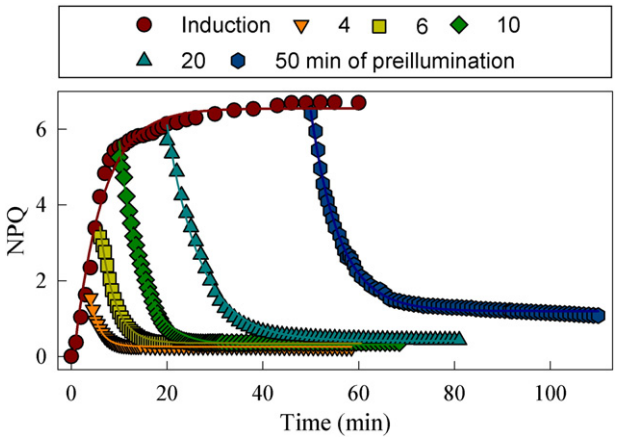

Fig. 6. Time course of nonphotochemical quenching (NPQ) induction and relaxation in *Macrocystis pyrifera* blades collected from the surface. Samples were exposed to approximately 60 min to 1000 μmol photon m^{-2} s^{-1} and samples (in triplicate) were collected at 4, 6, 10, 20 and 50 min. These subsamples were maintained in darkness to evaluate NPQ relaxation. The data represent the average of the three replicates.

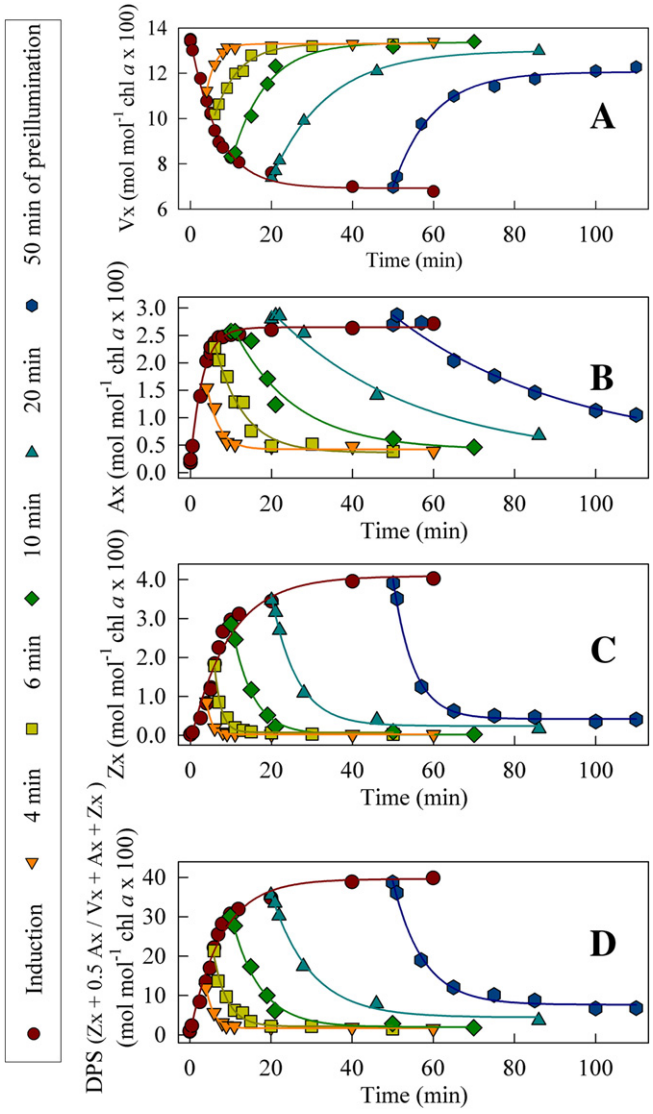

Fig. 7. Time course of the change of violaxanthin (Vx; Panel A), antheraxanthin (Ax; Panel B), zeaxanthin (Zx; Panel C) concentration and the de-epoxidation state of the xanthophyll cycle pool (DPS; Panel D) in *Macrocystis pyrifera* blades collected from surface. Triplicate samples of each depth were exposed to 1000 μmol photon m^{-2} s^{-1} for 4, 6, 10, 20, and 50 min and then the changes on xanthophyll pigment concentration were followed in darkness. The data represent the average of the three replicates.

An incomplete recovery of Vx concentration was also detected in all preillimnation treatments (Fig. 7). Vx concentration recovery was related to the preillumination period. In samples preilluminated for 4 min, Vx recovery was fast and almost complete while in samples preilluminated for 50 min, Vx recovery was approximately 20% of the concentration measured before the light treatment (Fig. 7). This incomplete Vx recovery was associated with the accumulation of Ax when the samples were exposed to longer preillumination periods (Fig. 7).

The reduction in darkness of DPS after high light exposure followed a monophasic exponential decay. The main phase of the DPS reduction had lifetime similar to the main NPQ relaxation phase (2 to 6 min; Table 2). Thus, a close relation between DPS and NPQ was observed in samples maintained in darkness after different periods of preillumination (Fig. 8B). NPQ in darkness was related to Zea concentration ($r^2 = 0.97$; Fig. 8A) but it was strongly dependent on DPS ($r^2 = 0.99$; Fig. 8B) and Vx concentration ($r^2 = 0.99$; Fig. 8C) that indicates that the appearance of this pigment is necessary for NPQ relaxation in *M. pyrifera*.

Table 2
Kinetic parameters of NPQ relaxation and DPS reduction in darkness after 4, 6, 10, 20 and 50 min of preillumination.

Preillumination time (min)	NPQ relaxation			DPS reduction		
	A_1*	τ_1 (min)	y_0	A_1	τ_1 (min)	y_0
4	1.2 ± 0.0	2.1 ± 0.2	0.27 ± 0.0	11.9 ± 0.3	1.8 ± 0.2	1.8 ± 0.2
6	3.1 ± 0.1	3.1 ± 0.3	0.35 ± 0.0	18.3 ± 0.3	4.1 ± 0.3	2.1 ± 0.2
10	5.4 ± 0.1	4.9 ± 0.4	0.33 ± 0.0	28.8 ± 1.3	5.6 ± 0.6	2.1 ± 0.1
20	5.4 ± 0.1	5.6 ± 0.7	0.45 ± 0.0	31.7 ± 1.2	5.7 ± 0.6	4.5 ± 0.9
50	5.5 ± 0.1	5.2 ± 0.8	1.2 ± 0.0	31.6 ± 1.1	6.5 ± 0.5	8.5 ± 0.6

* The parameters were calculated by fitting the NPQ and DPS data of Figs. 6 and 7 respectively to the function $y = A_1 * \exp(-t/\tau) + y_0$. SD represents the fitting error of the respective parameter, r^2 was >0.99 for NPQ data and up to 0.95 for DPS data in all preillumination treatments.

4. Discussion

We have demonstrated in previous reports [18,19] that qE is not present in *Macrocystis pyrifera* and NPQ is; further, NPQ is strongly related to the activity of the xanthophyll cycle. The major question that we posed in this paper was: How NPQ is controlled when the fast component of this process (qE) is absent. We found that NPQ induction was faster but the amplitude was similar in blades with a large xanthophyll cycle pigment pool (ΣXC). A fast NPQ induction was associated with a relatively fast conversion of Vx into Zx in blades that had a large ΣXC. Therefore, regulation of the size of the ΣXC is an important response to the light climate experienced by the photosynthetic tissue of *M. pyrifera*. Furthermore, we found that the ratio between de-epoxidated xanthophylls to total XC pigment concentration, but not the absolute concentration of Zx and Ax, explains NPQ formation in *M. pyrifera*. Therefore, there is an antagonist effect between Vx and de-epoxidated pigments for NPQ induction in this alga that could be also observed in the relaxation of this process in darkness.

4.1. ΣXC in blades from different depths

Light plays an important role in the regulation of the ΣXC size. Several factors affect the penetration of light in the water column. Absorption of light by water and by dissolved or particulate (mainly phytoplankton) matter affect the penetration of light in the water column [34]. Light intensity decreases exponentially with depth due to attenuation in the water column [30]. Shading and absorption from the canopy blades exacerbates the attenuation of light in kelp forests [35]. Therefore, a stronger exponential reduction of light is observed inside than outside of a kelp forest [35]. We found that ΣXC size responded to this strong reduction of the light intensity in the water column. Leaves from the same organism collected at different depths showed an exponential reduction in the size of the ΣXC with depth. For example, 1 m depth blades had 75% of the XC pigments than that measured in surface blades (canopy). Accumulation of photoprotective carotenoids is a common acclimation response to high light in phototrophs [25–28,36,37]. Similarly, the regulation of the ΣXC size is a major photoprotective response in *M. pyrifera*. The ΣXC size in surface blades of *M. pyrifera* was more than three-fold larger than in 6 m depth blades. Such variation in the size of the ΣXC has been previously described in several species of higher plants [25–27]. Thayer and Björkman [27] reported that shade-acclimated organisms had an average ΣXC size of ~4 mol mol^{-1} Chl $a \times 100$ while the average size was ~13 mol mol^{-1} Chl $a \times 100$ in sun-acclimated organisms. In this work, we measured a ΣXC size of ~20 mol mol^{-1} Chl $a \times 100$ in blades collected at surface. However, XC pigment concentration can be as large as 25 mol mol^{-1} Chl $a \times 100$ [24]. This concentration is almost two fold higher than the average ΣXC size reported for several sun-acclimated higher plants [27].

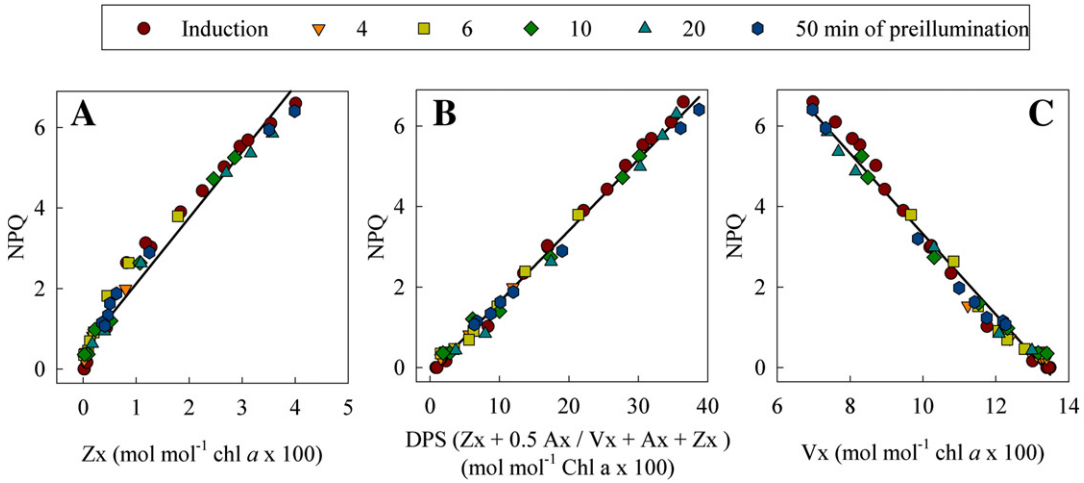

Fig. 8. Relation of nonphotochemical quenching (NPQ) of Photosystem II fluorescence, and zeaxanthin concentration (Panel A) and the de-epoxidation state of the XC pigment pool (DPS; Panel B) and the violaxanthin (Vx) concentration (Panel C) in *Macrocystis pyrifera* blades collected from surface. Samples were exposed to 1000 μmol photon m^{-2} s^{-1} and then maintained in darkness after different periods of preillumination (see legends of Figs. 6 and 7 for the description of the experiment).

4.2. ΣXC size effect on NPQ induction and de-epoxidation of XC pigments

The physiological implications of the increase of the ΣXC size in *M. pyrifera* were evident when the relationship between the size of this pool and NPQ induction was analyzed. In the absence of the fast qE component of NPQ, a large ΣXC represents a faster response to light stress in *M. pyrifera*. Although NPQ at 10 min was not significantly different between surface and 3 m blades, induction of this process was two times faster in surface blades. ΣXC in surface blades was more than two times larger than in 3 m blades. The effect of a large ΣXC size on the rate of NPQ induction is not clear in higher plants since the major proportion of this process is dominated by the ΔpH-PsbS driven antenna rearrangement that can be activated in the absence of Zx [38]. Therefore, NPQ does not match the kinetics of Zx formation during the first minutes of light exposure in higher plants. However, it has been documented that the induction of qE is slower in the *sChyB Arabidopsis thaliana* mutant, which shows an increase in the expression of β-carotene hydroxylase [28]. Slow qE induction was associated with a relatively slow change in the DPS since the ΣXC size is three-fold larger in the *sChyB* mutant than in the wild-type genotype [28]. In diatoms, the ΣXC size influences the NPQ extent [39]. However, a two-fold increase in the size of the ΣXC did not accelerate NPQ formation in *Phaeodactylum tricornutum* [40].

Similar to NPQ, the DPS was not significantly different between the surface and 3 m blades. The increase of the ΣXC in high light acclimated blades was not accompanied by an increase of the DPS. In contrast to this observation in our system, DPS increases as the size of XC pool size becomes larger in higher plants [3,13,27]. This is associated with the reduction of peripheral antenna size in high light acclimated plants and, as a consequence, an increase in the pool of free xanthophyll cycle pigments[41]. Probably in *M. pyrifera*, the increase of the ΣXC is co-regulated with xanthophyll binding proteins (most probably LHCSR proteins; see Section 4.3). This type of regulation has been observed in the green alga *Chlamydomonas reinhartdii* where in high light acclimated cells, PSII antenna size does not decrease but rather the concentration of LHCSR proteins increases [36].

In *M. pyrifera* similar maximum DPS can be established in tissue acclimated to different growth light conditions but the kinetics are different, DPS increase was faster in samples with a large ΣXC size. An increase in the rate of the Vx to Ax conversion seems to be associated with the faster accumulation of de-epoxidated xanthophylls. However, the accumulation of Zx during the first minutes of illumination was the dominant factor for the rapid DPS increase in surface and close to the surface samples. The more rapid conversion of Vx to Zx in blades grown under higher light intensities is likely to be related to an increased fraction of XC pigments that are (more easily) accessible to the Vx de-epoxidase enzyme (VDE). In higher plants, different pools of Vx can be identified according to the accessibility of this carotenoid to the VDE [41]. The de-epoxidation of Vx occurs in inverted hexagonal phase domains formed by monogalactosyldiacylglycerol (MGDG) in the thylakoid membrane [41,42]. Therefore, Vx must detach from the antenna protein pigment complexes to be converted into Ax and Zx [41]. Consequently, de-epoxidation of this pigment depends on the binding affinity to specific PSII antenna subunits [41]. In higher plants, Vx bound to some minor antenna complexes is not accessible to the VDE enzyme while the pigment associated with the peripheral antenna of PSII is rapidly de-epoxidated [43]. Schaller et al. [42] reported that a pool of Vx is not bound to any protein complex, but is found dissolved in the MGDG lipid that surrounds the PSII antenna of higher plants. A free MGDG-related pool of Dx has been also detected in diatoms and its size has been shown to increase in high light [44]. Therefore, a larger pool of easily accessible Vx can explain the increase in the de-epoxidation rate of this pigment and as consequence, the faster NPQ induction in *M. pyrifera* blades acclimated to high light. This Vx could be loosely bound to a specific protein complex important for photoprotection in this alga (see section below) or free in the thylakoid membrane (Fig. 9).

4.3. The de-epoxidation state of the XC pigments and NPQ induction

Important observations on the regulation of NPQ induction in the absence of a fast response are described in this paper. We found that in *M. pyrifera* a higher concentration of Ax and Zx is needed to induce the same NPQ in samples with a larger ΣXC size than in a tissue with a lower concentration of XC pigments. It seems paradoxical that photoprotective pigments are more efficient in inducing NPQ in low light-acclimated tissues than in tissues acclimated to high light conditions. However, this can be explained if we consider that low light acclimated samples might have a lower number of quenching sites and that they saturate at low concentration of de-epoxidated pigments (Fig. 9).

We found that NPQ induction in different light intensities could be explained by the de-epoxidation state of the XC pigment pool in blades with different acclimation characteristics (different sampling depths). Also, transient NPQ induction observed at subsaturating light intensities in samples with a large ΣXC is related to the interconversion of XC pigments.

Transient NPQ has been described in higher plants and its induction has been related to the transient accumulation of protons in

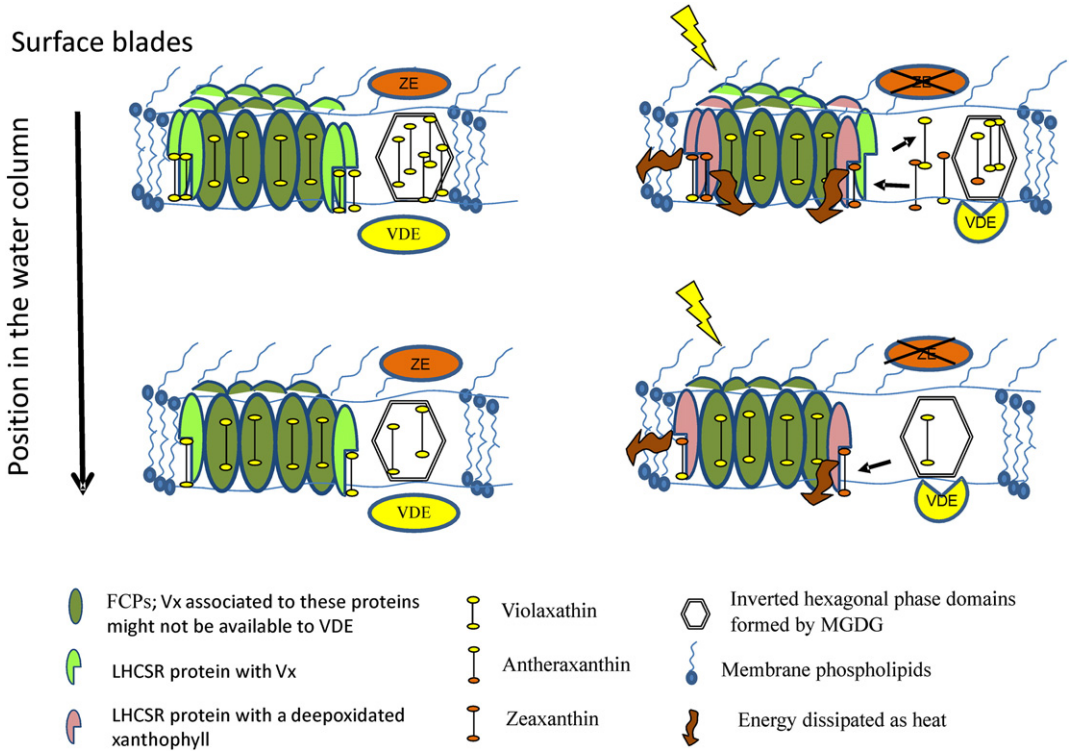

Fig. 9. A model of NPQ control in the brown alga *Macrocystis pyrifera*. The antenna macrodomain arrangement of *M. pyrifera* is shown. This arrangement differs from that of higher plants [60,61] since the fucoxanthin-binding proteins (FCPs) in brown algae are assembled in relatively large oligomers [61]. The antenna arrangement of blades from the surface (*top*) and from deeper strata (*bottom*) in the water column is shown. Blades from the surface have a large xanthophyll cycle pigment pool (ΣXC) associated with a large pool of violaxanthin (Vx) easily accessible to the Vx De-epoxidase enzyme (VDE). This pool of Vx is loosely bound to specific protein complexes or in monogalactosyldiacylglycerol (MGDG) domains of the thylakoid membrane (see text for full description). In darkness (*left column*) the VDE is inactive. In high light (*right column*), this enzyme becomes active (or increases its activity; see text) together with a reduction of the activity of the zeaxanthin (Zx) epoxidase (ZE). As a consequence, the concentration of antheraxanthin (Ax) and Zx increases in the MGDG domains. The less polar de-epoxidated pigments (Ax and Zx) diffuse to the lipid membrane domain and displace the Vx from their binding site in a protein of the *L*ight-*H*arvesting *C*omplex *St*ress Related (LHCSR) family. Therefore, there is a competition between epoxidated and de-epoxidated xanthophylls for the quenching site of these proteins. Vx bound to some FCPs are inaccessible to the VDE. The concentration of LHCSR proteins is high in surface blades and as a consequence, a high number of quenching sites exist in the antenna system. Low light acclimated samples have a low number of quenching sites and they saturate at a low concentration of de-epoxidated pigments. A large ΣXC and a high concentration of Vx accessible to the VDE, together with a large concentration of LHCSR proteins (quenching sites) in surface blades, are associated with a fast response of *M. pyrifera* to light stress conditions.

the lumen before the activation of the Calvin–Benson cycle [45–48]. In *M. pyrifera*, transitional NPQ was related to DPS changes during the illumination period. The close relationship between NPQ and DPS in this alga implies that there is an antagonist effect between Vx and de-epoxidated pigments for NPQ. This antagonist effect was also described in *Arabidopsis thaliana* since NPQ in wild type and *sChyB* genotypes was dependent on DPS instead of the concentration of Zx [28]. In this species, a high DPS has been associated with the stabilization of the quenched state since Vx inhibits and Zx promotes the qE component of NPQ [49]. Alternatively, if Zx is the direct quencher, either bound to PsbS, LHCII or to minor antenna complexes, there must exist competition between Vx and Zx for the quenching site [28]. In the absence of qE, the close relationship between DPS and NPQ in *M. pyrifera* supports the assumption that there must be a fixed number of quenching sites in the antenna system of this alga and that there must be competition between epoxidated and de-epoxidated xanthophylls for these sites (Fig. 9). Furthermore, as proposed by Johnson et al. [28], the binding and release rates of de-epoxidated xanthophylls from this site must be the rate-limiting factor for NPQ formation and relaxation, respectively. If Zx and Ax compete with Vx for the quenching sites in the antenna system of *M. pyrifera*, then NPQ must be controlled only by the activity of the enzymes involved in the XC (Fig. 9).

In higher plants, the VDE is active in saturating light while Zx epoxidase activity (ZE) seems to be constitutive [41]. One key observation of XC control in brown algae was made by Fernandez-Marin et al. [22]. They reported that in *Pelvetia canaliculata* there is a strong accumulation of Zx in darkness under stress conditions and when Zx epoxidase (ZE) is chemically inhibited [22]; they suggested that accumulation of de-epoxidated pigments is controlled by the activity of ZE since the VDE, in contrast to that in higher plants, has a basal constitutive activity. In *P. canaliculata*, Zx is not present in darkness because ZE prevents its accumulation, and when the activity of this enzyme is reduced or inhibited by stress conditions, Zx accumulation is detected [22]. It seems that in diatoms the epoxidation reaction is also important for DPS since the activity of the diatoxanthin epoxidase is strongly inhibited by the light-driven proton gradient [50]. We found that the level of irradiance modulated the maximum DPS in *M. pyrifera* and thereafter the NPQ detected at 10 min of light exposure. Maximum DPS and NPQ were higher in tissues exposed to higher irradiance. We suggest that VDE and ZE function must control the DPS level in *M. pyrifera*. If ZE activity is reduced or blocked in high light, the maximum DPS and also the kinetics of DPS increase should be determined by the VDE activity. A reduction of ZE activity in samples exposed to longer period to high light was observed in the XC pigment interconversion in darkness. The accumulation of Ax in darkness indicates that a reduction of ZE activity occurs in samples exposed to longer preillumination periods.

The amount and rate of formation of de-epoxidated pigments was important for NPQ induction in *M. pyrifera*. However, specific quenching sites must exist for NPQ to proceed. In *M. pyrifera*, a high NPQ in surface and near surface samples implies an increase in the number of quenching sites that must be associated with a higher concentration of the antenna complex involved in heat dissipation in this

alga (Fig. 9). In contrast, there must be fewer quenching sites in low light acclimated samples, resulting in their saturation at low concentrations of de-epoxidated pigments (Fig. 9). It is not yet known as to which proteins or complexes are involved in heat dissipation in brown algae. This group of organisms does not have homologous chlorophyll *a*/*b* binding proteins (CABs) [51,52]. Given that CABs are not present, photoprotective pigments must be associated with proteins different from those which play a role in qE control in higher plants. The candidates are proteins that belong to the light harvesting stress response protein family (LHCSR) [52]. It was shown recently [53] that of all the light harvesting complex genes, those that belong to the LHCSR clade showed the highest expression at surface compared to depth photosynthetic tissue of *M. pyrifera*.

LHCSR proteins are essential in the modulation of NPQ in other heterokonts as diatoms [54,55]. Specifically, two proteins of this family, the LHCX1 and LHCX6 seem to play an important role in the control of NPQ in diatoms. LHCX6 is strongly and rapidly induced in *Thalassiosira pseudonana* by high light; further, the steady state level of this protein parallels the increase in the qI component of NPQ [55]. In contrast to LHCX6, LHCX1 was not directly involved in NPQ development [55]; rather it has a structural function within the PSII-FCP supercomplex. However, LHCX1 is essential for NPQ development in *Phaeodactylum tricornutum* [54]. LHCX1-less mutants of this species showed a significantly reduced NPQ capacity and a reduced fitness [54]. Furthermore, the capacity of NPQ development was directly related to the expression of LHCX1 in different ecotypes of *P. tricornutum* [54]. How these proteins act in the NPQ process of diatoms is not clear. The evidence that two LHCSR proteins are important for NPQ indicates that diatoms have overlapping means to dissipate excess energy as heat [56]. Biochemical and structural information on LHCX proteins and their arrangement between the pigment–protein complexes is needed to know how the NPQ is controlled in diatoms [57,58]. Similar to those in diatoms, LHCSR proteins might be important for NPQ control in *M. pyrifera* (Fig. 9) and they must be identified and characterized biochemically and structurally. We propose that these proteins are the sites of quenching and the slow NPQ development might be controlled by the binding of de-epoxidated pigments to these proteins in *M. pyrifera* (Fig. 9).

The analysis of NPQ relaxation kinetics is a useful tool to characterize the components involved in this process. A two exponential decay model is necessary to explain NPQ relaxation in Arabidopsis since at least three different components are involved in NPQ development in higher plants: qE, qZ and qI [4]. The rapidly relaxing phase that is the dominating component in higher plants is absent in *M. pyrifera* and as a consequence, NPQ relaxation can be described by a single exponential decay model. In samples exposed to longer illumination periods a long-lasting component was observed that is similar to the qI component described in higher plants [5]. This last component was related to a reduction of the conversion of Ax into Vx after long preillumination periods. This component represents a minor proportion of the relaxation kinetics of NPQ. The major proportion of NPQ formed in light disappears in minutes and is related to the re-appearance of Vx in darkness. The similar lifetime of the main NPQ relaxation phase and DPS reduction in darkness confirms the antagonist effect between violaxanthin and de-epoxidated pigments for NPQ development. Relaxation kinetics of this NPQ phase resembles the ones reported for the qZ component described in higher plants [4,5]. Probably a qZ type NPQ (or the one observed in *M. pyrifera*) represents an ancient response to high light. It has been speculated that LHCSR probably evolved a function different from that of light harvesting and they may have appeared in ancestral chlorophyll *c* fucoxanthin-containing organisms and were possibly acquired by green algae later [52]. The ancestral LHCSR mechanism found in green algae and mosses was probably functionally replaced by PsbS during the evolution of land plants [59]. However, a most ancient mechanism of NPQ control related to these proteins might be present in brown alga that is not related to allosteric changes in the PSII antenna and is controlled mainly by the presence of Zx and Ax (Fig. 9).

5. Conclusions

From the data presented in this paper, it is obvious that ΣXC size modulates the NPQ kinetics of *M. pyrifera*. In the absence of a rapid inducible NPQ component controlled by the ΔpH-PsbS driven antenna rearrangements as in higher plants, *M. pyrifera* relies on the increase of the ΣXC size to accelerate the formation of the photoprotective pigments zeaxanthin and antheraxanthin. The relationship between epoxidated and de-epoxidated xanthophylls, but not to the absolute concentration of zeaxanthin and antheraxanthin, explains the NPQ formation in *M. pyrifera*. Therefore, there must be an antagonist effect between violaxanthin and de-epoxidated pigments for NPQ. NPQ induction was faster in blades with a large ΣXC and the size of this pool follows closely the light conditions experienced by the photosynthetic tissue.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbabio.2012.12.006.

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