Journal of Photochemistry and Photobiology B: Biology xxx (2011) xxx–xxx

Contents lists available at ScienceDirect

Journal of Photochemistry and Photobiology B: Biology

journal homepage: www.elsevier.com/locate/jphotobiol

# Photoprotection in the brown alga *Macrocystis pyrifera*: Evolutionary implications

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ARTICLE INFO

*Article history:*
Available online xxxx

*Keywords:*
Brown algae
Evolution
*Macrocystis pyrifera*
Non-photochemical quenching
Photoprotection
Xanthophyll cycle

ABSTRACT

The dissipation of energy as heat is essential for photosynthetic organisms to protect themselves against excess light. We compared Photosystem II florescence changes (non-photochemical quenching, NPQ) in the brown alga *Macrocystis pyrifera* with that of *Ficus* sp., a higher plant to examine if the mechanism of heat dissipation (energy-dependent quenching, qE) differs between these evolutionary distant groups of phototrophs. We discovered that *M. pyrifera* had a slower rise of NPQ upon illumination than the *Ficus* sp. Further, the NPQ relaxation phase that takes place in the first minutes after light to dark transition is absent in this brown alga. We found that the NPQ induction rate in this alga was 1.5 times faster in pre-illuminated samples than in dark-adapted samples; this was associated with an increase in the rate of accumulation of the carotenoid zeaxanthin. Therefore, we conclude that NPQ in *M. pyrifera* is associated only with the formation of zeaxanthin. These results indicate that *M. pyrifera* lacks the fast component of qE that is related to allosteric changes in the light harvesting complexes of *Ficus* sp., a representative of higher plants. Although the xanthophyll cycle of this brown alga is similar to that of *Ficus* sp., yet, the transthylakoid proton gradient (ΔpH) does not influence NPQ beyond the activation of the violaxanthin de-epoxidase enzyme. These findings suggest that NPQ control mechanisms are not universal and we suggest that it may have diverged early in the evolution of different groups of eukaryotic phototrophs.


## 1. Introduction

Too little light is not enough for plants, but too much light is not good [1,2]. The regulation of photosynthesis requires the fine-tuning of absorption process and utilization of excitation energy by photochemical processes (for background, see [3,4]). Specifically, when light is in excess, the probability of formation of long-lived excited states of chlorophyll (Chl) *a* increases and reactive oxygen species are produced [5]. As a consequence, plants can be photoinhibited and lose their photosynthetic capacity [6]. Phototrophic organisms have adopted mechanisms to cope with excess light. The dissipation of excess energy as heat is one of the most important photoprotection mechanism of higher plants and algae [7–11]. This mechanism brings tolerance to variations in light intensity and confers a strong fitness to higher plants under field conditions [12,13].

A decrease of Photosystem II (PSII) Chl *a* fluorescence independent of photochemistry (i.e., non-photochemical quenching, NPQ, of chlorophyll a excited state) has been used as a proxy to measure thermal dissipation [14,15]. The energy-dependent quenching (qE) or NPQ represents an efficient mechanism to reduce the damaging consequences for the photosynthetic apparatus [17]. (State transitions and photoinhibition could affect PSII emision; however, they are not NPQ processes, as explained by Papageorgiou and Govindjee [16], this issue.) Three elements are necessary for the full development of qE: the formation of ΔpH [18], the presence of the PSII antenna subunit, such as PsbS [19] and the synthesis of zeaxanthin (Zea) through the xanthophyll cycle. The xanthophyll cycle involves the de-epoxidation of the carotenoid violaxanthin (Vio) into Zea via the intermediate pigment antheraxanthin (Anth) in saturating light and the reverse reaction in low light or in darkness [20]. qE occurs when the acidification of the lumen in high light (establishment of ΔpH) protonates one or more PSII light harvesting antenna complexes (LHCs) [18], but especially, in many cases, of PsbS [21]. Protonated PsbS induces the dissociation of part of the PSII peripheral antenna complex, where most of the quenching of chlorophyll fluorescence takes place [17]. Reorganization of PSII antenna system promotes thermal dissipation within seconds [17,22]. In parallel, a low pH in the lumen activates the

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doi:10.1016/j.jphotobiol.2011.04.004

violaxanthin de-epoxidase enzyme (VDE), which converts Vio into Zea. This pigment might bind to minor antenna light harvesting complex (Lhcb4–Lhcb6) to form a Chl–Zea pair; a charge-transfer process may dissipate energy here [23]. Alternatively, Zea might act as an allosteric effector to form a quenching complex and energy transferred from the lowest excited state of Chl *a* to the $S_2$ excited state of lutein [24,25]. Further, in this issue, Matsubara et al. [26] demonstrate, using fluorescence lifetime imaging microscopy, that both lutein and Zea cycles are involved in photoprotection in avocado leaves. (See [27] for the relation of the lutein epoxide cycle to other xanthophyll cycles.) Of the different elements involved in qE, the ΔpH is a major controlling factor since it allows a flexible and rapid switching between a light harvesting, and an energy dissipation function of the light harvesting antenna system of higher plants [22].

It is often assumed that the molecular control of qE is similar in all eukaryotic phototrophs that show xanthophyll cycle activity. However, there are differences in the induction and dissipation characteristics of NPQ in different groups of algae [28]. For example, NPQ induction and dissipation characteristics in the brown alga *Macrocystis pyrifera* (Heterokontophyta, Phaeophyceae) suggest different controlling mechanisms than the ones known to be present in higher plants. The extremely high NPQ [29] of this alga is blocked completely when Zea synthesis is chemically inhibited [30]. Further, when ΔpH is disrupted, NPQ dissipates slowly and this dissipation is related to the epoxidation of Zea [30]. It seems that the ΔpH in *M. pyrifera* does not influence NPQ beyond the activation of the VDE. This challenges the well-established idea that the ΔpH is obligatory for NPQ. Beside its physiological significance, a dissimilar control of NPQ among divergent groups of organisms would have profound evolutionary implications since it would suggest that one of the most important photosynthetic regulation mechanisms might have evolved differently in eukaryotic phototrophs. Here, by monitoring PSII Chl *a* fluorescence, a highly sensitive and non-invasive signal of photosynthesis and quenching processes in this system ([31–36]; also see [16,37], this issue), we have characterized the NPQ induction kinetics of *M. pyrifera* in different light acclimation conditions. We establish here that ΔpH does not control qE in this brown alga.

## 2. Materials and methods

### 2.1. Plant material

Samples of the brown alga *M. pyrifera* (L) C. Agardh were collected in a kelp forest near Ensenada (31°41.96 N; 116°40.90 W), Baja California, Mexico. Surface blades (acclimated to full sunlight) were collected early in the morning, tagged, kept in darkness and transported to the laboratory in 'coolers' with seawater. Tissue disks (1.2 cm diameter) were cut with a cork borer 10 cm above the pneumatocyst, along the central axis of each blade that was free of visible epiphytes. The disks were maintained in Erlenmeyer flasks with 200 mL of filtered seawater at constant temperature (17 ± 0.5 °C) with vigorous air bubbling. The experiments for NPQ induction and pigment quantification were performed the day of collection of the organisms. Samples of a higher plant *Ficus* sp. were collected from a garden of our institute, CICESE (*Centro de Investigación Científica y de Educación Superior de Ensenada*), early in the morning. After the collection of the *Ficus* sp. blades, and during the experiments, the petioles of the leaves were kept in water.

### 2.2. Non-photochemical quenching (NPQ) induction experiments

Photosystem II (PSII) Chl *a* emission was measured in tissue disks of *M. pyrifera* and in whole blades of *Ficus* sp., using a pulse amplitude modulated (PAM) fluorometer (Diving-PAM; Heinz Walz, Effeltrich, Germany). The *M. pyrifera* disks were maintained in a homemade acrylic temperature-controlled chamber (17 °C) with constant flow of seawater. PSII florescence was monitored by placing the PAM fiber optic at a 60° angle in relation to the light exposed side of the disks and this optical geometry was maintained during all the measurements. Both the organisms were dark-adapted for at least one hour before the measurement of PSII Chl *a* fluorescence changes during dark to light (1300 μmol quanta $m^{-2}$ $s^{-1}$) and light to dark transitions (Fig. 1A and B). Saturating light pulses were fired continuously every 30 s to measure the maximum florescence when all the PSII reaction centers were closed ($F_m$ or $F'_m$; without or with non-photochemical quenching, respectively). NPQ was calculated as $NPQ = (F_m - F'_m)/F'_m$.

The NPQ induction was also characterized in preilluminated (PRE) *M. pyrifera* (hereafter, $NPQ_{PRE}$) samples for different time periods. Dark-adapted disks were illuminated with high light (HL) (HL1; see Fig. 1A and B) for 0, 1, 3, 5, 10 and 15 min, then maintained in darkness for 5 min before exposing them again to high light (HL2; see Fig. 1A and B). The light intensity for both HL1 and HL2 was 1300 μmol quanta $m^{-2}$ $s^{-1}$. Alternatively, the first period of illumination was constant (10 min) and the subsequent period of darkness was varied from 1 to 15 min before the exposure of the samples to HL2. In parallel, three disks were exposed to the same light conditions and then they were used for pigment analysis. In both the protocols, the disks for pigment analysis were sampled before the HL2 exposure period.

### 2.3. Pigment measurement and determination of the rate of xanthophyll cycle pigment interconversion

Using High Performance Liquid Chromatography (HPLC), we measured the pigment content of the tissue, as described by Colombo-Pallotta et al. [29]. The rate of Zea accumulation was measured in blades, collected from the surface to 9 m depth, which had different concentrations of Anth before they were exposed to high light. These blades were acclimated to different light environmental conditions [29]. In addition, the rate of Zea accumulation was measured in dark-adapted *M. pyrifera* tissue disks obtained from the blades collected at the surface. These samples were preilluminated (HL1) for 3, 5, 8, 10 and 15 min. These disks were then maintained in darkness for 5 min before they were exposed again to high light (HL2). To characterize the accumulation of Zea in preilluminated samples, three disks were sampled at different time periods (0, 1, 3, 5, 10, 15 min) after exposure to HL2.

### 2.4. NPQ induction in the presence of dithiothreitol

$NPQ_{PRE}$ induction was measured in samples incubated with dithiothreitol (DTT) in darkness before exposing them to high light. Dark-adapted *M. pyrifera* tissue disks were exposed to HL1 for 10 min. Subsequently, the disks were immersed in a 1 mM DTT solution. The disks were kept in darkness for 10 min before exposing them again to high light conditions (HL2). PSII florescence in *Ficus* sp was measured in tissue disks treated in the same way as *M. pyrifera* disks, but here distilled water was used instead of filtered seawater. Control samples were treated in the same manner as the samples treated with DTT but they were incubated in darkness without the presence of the inhibitor in the water. Inhibition of Zea formation by DTT was confirmed by pigment quantification in the samples. Zea concentration did not increase in high light exposed samples of *Ficus* sp. and in *M. pyrifera* treated with DTT.

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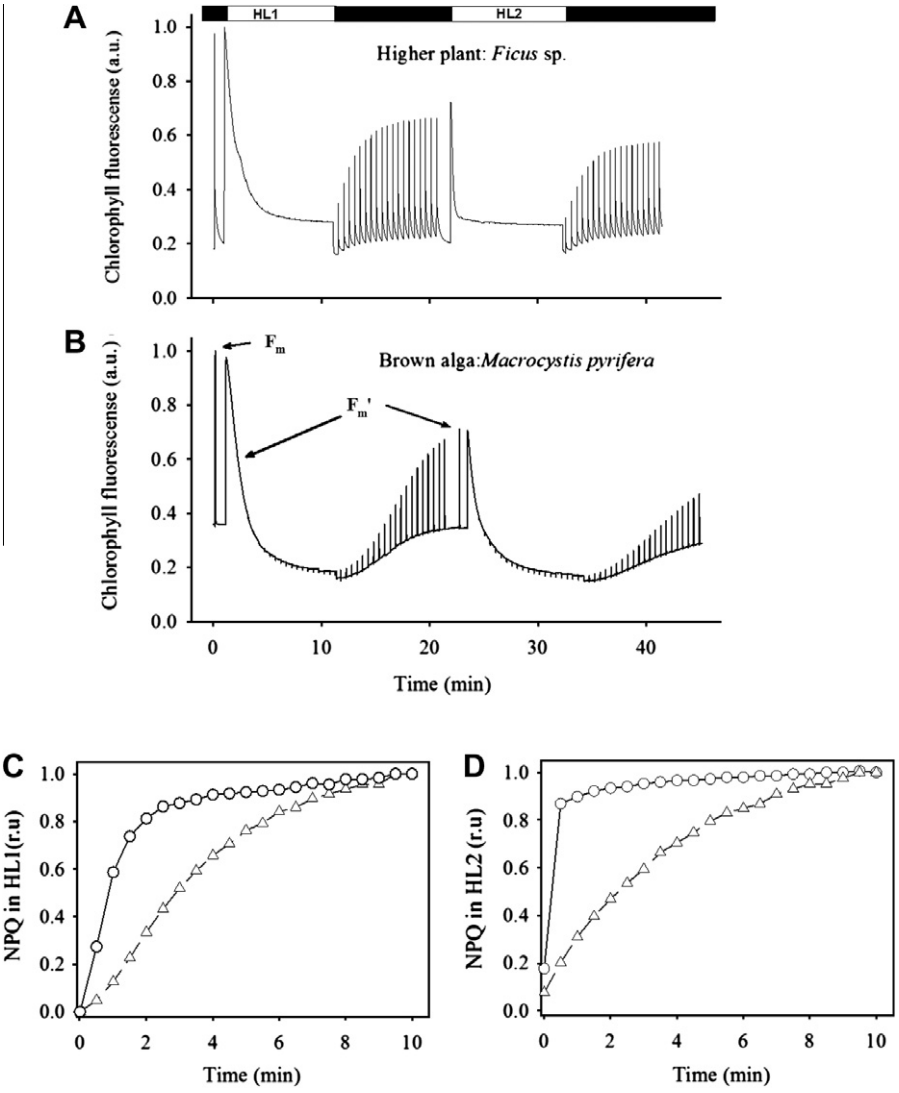


**Fig. 1.** Photosystem II (PSII) chlorophyll *a* florescence changes in *Ficus* sp. (a higher plant; Panel A) and *Macrocystis pyrifera* (a brown alga; Panel B) during dark to light and light to dark transitions. Dark rectangles represent dark periods, while white rectangles the high light (HL) periods (HL1 and HL2: 1300 μmol quanta $m^{-2}$ $s^{-1}$). Saturating light pulses were fired continuously every 30 s to measure the maximum florescence emission when all PSII reaction centers are closed ($F_m$ or $F'_m$ without or with non-photochemical quenching, NPQ). Panel C shows the relative (maximum normalized to one) non-photochemical quenching of fluorescence (NPQ = $F_m - F'_m/F'_m$) of dark-adapted samples during the HL1 period, and Panel D, the NPQ in samples exposed to HL2, (preilluminated samples). The NPQ in *Ficus* sp. is shown by circles and in *M. pyrifera* by triangles. Maximum NPQ of chlorophyll fluorescence was 4.5 in *M. pyrifera* samples and 2.3 in *Ficus* sp.

## 3. Results

### 3.1. NPQ induction and relaxation in M. pyrifera and Ficus sp

We observed important differences in PSII fluorescence changes between a brown alga *M. pyrifera* and a higher plant (e.g., *Ficus* sp.) exposed to high light (1300 μmol quanta $m^{-2}$ $s^{-1}$) and during light to dark transition (Fig. 1A and B). NPQ formation in dark-adapted samples ($NPQ_{DA}$) of *M. pyrifera* was approximately 3 times slower than in the *Ficus* sp. (Fig. 1C). However, *M. pyrifera* had higher NPQ (approximately 4.5) than *Ficus* sp. (approximately 2.3) after 10 min exposure to high light. The half-time ($t_{1/2}$) of $NPQ_{DA}$ induction in *M. pyrifera* blades collected from the surface was between 3.2 to 4.5 min ($n = 5$; Fig. 1C) while in *Ficus* sp. the $t_{1/2}$ was between 1.1 and 0 min ($n = 5$; Fig. 1C). Also, the relaxation of fluorescence quenching in darkness was slower in *M. pyrifera* than in *Ficus* sp. (Fig. 1A and B). The $t_{1/2}$ of NPQ relaxation of *M. pyrifera* in darkness was between 3.1 and 3.6 min.

The most important difference in NPQ induction between *M. pyrifera* and *Ficus* sp. was observed in samples that had been previously exposed to light (Fig. 1A and B). Preillumination accelerated significantly the formation of NPQ in *Ficus sp*. In this organism, significant increase in the rate of NPQ formation was observed in preilluminated samples and maximum $NPQ_{PRE}$ was reached within a minute upon illumination (Fig. 1C). The rapid $NPQ_{PRE}$ induction observed in *Ficus* sp. was not present in *M. pyrifera* (Fig. 1D). $NPQ_{DA}$ was quite similar to $NPQ_{PRE}$ in this brown alga (cf. Fig. 1C with Fig. 1D).

### 3.2. NPQ induction and Zea accumulation in M. pyrifera preilluminated samples

The increase in the rate of NPQ induction in preilluminated *Ficus* sp. could be associated with the presence of zeaxanthin formed during the previous light period, as has been observed in

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other higher plants [38]. We investigated if the presence of this pigment affects $NPQ_{PRE}$ formation in *M. pyrifera*. We characterized $NPQ_{PRE}$ in samples with different concentrations of Zea. To induce the formation of this pigment, *M. pyrifera* samples were exposed to different periods of illumination (HL1) followed by a dark period (5 min) before $NPQ_{PRE}$ was measured in high light (see Section 2 for details). Fig. 2 shows $NPQ_{PRE}$ induction in samples preilluminated for different time periods. Maximum $NPQ_{PRE}$ after 10 min in high light (HL2) was similar in all samples (Fig. 2A). Therefore, maximum $NPQ_{PRE}$ did not increase by the presence of preformed Zea before the light exposure (Fig. 2B). In contrast, the amount of NPQ detected in darkness before the measurement of $NPQ_{PRE}$ (at $t = 0$ in Fig. 2A) was related to the preillumination time period. NPQ as high as 4.2 was detected in samples exposed for 15 min to HL1 (Fig. 2A). The high NPQ detected in darkness just before the exposure of the samples to high light was associated with the amount of Zea detected at this time point (Fig. 2B). We found that longer the preilluminaton period, the higher amount of Zea was present in the tissue before the NPQ measurement. A linear relationship between NPQ and Zea concentration in darkness after high light exposure ($r^2 = 0.96$, $n = 28$; Fig. 2B) was found in samples exposed to two preillumination protocols (see Section 2. Material and Methods; Fig. 2B). The relationship between NPQ and the de-epoxidation state of the xanthophyll cycle pigments (Anth[0.5] + Zea/Vio + Anth + Zea) was not better than the NPQ and Zea relationship ($r^2 = 0.95$, $n = 28$).

The sustained NPQ in darkness observed in *M. pyrifera* could be attributed to the reduction of the plastoquinone pool [39]. Plastoquinone pool reduction during the dark-adaptation period might induce NPQ formation and Vio to Zea conversion [28]. Therefore, we tested if the dark reduction of PQ pool is present in *M. pyrifera*. We observed that there was no NPQ induction or Zea accumulation in light when PSII was inactivated with 20 μM 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) (data not shown). Furthermore, we found that illumination of *M. pyrifera* samples with far red light previous to high light exposure did not affect the rate and the extent of NPQ formation. There was no increase in the maximum fluorescence ($F_m$) or decrease of the minimum fluorescence ($F_o$) in the presence of far red light (data not shown). Therefore, reduction of plastoquinone pool independent of PSII activity was not present in this alga and no process that could induce NPQ or Zea accumulation in darkness was detected.

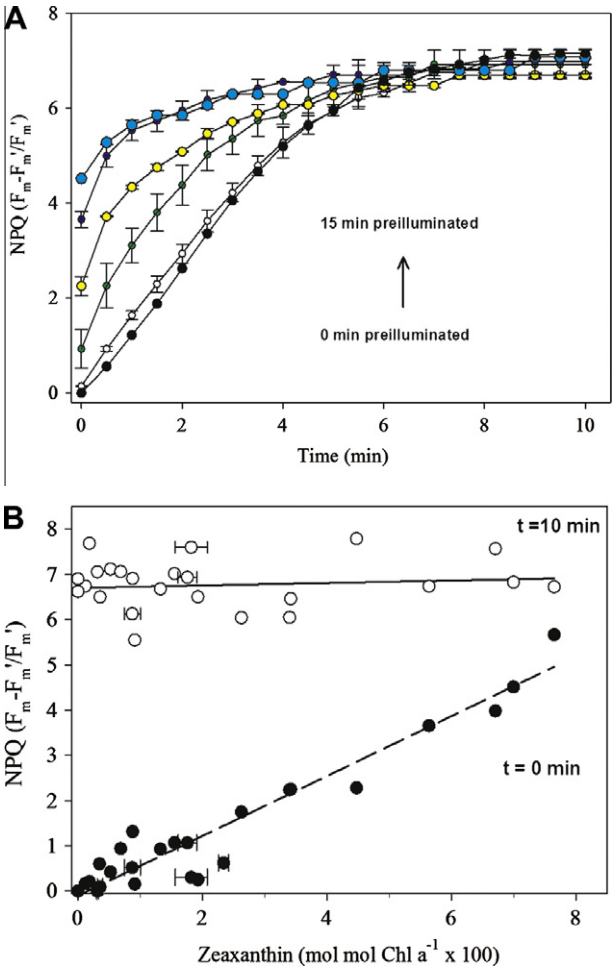


**Fig. 2.** Non-photochemical quenching of PSII fluorescence (NPQ) in *Macrocystis pyrifera* preilluminated samples. Panel A shows NPQ kinetics in high light (HL2, see Fig. 1) of samples that were previously exposed to HL1 (see Fig. 1) for 1–15 min and maintained in darkness for 5 min before the measurement of NPQ. Panel B shows NPQ in darkness ($t = 0$ min, dark circles) as a function of the zeaxanthin concentration present before the *M. pyrifera* samples were exposed to high light (HL2). Also, maximum NPQ induced in 10 min of exposure to HL2 is shown here ($t = 10$ min, empty circles). Panel B shows data from samples exposed to (1) HL1 for 1–15 min (cf. Panel A), followed by darkness for 5 min, or (2) to 10 min to HL1 followed by 1–15 min of darkness. NPQ and zeaxanthin were measured before HL2 was given. Both sets of measurements are pooled together and each point represents an independent measurement.

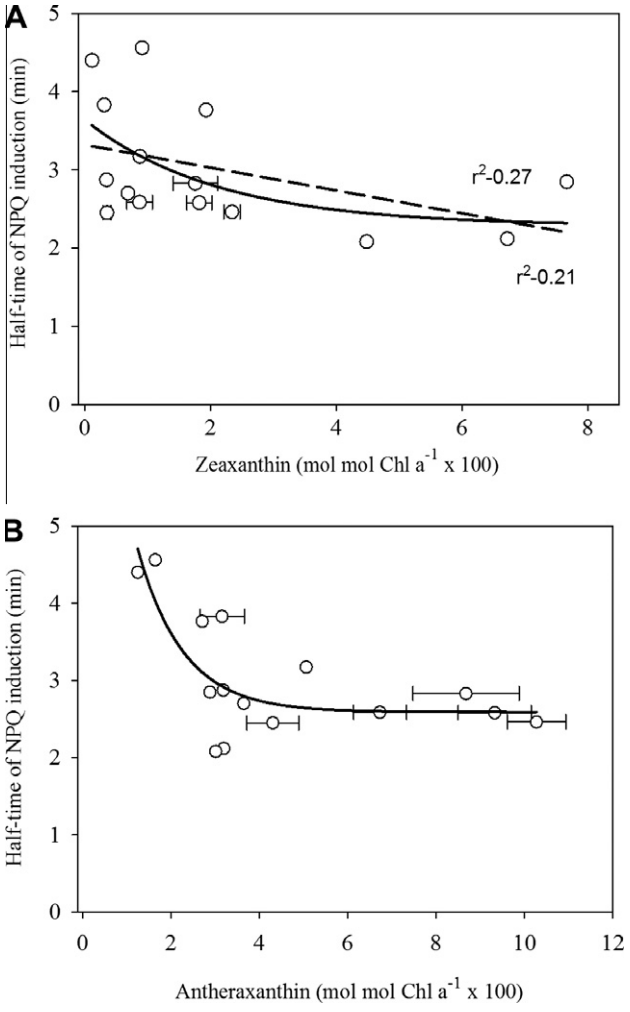


**Fig. 3.** The half-time ($t_{1/2}$) of non-photochemical quenching (NPQ) induction in preilluminated *Macrocystis pyrifera* samples as a function zeaxanthin (Panel A) and antheraxanthin (Panel B) concentration present before the exposure of the samples to high light. The continuous line represents the fit of data to an exponential model. The broken line represents the fit of the data to a linear model.

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In *M. pyrifera*, the rate of $NPQ_{PRE}$ formation was faster in preilluminated samples. The half-time ($t_{1/2}$ = 2.55 min ± 0.17 min, $n$ = 5) of $NPQ_{PRE}$ formation in samples preilluminated for 15 min was approximately 1.5 times lower than the $t_{1/2}$ observed in dark-adapted samples ($t_{1/2}$ = 3.89 min ± 0.15 min; $n$ = 5). The increase in the rate of NPQ formation was not associated with the Zea content of preilluminated samples (Fig. 3A) but to the amount of Anth present in these samples (ANOVA, $p$ = 0.006, $r^2$ = 0.74; Fig. 3B). There was a sharp decrease in the $t_{1/2}$ of $NPQ_{PRE}$ formation when the concentration of Anth increased from 0.018 to 0.03 mol Anth mol Chl $a^{-1}$ (Fig. 3B). At higher concentrations of Anth, no further decrease in the $t_{1/2}$ of $NPQ_{PRE}$ formation was observed (Fig. 3B).

We speculate that an increase in the rate of NPQ induction in preilluminated samples may be associated with an increase in the rate of Zea production when Anth is present. To test this, we measured the rate of Zea accumulation upon exposure to high light in samples that had different concentrations of Anth. The half-time of Zea accumulation was calculated in samples collected from the surface to 9 m in the original location (empty circles in Fig. 4). Also, the $t_{1/2}$ of Zea synthesis was measured in samples that were preilluminated for different time periods (3, 5, 8, 10 and 15 min; filled circles in Fig. 4), before de-epoxidation reaction was induced by high light (HL2). We found that Zea formation rate was related to the amount of Anth present before high light exposure. The $t_{1/2}$ of Zea accumulation decreased exponentially (ANOVA, $p$ = 0.0005, $r^2$ = 0.92) with the concentration of Anth before the de-epoxidation reaction was induced in high light (Fig. 4). The $t_{1/2}$ of Zea accumulation was approximately 8 min at the lowest Anth concentration (0.002 mol mol $Chla^{-1}$) detected in samples collected from 9 m depth. The half-time of Zea formation decreased to 2.16 min when Anth concentration was higher than 0.03 mol mol $Chla^{-1}$. These high Anth concentrations were detected in samples preilluminated for longer periods (from 8 to 15 min) before they were exposed to HL. The fastest Zea formation rate was not different ($t$-test; $p$ = 0.11) than the fastest rate of $NPQ_{PRE}$ induction ($t_{1/2}$ = 2.55 min) detected in the preilluminated samples. Therefore, the relationship between Anth and the increase in the rate of NPQ induction in preilluminated samples (c.f Fig. 3B) could be explained by the increase in the rate of Zea accumulation when Anth was present before high light exposure.

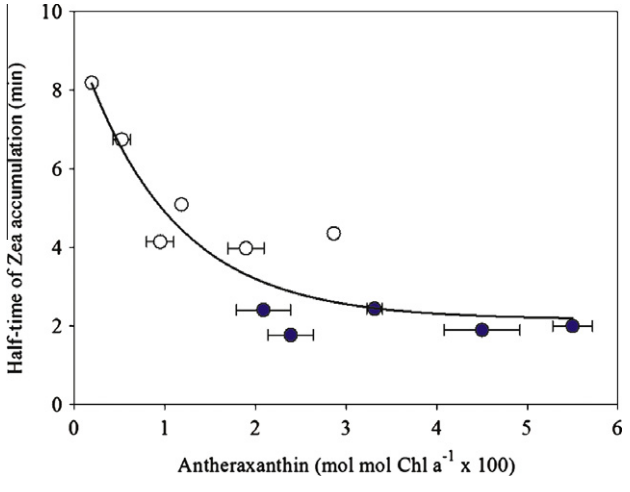


**Fig. 4.** The half-time ($t_{1/2}$) of Zeaxanthin (Zea) accumulation in *Macrocystis pyrifera* samples with different concentration of antheraxanthin before the exposure of the samples to high light. The $t_{1/2}$ of Zea accumulation was calculated in samples collected from the surface to 9 m depth (empty circles). Also, the $t_{1/2}$ of Zea synthesis was measured in samples that were preilluminated for different time periods (filled circles), before de-epoxidation reaction was induced by high light. The standard errors were less than 10% of the estimated $t_{1/2}$ of $NPQ_{PRE}$ formation and of Zeaxanthin synthesis. The continuous line represents the fit of data to an exponential model.

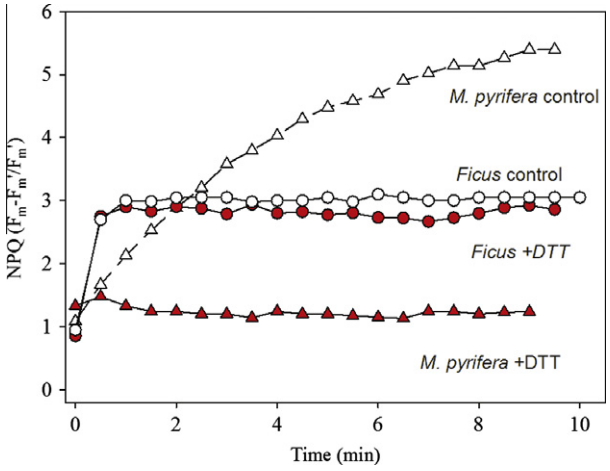


**Fig. 5.** Effect of dithiothreitol (DTT) on the non-photochemical quenching of fluorescence (NPQ) development in preilluminated *Macrocystis pyrifera* (triangles) and *Ficus* sp. (circles). Samples were illuminated for 10 min (HL1) and then incubated in darkness for 10 min with (solid symbols) or without (open symbols) 1 mM DTT. After this incubation period, the samples were exposed again to high light (HL2). Five independent experiments confirmed the results shown here.

### 3.3. NPQ induction in preilluminated samples in the presence of dithiothreitol

The dependence of NPQ formation on Zea accumulation in *M. pyrifera* was further tested in samples incubated with 1 mM dithiothreitol (DTT) that inactivates the violaxanthin de-epoxidase enzyme (VDE) [7]. The inhibition of Zea accumulation with DTT eliminated the increase of $NPQ_{PRE}$ in this alga (Fig. 5). $NPQ_{PRE}$ did not increase from the value detected in darkness before the exposure to high light. In contrast, DTT did not affect $NPQ_{PRE}$ induction in *Ficus* sp. (Fig. 5). In *M. pyrifera* there was a small rise in NPQ in DTT-treated samples. However, this rise was significantly smaller than the one observed in *Ficus sp.* (see Ficus + DTT in Fig. 5). In preilluminated plants, qE relies mainly on ΔpH and inhibition of the xanthophyll cycle does not affect $NPQ_{PRE}$ development. In contrast, in *M. pyrifera* the activity of the xanthophyll cycle is essential for the increase of NPQ even in the presence of preformed Zea. In higher plants, retention of Anth can accelerate ΔpH-dependent NPQ formation [26]. In *M. pyrifera* this pigment might contribute in some proportion to qE, but it is also by a ΔpH–independent mechanism. Anth was present at concentrations similar to Zea in the preilluminated samples. However, the lack of NPQ formation observed in DTT treated samples (Fig. 5) indicates that de-epoxidation of xanthophyll cycle pigments is needed for qE development in *M. pyrifera*.

## 4. Discussion

In a previous report [30] we suggested that the ΔpH does not control NPQ formation in *M. pyrifera* and this process activated only the accumulation of Zea. In this paper we confirmed that the NPQ in the brown alga *M. pyrifera* depends only on the activity of the xanthophyll cycle. The xanthophyll cycle of this brown alga appears to be similar to that of the higher plants; however, the control of qE in *M. pyrifera* is different: In contrast to known results on higher plants, ΔpH does not influence NPQ independently of the activation of the violaxanthin de-epoxidase enzyme.

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The kinetics of fluorescence changes showed that *M. pyrifera* has a slow induction of NPQ upon exposure to high light. Also, the fast NPQ relaxation phase that takes place in the first minutes after light to dark transition is absent in the alga. The slow NPQ induction and its relaxation kinetics (see Table 1 for comparison with available data on higher plants) indicate that *M. pyrifera* lacks the fast component of qE. In higher plants [38] and in green algae [40], the major proportion of NPQ is formed rapidly in high light and this dissipates almost immediately in darkness since qE is controlled by ΔpH. The importance of ΔpH on qE control is particularly evident in higher plants, examined thus far, that have been exposed previously to high light. Preillumination accelerates the formation of qE and high NPQ ($NPQ_{PRE}$) is reached within a minute upon illumination (see Fig. 1C) since the system is "light activated" [22]. Light activation implies that Zea is present (formed during the previous illumination period) in the PSII antenna and $NPQ_{PRE}$ depends mainly on ΔpH [41]. The fast $NPQ_{PRE}$ formation is associated with allosteric changes of specific LHCs caused by ΔpH [17,22,41]. Such activation appears to be absent in *M. pyrifera*.

If ΔpH control of qE is not present in *M. pyrifera*, then NPQ should be related only to xanthophyll cycle activity. Consequently, increases in NPQ must follow the accumulation of Zea upon illumination after any pre-acclimation condition(s). We have found that NPQ induction and its relaxation are dependent on the formation and the disappearance of Zea, respectively. The close connection of qE to xanthophyll cycle activity was supported by the observation that the sustained NPQ in darkness was related to the presence of Zea. A sustained NPQ in darkness could be associated with the dark reduction of plastoquinone pool (PQ) and the build-up of ΔpH [39]. For example, in diatoms, the reduction of $F_m$ in light in DCMU-treated cells indicates that chlororespiration could induce NPQ independent of PSII activity [39]. In these organisms, non-linear electron transport pathways might activate their xanthophyll cycle [28]; the formation of a proton gradient across the thylakoid membrane due to chlororespiration is strong enough to activate the diadinoxanthin de-epoxidase enzyme [28], and accumulation of diatoxanthin and NPQ induction could take place in darkness [42,43]. We suggest that in *M. pyrifera*, reduction of plastoquinone pool, independent of PSII activity, is not present since there was no NPQ induction or Zea accumulation in light when PSII was inactivated in DCMU treated samples. Another indication of the lack of PQ reduction independent of PSII activity is that no effects of far red light on the rate and extent of NPQ formation were observed (data not shown). Therefore, we conclude that NPQ in darkness is related only to the presence of Zea formed during the previous light exposure period.

To test the dependence of NPQ on xanthophyll cycle, the induction of this process was probed in samples with different concentrations of Zea. In dark-adapted *Arabidopsis thaliana*, the $t_{1/2}$ of NPQ development is 56 s, but it decreases to 8 s in samples preilluminated for 15 min [38]. The increase in the rate of NPQ formation was associated with the amount of Zea present before $NPQ_{PRE}$ was probed [38]. We found that in *M. pyrifera*, the rate of NPQ formation in preilluminated samples was only 1.5 times faster than in dark-adapted samples. The faster $NPQ_{PRE}$ induction was not related to the amount of Zea but to the Anth concentration present before the exposure of the samples to high light. It is known that the conversion of Anth to Zea is faster than of the Vio to Anth conversion

**Table 1**
Non-photochemical PSII fluorescence quenching, xantophyll pigment interconversion and antenna characteristics of *M. pyrifera* (a brown alga) compared to some higher plants. DTT = dithiothreitol; NPQ = non-photochemical quenching of chlorophyll fluorescence.

| | *Macrocystis pyrifera* | Higher plants |
|---|---|---|
| *NPQ kinetics* | | |
| Maximum NPQ | "Super high" = 6–10 | 3–4 |
| $t_{½}$ of NPQ development | Slow: 3–4 min | 1 min[a] |
| NPQ dissipation in darkness | Slow, depends on Zea epoxidation. | Fast, qE component dissipates in 0.5–1 min[a] |
| Increase in the rate of NPQ induction in preilluminated samples | 1.5 times | 8-fold: from 1 min to 8 s in *Arabidopsis thaliana*[a] |
| DTT effect on NPQ formation | Highly susceptible, no NPQ development | DTT blocks 30% of total NPQ induction[b] |
| Uncoupler effect: $NH_4Cl$ | Slow dissipation of NPQ: depends on Zea epoxidation | Disrupts NPQ immediately[c] |
| Uncouplers effect: Nigericin | Slow dissipation of NPQ: depends on Zea epoxidation | Disrupts NPQ immediately |
| *Xanthophyll cycle* | | |
| Type of xanthophyll cycle (XC) | Vio ↔ Anth ↔ Zea | Vio ↔ Anth ↔ Zea |
| Xanthophyll cycle pigment pool (ΣXC) | Large: 0.25 mol mol $Chla^{-1}$ | Small: 0.05 mol mol $Chla^{-1}$[,b] |
| De-epoxidation reaction, $t½$ | $t½$ = 3–4 min | $t½$ = 1–3 min[a,d] |
| Epoxidation reaction | $t½$ = 3.5–5 min | $t½$ = 9–14 min[e] |
| Major carotenoid pigment | Fucoxanthin | Lutein |
| Accessory Chl pigments | Chla; chl *c* | Chla; chl *b* |
| *Antenna characteristics* | | |
| Minor antennas | CP 24 and 26 are nor present | CP 24 and 26 important for NPQ[f,g] |
| PsbS | Not present | Key element for NPQ[h] |
| LHCRS (LI818) | Present[j] | Not present[i,j] |
| Structural organization | Large oligomeric structure | Several subunits |
| State transitions | Not present | Present: disassociation of specific LHCIIb subunits |

[a] Johnson et al. [38].
[b] Pfündel and Bilger [7].
[c] Ruban et al. [25].
[d] Jahns [59].
[e] Nikens et al. [58].
[f] Horton et al. [22].
[g] de Bianchi et al. [17].
[h] Li et al. [19].
[i] Peers et al. [55].
[j] Dittami et al. [50].

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[44]. The release of Vio from its binding site and the subsequent diffusion into the thylakoid membrane are the rate limiting steps for the de-epoxidation reaction of the xanthophyll cycle [44]. Therefore, Anth to Zea conversion could be twofold faster than the Vio to Anth reaction [45]. We have found that the rate of Zea accumulation increases when Anth is present in preilluminated samples or in high light acclimated organisms. Therefore, the presence of Anth in the antenna system of *M. pyrifera* accelerates the accumulation of Zea and as consequence the rate of NPQ induction.

In the presence of preformed Zea or Anth, NPQ did not increase in high light if the xanthophyll cycle de-epoxidation reaction was inhibited. Since *M. pyrifera* does not show a fast induction and relaxation of NPQ, and the xanthophyll cycle pigment interconversion is the only factor that affects this process, we suggest that LHCs allosteric changes, related to qE induction, are not present in this alga. Pigment content and different absorption characteristics might have resulted in different light-absorbed dose dependence between *Ficus* sp. and *M. pyrifera* However, the light intensity used in our experiments was high enough to induce saturation of photosynthetic activity, NPQ and xanthophyll cycle activity. Also, we used a light intensity that is more than 5 times the saturating light intensity for photosynthesis in *M. pyrifera* [29]. However, NPQ induction has to be characterized at different light intensities to investigate if a Zea-independent qE component exists in this alga under different illumination conditions.

Although the exact molecular details of qE control in higher plants have not been fully elucidated, it is clear that specific PSII light harvesting complexes (LHCII) and conformational changes in the PSII–LHCII supercomplex are essential for the induction of this process [17,22]. Mutagenic analyses have shown that Lhcb4 (CP29), Lhcb5 (CP26), and probably Lhcb6 (CP24) are important for qE development [23,46]. After reorganization of PSII–LHCII, PSII supercomplex, quenching is suggested to occur in CP29 and CP26, whereas quenching of a detached part of the LHCII antenna (a pentameric complex) is by CP24 [47]. This could take place either by a charge-transfer process between a Chl *a*-carotenoid pair [48] or by direct transfer of energy from the lowest excited state of Chl *a* to an excited state of lutein or zeaxanthin [24,25]. Therefore, heat dissipation in the photosynthetic apparatus is a property of the PS II supercomplex [17], in which conformational changes, associated probably with PsbS and $\Delta$pH, might stabilize the dissipative state of the antenna of PSII [48].

The higher plant model of qE control seems not to apply to *M. pyrifera*. The higher plant model of qE control has been postulated after more than three decades of work performed on this group of organisms. Models have been postulated after the investigation of the qE process in different species, different approximations and by using genetically modified organisms. We used *Ficus* sp. as a species representative of higher plants. The florescence kinetics and changes in NPQ follows the higher plants model in this species. With respect to brown algae, much less work has been performed on this group of phototrops. Characterization of NPQ controlling mechanisms in brown algae is minimal, and further work is needed. However, it is well known that antenna apoprotein composition of brown algae is different from that of higher plants and they do have different evolutionary origin. Therefore, extrapolation of antenna characteristics described in other organisms of the same group as *M. pyrifera* has allowed us to postulate that the different antenna arrangement might influence qE differently in higher plants and in brown algae.

Brown algae do not have homologous chlorophyll a/b binding proteins (CABs); thus, CP24, CP29 and CP26 are not present in this group of phototrophic organisms [49–51]. Also, PsbS is not present in brown algae [49]. Furthermore, antenna macrodomain arrangement differs notably between higher plants and brown algae, examined thus far (see Table 1). The fucoxanthin-binding proteins (FCPs) in brown algae are assembled in relatively large oligomers that are not organized differentially in association with either PSI or PSII [52,53]; thus, these systems share the same antenna [49]. Probably, PSII antenna of brown algae lacks the flexibility (disassociation or allosteric changes) that permits a rapid rearrangement of the system in response to variations of light conditions; thus, NPQ must rely on the accumulation of photoprotective pigments through the xanthophyll cycle.

Given that CABs are not present in brown algae, photoprotective pigments must be associated with proteins different from those which play a role in qE control in higher plants. The *Ectocarpus siliculosus* genome (first brown alga genome sequenced) codes for a total of 53 chlorophyll binding proteins (CBPs) representing different families [54]. Thirteen copies belong to the light harvesting stress response protein family (LHCSR) that were formerly known as LI818 or LI818-like proteins [50]. Peers et al. [55] have reported that a LHCSR protein is essential for qE in the green alga *Chlamydomonas reinhardtii*. Moreover, in the diatom *Cyclotella meneghiniana*, chromoproteins of this family have been suggested to bind diadinoxanthin and diatoxanthin [56], and to be involved in the quenching of fluorescence [57]. LHCSR proteins are poorly represented within the green lineage, but numerous proteins of this family are present in haptophytes and heterokonts, which indicate that probably they are important for the response to stress conditions in these organisms [50]. Furthermore, these proteins probably evolved a function different from that of light harvesting and they may have appeared in an ancestral Chl *c* fucoxanthin-containing organism and were possibly acquired by green algae later [50]. The role of these proteins in photoprotection related to the different control of qE observed in *M. pyrifera* requires further investigation. For example, *M. pyrifera* that might posses several copies of the LHCSR proteins (as observed in *E. siliculosus* [50]) does not show the fast component of qE. In contrast, the lack of LHCSR in mutants of *C. reinhardtii* [55] and in the moss *Physcomitrella patens* [58] affects not only the slow phase but also the fast phase of NPQ induction. Comparative analysis between distant evolutionary organisms might help to decipher the molecular mechanisms of LHCSR role on qE development that might represent an ancient response to high light [58].

There are important evolutionary implications of the differential control of qE between higher plants (see e.g., Nilkens et al. [59]; Jahns [60]) and brown algae (this paper) (Table 1). Green [61] has suggested that the evolution of antenna systems must be intimately connected with the evolution of mechanisms for getting rid of excess excitation energy in the photosynthetic apparatus. Thus, it is reasonable to suggest that dissipation of heat in the photosynthetic apparatus must differ between organisms with different antenna arrangement even if they have a similar xanthophyll cycle, as demonstrated here in this paper. The xanthophyll cycle is highly conserved since it is present in 9 out of the 11 divisions of eukaryotic phototrophs. We believe that the xanthophyll cycle and its related photoprotective role must have been tightly coupled to the evolution of the antenna systems of the different groups of eukaryotic phototrophs. Light harvesting antenna families must have evolved independently in green and red algae lineages [50,62]. After the disappearance of the phycobilisomes (PBS; ancient antenna associated with PSII), divergence of PSI and PSII type antennae must have taken place early in the evolution from the green lineage [62]. In contrast, red algae may have maintained their PBS antenna complex, where its loss occurred after a secondary endosymbiotic event and a duplication of the PSI antenna polypeptide *Lhca* gene to form the PSII antenna of Chromophytes [58,62]. Probably, early diversification of antenna function allowed the members of the green lineage to have a greater flexibility and specialization of their LHCs in photoprotection. We postulate that xanthophyll cycle appeared before the

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separation of the green and red algae lineages and LHCs evolutionary events must have influenced the mechanisms of photoprotection related to this cycle.

## 5. Abbreviations

| | |
|---|---|
| Anth | antheraxanthin |
| Chl | chlorophyll |
| DA | dark-adapted sample |
| DCMU | 3-(3,4-dichlorophenyl)-1,1-dimethylurea |
| DTT | dithiothreitol |
| $F_m$ | maximum fluorescence in dark-adapted samples |
| $F'_m$ | maximum fluorescence in light-adapted samples |
| LHCs | harvesting antenna complexes |
| NPQ | non-photochemical quenching of chlorophyll a fluorescence (measured as $(F_m–F'_m)/F'_m$, where $F_m$ is maximum fluorescence in dark and $F'_m$ is maximum fluorescence in light) |
| PRE | preilluminated sample |
| PSII | Photosystem II |
| PSI | Photosystem I |
| qE | energy-dependent quenching of chlorophyll fluorescence |
| VDE | violaxanthin de-epoxidase enzyme |
| Vio | violaxanthin |
| Zea | zeaxanthin |

## Acknowledgements

This work was supported by the SEP-CONACYT project CB-2005-01-49615. O-A received a scholarship from CONACyT, and project SEP-CONACYT CB-2005-01-49615 for his Ph.D. studies. Also, the support of CICESE postgraduate department is acknowledged. Govindjee thanks the Department of Plant Biology of the University of Illinois at Urbana-Champaign for their continual support. We thank Dr. Jose I. Garcia-Plazaola for discussing ideas related to the manuscript and for his comments on our paper. We thank also three anonymous reviewers for their suggestions that have improved our paper.

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Please cite this article in press as: E. Garcia-Mendoza et al., Photoprotection in the brown alga *Macrocystis pyrifera*: Evolutionary implications, J. Photochem. Photobiol. B: Biol. (2011), doi:10.1016/j.jphotobiol.2011.04.004