

# Kinetic study of the plastoquinone pool availability correlated with H<sub>2</sub>O<sub>2</sub> release in seawater and antioxidant responses in the red alga *Kappaphycus alvarezii* exposed to single or combined high light, chilling and chemical stresses

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## Abstract

Under biotic/abiotic stresses, the red alga *Kappaphycus alvarezii* reportedly releases massive amounts of H<sub>2</sub>O<sub>2</sub> into the surrounding seawater. As an essential redox signal, the role of chloroplast-originated H<sub>2</sub>O<sub>2</sub> in the orchestration of overall antioxidant responses in algal species has thus been questioned. This work purported to study the kinetic decay profiles of the redox-sensitive plastoquinone pool correlated to H<sub>2</sub>O<sub>2</sub> release in seawater, parameters of oxidative lesions and antioxidant enzyme activities in the red alga *Kappaphycus alvarezii* under the single or combined effects of high light, low temperature, and sub-lethal doses of 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) and 2,5-dibromo-3-methyl-6-isopropyl-p-benzoquinone (DBMIB), which are inhibitors of the thylakoid electron transport system. Within 24 h, high light and chilling stresses distinctly affected the availability of the PQ pool for photosynthesis, following Gaussian and exponential kinetic profiles, respectively, whereas combined stimuli were mostly reflected in exponential decays. No significant correlation was found in a comparison of the PQ pool levels after 24 h with either catalase (CAT) or ascorbate peroxidase (APX) activities, although the H<sub>2</sub>O<sub>2</sub> concentration in seawater ( $R=0.673$ ), total superoxide dismutase activity ( $R=0.689$ ), and particularly indexes of protein ( $R=0.869$ ) and lipid oxidation ( $R=0.864$ ), were moderately correlated. These data suggest that the release of H<sub>2</sub>O<sub>2</sub> from plastids into seawater possibly impaired efficient and immediate responses of pivotal H<sub>2</sub>O<sub>2</sub>-scavenging activities of CAT and APX in the red alga *K. alvarezii*, culminating in short-term exacerbated levels of protein and lipid oxidation. These facts provided a molecular basis for the recognized limited resistance of the red alga *K. alvarezii* under unfavorable conditions, especially under chilling stress.

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**Keywords:** Plastoquinone pool; Hydrogen peroxide; Alga; Oxidative stress; Antioxidant; *Kappaphycus alvarezii*

**Abbreviations:** APX, ascorbate peroxidase; ASW, artificial seawater; CAT, catalase; CD, conjugated dienes; CHL a, chlorophyll a; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; DBMIB, 2,5-dibromo-3-methyl-6-isopropyl-p-benzoquinone; DTNB, 5,5'-dithio-bis(2-nitrobenzoic acid);  $F_0$ , basal fluorescence of a dark-acclimated alga;  $F_m$ , maximal fluorescence of a dark-acclimated alga; FW, fresh weight; GSH, glutathione; HL, high light treatment; LL, low light treatment; PAR, photosynthesizing active radiation; PQ pool, plastoquinone pool; PQH<sub>2</sub>, reduced plastoquinone; PS I, photosystem I; PS II, photosystem II; ROS, reactive oxygen species; SOD, superoxide dismutase; TBARS, thiobarbituric acid reactive substances

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## 1. Introduction

Wild and farmed crops of red algal species *Kappaphycus alvarezii* (Doty) Doty and *Eucheuma denticulatum* are primary sources of a commercially valuable family of anionic hydrocolloids called carrageenans [1]. In the food industry, structural isomeric forms of kappa- and iota-carrageenans are widely used as gelling, stabilizing and viscosity-building agents (thickeners) for the preparation of several products, including chocolate-flavored milk, frozen desserts, soymilk, cottage cheese dressings

and some diet products [2]. The worldwide demand for carrageenan has recently increased, encouraging the commercial production and cultivation of *K. alvarezii* and other carrageenophytes outside their region of origin [3]. While the role of redox-sensitive molecules in subcellular communications of cultivated plants under adverse environmental conditions has attracted the attention of many plant physiologists for years, the study of the adaptive mechanisms mediated by reactive oxygen species (ROS) in economically valuable algae is still only marginally explored [4,5].

In terrestrial plants, photo- and thermoacclimation appear to be triggered by fluctuations in the redox state of the plastoquinone pool (PQ pool) within chloroplasts [6,7], with putative coadjutant participation of hydrogen peroxide ( $\text{H}_2\text{O}_2$ ), ascorbate and glutathione pools [8]. Low-scale accumulation of chloroplast-originated  $\text{H}_2\text{O}_2$  within plant tissues is postulated as a crucial event in initiating adequate antioxidant responses in higher plants [9]. In contrast, red seaweeds of the genus *Kappaphycus* and *Euclima* exposed to abiotic/biotic stresses apparently release  $\text{H}_2\text{O}_2$  into seawater (by a proper mechanism or simple diffusion) as a primary response to prevent subsequent oxidative lesions provoked by fast and massive production of peroxide within algal tissues [10,11]. Such a mechanism – unfeasible for terrestrial plants – was suggested to explain the apparent late onset of the induction of the  $\text{H}_2\text{O}_2$ -scavenging enzymes catalase (CAT) and ascorbate peroxidase (APX) when *K. alvarezii* cultures were treated with clofibrate, a xenobiotic that induces  $\text{H}_2\text{O}_2$  production in peroxisomes [12]. In contrast, significantly higher activities of total superoxide dismutase (SOD) were immediately observed within 4 h of clofibrate addition. This particular feature has raised questions concerning the role and efficiency of  $\text{H}_2\text{O}_2$  as a redox signal in aquatic photosynthetic organisms.

Thus, this work involved a study of the correlations between redox-sensitive PQ pool (as a parameter of ROS overproduction within chloroplasts), the  $\text{H}_2\text{O}_2$  concentration in seawater, indexes of oxidative lesions (conjugated dienes and thiol group measurements) and antioxidant enzyme activities (total SOD, CAT and APX) in the red alga *Kappaphycus alvarezii* in response to single or combined effects of high light, low temperature, and sub-lethal doses of 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) and 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone (DBMIB), which are blockers of the thylakoid electron transport system. The use of DCMU and DBMIB in combination with high light and/or chilling stresses allows the redox state of the PQ pool to be artificially modified at different time-dependent levels [13].

## 2. Materials and methods

### 2.1. Chemicals

All the chemicals were purchased from Sigma-Aldrich (Sweden AB), except for  $\text{FeSO}_4 \cdot 12\text{H}_2\text{O}$ ,  $\text{FeCl}_3$ , NaOH, HCl,  $\text{HClO}_4$ , ethanol, and ethyl acetate from Merck Co. (Darmstadt, Germany), and ascorbic acid and Perdrogen® (30%  $\text{H}_2\text{O}_2$  solution) from Riedel-deHaën Laborchemikalien (Seelze, Germany).

### 2.2. Algal stock material

The red algae specimens were clones of *Kappaphycus alvarezii* (Doty) Doty ex Silva originally from the Philippines, which were first cultivated in the laboratory by Prof. E.J. Paula (University of São Paulo) in Brazil and subsequently used in field cultivations in Ubatuba (SP, Brazil). Stock cultures of tetrasporophytes of a brown variant of *K. alvarezii* originating from Ubatuba crops were grown in a reserved laboratory at the Botany Institute, Stockholm University, Sweden. The algae were cultured in sterile seawater, 33 psu at  $(24 \pm 2)^\circ\text{C}$  and supplied with bubbling atmospheric air for at least 2 months prior to the experiments. The cultures were irradiated with white fluorescent light ( $55 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ ) in a light:dark (L:D) cycle of 12:12 h. The seawater medium (Guillard f/2) was changed every 2 weeks during the maintenance of cultivars in the laboratory and was replaced by freshly prepared f/2 medium 48 h before the experiments [14].

For PQ pool estimations (by chlorophyll fluorescence techniques), open field cultivars from Ubatuba were collected 5 days before the experiment, cleaned, blotted dry, weighted on the day of collection and kept in 2.8 L Erlenmeyer flasks filled with 2 L of autoclaved natural seawater under constant temperature and irradiance conditions ( $26^\circ\text{C}$ ,  $50 \mu\text{mol photon. m}^{-2} \text{s}^{-1}$ ). In order to provide gradual adaptation of natural grown algae to the experimental conditions, the composition of the cultivation medium was daily changed from autoclaved natural seawater (ASW) to Guillard f/2 medium, following the ratios (ASW:Guillard f/2): day 1—(80:20); day 2—(60:40); day 3—(40:60); day 4—(20:80); and day 5—(0:100).

### 2.3. Light and temperature conditions

Algal cultures (1.5 g FW in 100 mL medium) were placed in two temperature-controlled chambers maintained at  $26^\circ\text{C}$  and  $12^\circ\text{C}$ , in which two isolated benches were constructed to allow two different intensities of light exposure:  $(46 \pm 11) \mu\text{mol photon m}^{-2} \text{s}^{-1}$  (low light; LL) and  $(504 \pm 13) \mu\text{mol photon m}^{-2} \text{s}^{-1}$  (high light; HL). Both chambers were equipped with high-capacity air-circulating systems, which efficiently prevented temperature oscillations due to the proximity of the fluorescent lamps to the culture flasks or irregular airflow inside the room. The light intensity in each experimental flask was checked with a portable luminometer to correct the predetermined irradiances. To avoid systemic errors in the procedure, each replicate flask was randomly repositioned on the bench during the experiment (72 h), respecting the designated light treatment ( $50$  or  $500 \mu\text{mol photon m}^{-2} \text{s}^{-1}$ ).

For PQ pool evaluation, experimental groups were exposed to low or high irradiances in temperature-controlled incubators ( $26^\circ\text{C}$  or  $12^\circ\text{C}$ ) upon the adjustment of flasks' distance from the lamps and/or covering them with neutral black meshes (50% irradiance reduction). The average irradiances for LL and HL incubations measured with a Li-Cor LI-189 quantummeter with a spherical quantum sensor LI-193 SA were  $(54.3 \pm 4.8) \mu\text{mol photon m}^{-2} \text{s}^{-1}$  and  $(505 \pm 34) \mu\text{mol photon m}^{-2} \text{s}^{-1}$ , respectively.

### 2.4. Treatment with inhibitors of the thylakoid electron transfer system

To disrupt electron transfer within thylakoid membranes, single-sublethal doses of 3'-(3,4-dichlorophenyl)-1',1'-dimethyl urea (DCMU) or 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone (DBMIB) were added to the algal cultures under light and/or temperature treatments at time zero ( $t_0$ ). The inhibitors were administered directly to the culture seawater from stock solutions prepared in acetone and methanol, respectively, and then diluted in sterile seawater to final concentrations of  $0.05 \mu\text{M}$  DCMU and  $8 \mu\text{M}$  DBMIB, as suggested by previous studies and colleagues (Chow, F. personal communication) [14,15].

### 2.5. Determination of hydrogen peroxide in seawater

Total concentrations of  $\text{H}_2\text{O}_2$  released in seawater were evaluated by integrating the area under the kinetic curve of  $\text{H}_2\text{O}_2$  release from time zero ( $t_0$ ) – when light, temperature and/or inhibitor treatments were initiated – to 8 h ( $t_{8 \text{ h}}$ ). Briefly, a stock solution of luminol (5-amino-2,3-dihydro-1,4-phthalazinedione) was prepared by dissolving the fluorescent compound in 1 M NaOH and then

mixing with 0.4 M MOPS (3-[*N*-morpholino]propanesulfonic acid), pH 7.0, to a final volume of 10 mL. Then, luminol (5  $\mu$ M) and horseradish peroxidase (HRP; 11  $\mu$ kat mL<sup>-1</sup> in 0.1 M phosphate buffer pH 7.0) were mixed and added to 1.0 mL of seawater sample. A standard curve for H<sub>2</sub>O<sub>2</sub> was prepared under the same experimental conditions by diluting 30% H<sub>2</sub>O<sub>2</sub> (Peridrol®) in seawater, as described by Glazener et al. [16] with the modifications reported by Collén and Pedersén [11]. Luminol-chemiluminescence was measured using an LKB Wallace 1250 luminometer and a flatbed recorder and final results were normalized to biomass as  $\mu$ M H<sub>2</sub>O<sub>2</sub>(g FW)<sup>-1</sup>.

## 2.6. Crude extracts

The crude extracts were prepared by grinding 1.5 g wet weight of algae (stored frozen at -86 °C) to powder in a mortar in liquid N<sub>2</sub> and then homogenizing it in 3 mL of the assay-specific extraction buffer by sonication in an ice-water bath for 3 min, 70% duty cycle in a Cole-Parmer Model 4710 Sonicator. For the determination of total SOD and CAT activities, the extraction buffer was composed of 0.10 M potassium phosphate buffer, pH 7.4, containing 1 mM dithiothreitol and 0.1 mM EDTA. For the APX analysis, the pulverized samples were homogenized in 3 mL of 50 mM phosphate buffer, pH 7.0, with 10% polyvinylpyrrolidone (PVP-40), 0.25% Triton X-100, and 0.5 mM ascorbate. To determine the protein-oxidized products, cell debris was removed from all crude homogenates (prepared with 0.1 M phosphate buffer, pH 7.4) by centrifugation at 12,000×g for 10 min at 4 °C and the supernatant used for each specific analysis. The preparation of samples for chlorophyll *a* (CHL *a*) and conjugated dienes (CD) determinations followed the procedures described above, but used 3 mL of pure methanol as the extracting solvent.

## 2.7. Antioxidant enzyme determinations

Total SOD activity was measured by the indirect cytochrome *c* (cyt. *c*) method, in which a constant flux of O<sub>2</sub><sup>•-</sup> is generated by the xanthine/xanthine oxidase system and promptly scavenged by SOD present in the sample [17]. To check for O<sub>2</sub><sup>•-</sup> consumption rates in the system, the reduction of cyt. *c* was monitored by spectrophotometry at 550 nm for 3 min (25 °C), using various amounts of the supernatant compared to a blank system (in the absence of sample). One SOD unit is defined here as the enzyme concentration required for 50% inhibition of cyt *c* reduction at 25 °C. CAT activity was measured by following the absorbance decay of H<sub>2</sub>O<sub>2</sub> at 240 nm and 30 °C [18]. APX was evaluated as described by Nakano and Asada [19]. Briefly, the reaction was triggered by the addition of 0.1 mM H<sub>2</sub>O<sub>2</sub> to the solution containing 5–10% (v/v) sample, 0.1 mM EDTA and 0.5 mM ascorbate in 50 mM phosphate buffer, pH 7.0. Ascorbate consumption was monitored by spectrophotometry at 290 nm (25 °C), with a molar extinction coefficient of  $\epsilon=2.8$  mM<sup>-1</sup> cm<sup>-1</sup>.

## 2.8. Parameters of oxidative lesions

The content of thiol groups was quantified as indexes of protein oxidation in the total protein fraction, which was isolated from crude homogenate by precipitation with 20% trichloroacetic acid solution in ice. After washing once with 0.3 M HClO<sub>4</sub>, 5 mM EDTA and 0.06% bipyridine solution and twice with a 1:1 ethyl acetate:ethanol (v/v) mixture, the protein precipitate for thiol assay was dried in a DNA plus/Maxi Dry Heto® System to remove traces of organic solvent. The pellet was then dissolved in 500  $\mu$ L 6 M guanidine-HCl and reduced thiol groups were detected by the formation of colored adducts upon reaction with 2 mM 5,5'-dithio-bis(2-nitrobenzoic acid) solution (DTNB). A control treatment with 10 mM *N*-ethylmaleimide solution – a specific thiol-blocking compound – was performed to discount non-specific reaction of DTNB with other organic groups present in the samples. The absorbance of DTNB-treated samples at 412 nm was calculated using reduced glutathione (GSH) as the standard [20].

In order to evaluate the extension of lipid peroxidation in algal cells, conjugated dienes levels (CD) were measured in methanol homogenates by UV-visible spectrophotometry. Methanol extracts were treated with 0.1% NaCl solution to remove residual hydrophilic substances that could interfere with the UV absorption. The organic fraction was totally evaporated under a N<sub>2</sub> flow for 5 min and under vacuum for an additional 10 min (in the dark), leaving a lipid

film adhering to the inner glass surface of the round-bottom flask. The lipid film was dissolved in isoctane for absorbance measurements of CD absorption at 234 nm. Conjugated dienes concentration (normalized to chlorophyll *a* content) was calculated using a molar extinction coefficient of 26,000 M<sup>-1</sup>cm<sup>-1</sup> [21].

## 2.9. Chlorophyll *a* and protein determinations

CHL *a* was measured, as described by Mackinney [22], on 500  $\mu$ L methanol-extracted samples prepared as described earlier. The CHL *a* concentrations were estimated by measuring the corrected absorbance at 665 nm (subtracting the absorbance measured at 750 nm), using the mass extinction coefficient of  $\epsilon=27.6$   $\mu$ g chlorophyll *a*<sup>-1</sup> cm<sup>-1</sup>. Protein concentrations were estimated by the method of Lowry et al. [23] using bovine serum albumin as standard.

## 2.10. Size estimation of the plastoquinone pool

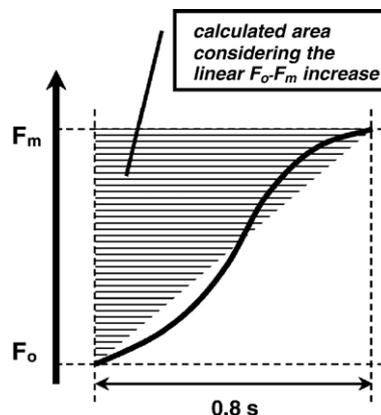
Chlorophyll fluorescence was measured using a Walz Diving-PAM underwater fluorometer (Walz, Effeltrich, Germany), at Prof. Orlando Necchi laboratory, Dept. of Botany, Universidade Estadual Paulista (Unesp), São José do Rio Preto, SP, Brazil. Apices of algal thalli were placed directly on the tip of the fluorometer fiberoptic using the magnet sample holder supplied. Rapid light curves [24] were generated and consisted of the fluorescence responses to eight increasing photosynthesis active radiation levels within the range of 0–690  $\mu$ mol photon m<sup>-2</sup> s<sup>-1</sup>. This device used a halogen lamp to provide actinic and saturating-pulse light. The exposure time at each PAR level was 15 s, each separated by a 0.8 s saturating flash ( $\sim 6000$   $\mu$ mol photon m<sup>-2</sup> s<sup>-1</sup>). The Diving-PAM data were processed using the WinControl software supplied.

The transition from modulated fluorescence of a dark-acclimated sample ( $F_o$ ) to maximal fluorescence ( $F_m$ ) is mostly observed due to the net decrease in the concentration of the quencher (plasto)quinone A ( $Q_A$ ) and, thus, the net accumulation of reduced (plasto)quinol ( $Q_A^{\bullet-}$ ). The values of  $F_o$  and  $F_m$  applied in PQ pool calculations were exclusively obtained from a single light intensity – from the total of 8 provided by the equipment during the light-curves analysis – which was the highest irradiance that did not result in photoinhibition in the algae. Thus, the area over a hypothetical linear increase of fluorescence (the difference between  $F_o$  and  $F_m$ ) was calculated after the saturating pulse of 0.8 s. The estimated area over the CHL fluorescence rise curve has been accurately used to measure the availability of the plastoquinone (PQ) pool as an electron acceptor of PSII [25,26]. The PQ pool estimation is illustrated by Scheme 1 and was calculated by (Eq. (1)):

$$\text{PQ pool (area)} = [(F_m - F_o) \times 0.8 \text{ s}] / 2 \quad (1)$$

where:  $F_o$ =basal fluorescence of a dark-acclimated sample,  $F_m$ =maximal fluorescence of a dark-acclimated sample, assuming that  $F_m$  (from initial  $F_o$ ) was fully obtained after the light saturating pulse (0.8 s).

In the sake of simplicity, the data here reported will be presented as the PQ pool size or availability, rather than specifying 'predictable' redox states of PQ



Scheme 1.

molecules upon temperature, light or chemical treatments. The endogenous pool capacity for scavenging  $Q_A^{\bullet-}$  radicals from PSII (PQ pool availability) could be interpreted as an alternative index to quantify the relief of excitatory pressure in the thylakoids [25–27].

### 2.11. Statistical analysis

Data are presented as (mean±S.D.) for at least three independent measurements (for a cleaner presentation of the graphs). Fitting curves and correlation tests between plastoquinone pool size and antioxidant activities or parameters of oxidative lesions were performed by Origin 7.0 Software.

## 3. Results

The measurement of chlorophyll a (CHL a) content in homogenates could be used as a parameter of adequate adaptation of algal cells under unfavorable experimental conditions. As reported in Table 1, most of the applied stimuli did not result in substantial variations in the CHL a content of algal cells, except for the HL/12 °C-acclimated cultures (33% lower CHL a content than LL/12 °C algae) and DCMU/HL/26 °C samples (13% lower than control).

Single or combined effects of high light (HL), chilling (12 °C), and/or addition of sub-lethal doses of specific inhibitors of the thylakoid electron transport system (DCMU and DBMIB) appeared to induce redox imbalances in the chloroplasts of the red alga *Kappaphycus alvarezii*, as estimated based on significant variations in the availability of the plastoquinone (PQ) pool within 24 h. Regarding HL stress at 26 °C, an apparent increase in PQ pool availability in the first 8 h of the experiment (20% higher than at  $t_0$ ) was followed by a gradual decrease in the remaining 16 h of HL treatment, reaching 60% of the maximum level observed at  $t_{8\text{ h}}$  (Fig. 1A). Indeed, the variation of the PQ pool availability over time in the red alga *K. alvarezii* exposed to HL at 26 °C was better fitted by a Gaussian-function ( $y=y_0+[A/(w(\pi/2)^{1/2})]e^{-2[(x-x_c)2/w^2]}$ ), specially when an apparent “effect lag phase” of 2 h (from  $t_0$  to  $t_{2\text{ h}}$ ) was ignored in the fitting curve:  $y_0=(98.1\pm 12.2)$ ;  $x_c=(7.11\pm 1.54)$ ;  $w=(12.38\pm 5.11)$ ;  $A=(2,253\pm 885)$ ;  $R^2=0.99991$ . No significant variation was observed in the red algal cultures kept under LL regimen at 26 °C.

Table 1

Chlorophyll a content in the red alga *Kappaphycus alvarezii* under single or combined effects of high light, chilling and chemical stresses<sup>a</sup>

Algal samples	26 °C		12 °C	
	LL <sup>b</sup>	HL <sup>c</sup>	LL <sup>b</sup>	HL <sup>c</sup>
Control	33±1	31±4	33±6	22±2
+0.05 μM DCMU	37±14	27±1*	37±2	36±6*
+8.0 μM DBMIB	32±4	32±11	31±13	32±2*

Values represent mean±S.E. of three experiments.

<sup>a</sup> Concentration of chlorophyll a (μg chlorophyll a. FW g<sup>-1</sup>) in methanol extracts of the red alga *K. alvarezii* submitted to single- or combined effects of regular (26 °C) or low temperatures (12 °C), low (LL=46±11 μmol photon m<sup>-2</sup> s<sup>-1</sup>) or high irradiances (HL=504±13 μmol photon m<sup>-2</sup> s<sup>-1</sup>) in the presence or absence of inhibitors of thylakoid electron transport chain (DCMU 0.05 μM or DBMIB 8.0 μM).

\* Statistically significant values compared to respective control group (same light and temperature conditions).

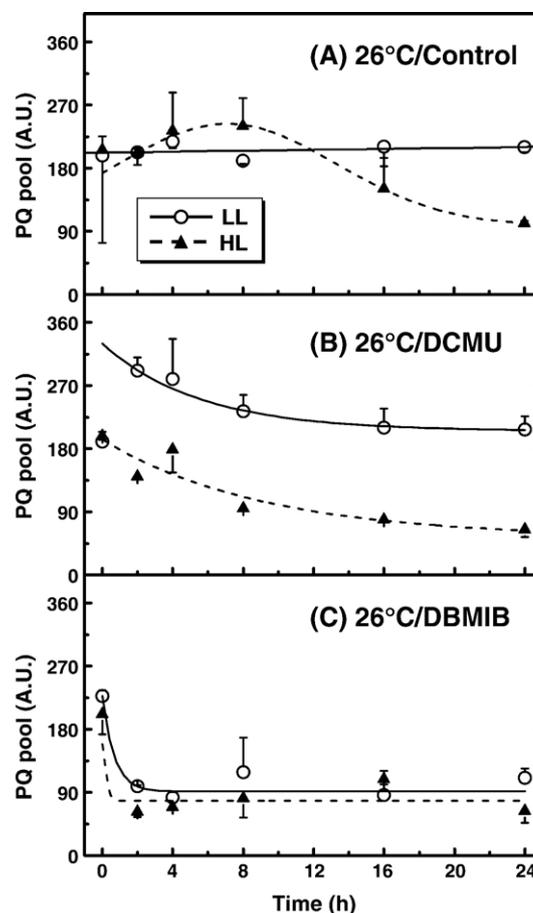


Fig. 1. The 24 h kinetics of plastoquinone pool availability (in Arbitrary Units, A.U., as defined in Materials and Methods) in *Kappaphycus alvarezii* cultures exposed to (○) low light (open circle/solid line)<sup>a</sup> or (▲) high light (closed triangle/dashed line)<sup>b</sup> at 26 °C in: (A) control group; (B) 0.05 μM DCMU-treated group; or (C) 8 μM DBMIB-treated group. Values represent mean±S.E. ( $n\geq 3$ ). <sup>a</sup>LL; ( $54.3\pm 4.8\ \mu\text{mol photon m}^{-2}\ \text{s}^{-1}$ ). <sup>b</sup>HL; ( $505\pm 34\ \mu\text{mol photon m}^{-2}\ \text{s}^{-1}$ ).

On the other hand, DCMU- and DBMIB-induced stresses (under LL and 26 °C) drastically reduced the availability of PQ pool by exponential functions ( $y=y_0+A e^{-kt}$ ), as illustrated by the straight lines in Fig. 1B ( $y_0=205.1\pm 21.6$ ;  $A_1=124.7\pm 45.3$ ;  $k=5.5\pm 5.3$ ;  $R^2=0.994$ ) and Fig. 1C ( $y_0=91.1\pm 7.3$ ;  $A_1=136.5\pm 10.7$ ;  $k=0.68\pm 0.23$ ;  $R^2=0.986$ ), respectively. Interestingly, the previously argued “effect lag phase” is also evident in the DCMU/LL/26 °C group, since a sudden increase in the PQ pool was observed from  $t_0$  h to  $t_{2\text{ h}}$  in these samples, followed by a smooth exponential decay (Fig. 1B). After 24 h under regular temperatures, the combined DCMU and HL stresses (dashed lines in Fig. 1B) resulted in a significantly lower-and-stabilized level of the PQ pool compared to DCMU/LL/26 °C (70% lower), although no significant difference was observed in DBMIB-added groups (Fig. 1C). Moreover, exponential functions were also applied to explain the variation of the PQ pool availability over time in the red alga *K. alvarezii* under concomitant HL and chemical stresses at 26 °C: (i) DCMU/HL/26 °C:  $y_0=77.6\pm 0.6$ ;  $A_1=112.2\pm 4.3$ ;  $k=3.6\pm 0.3$ ;  $R^2=0.991$ ; and (ii) DBMIB/

HL/26 °C:  $y_0=78.2\pm 16.7$ ;  $A_1=80.3\pm 37.4$ ;  $k=0.082\pm 6.4\times 10^{-7}$ ;  $R^2=0.768$ ).

The severity of the chilling stress to the tropical red alga *K. alvarezii* was evident by the substantial depletion of the PQ pool levels in all circumstances, especially under concomitant HL or chemical stresses (Fig. 2). Even under LL intensity (straight line in Fig. 2A), the PQ pool level of chilling-acclimated *K. alvarezii* was also exponentially diminished with time of exposure:  $y_0=110.2\pm 54.1$ ;  $A_1=225.5\pm 28.0$ ;  $k=13.0\pm 9.3$ ;  $R^2=0.967$  (excluding the deviating point  $t_{8\text{ h}}$ ). Concomitant HL treatment accelerated the chilling-induced exponential depletion of the PQ pool ( $y_0=57.8\pm 12.3$ ;  $A_1=163.5\pm 25.6$ ;  $k=1.2\pm 0.6$ ;  $R^2=0.932$ ), leading to distinguished steady-state levels after 24 h (Fig. 2A).

The synergistic effect of the chilling stress of accelerating the exponential decrease of PQ pool availability in DCMU- and DBMIB-treated algae is clearly shown in Fig. 2B and C, especially when compared with the same conditions at regular temperatures (Fig. 1B and C). Except for the combined DCMU/LL/12 °C treatment (straight line in Fig. 2B), all the

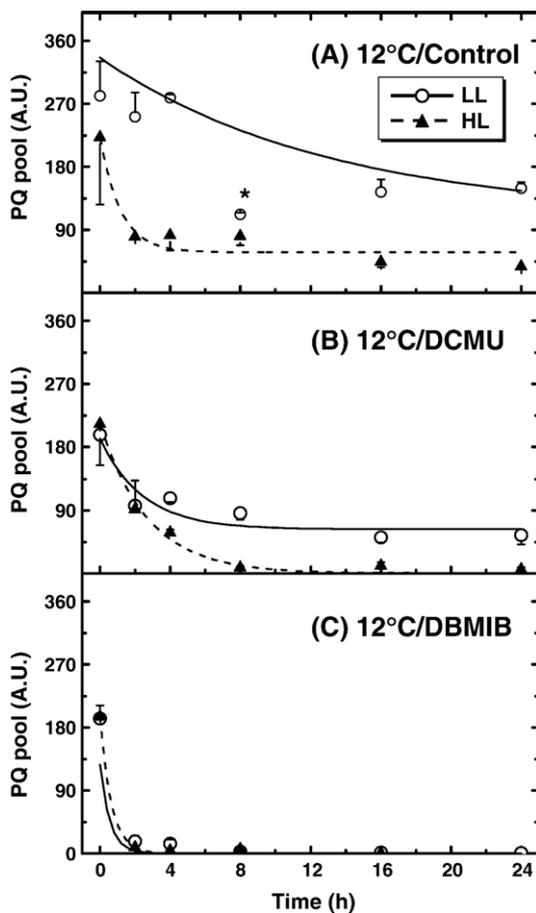


Fig. 2. The 24 h kinetics of plastoquinone pool availability (in Arbitrary Units, A.U., as defined in Materials and methods) in *Kappaphycus alvarezii* cultures exposed to (○) low light (open circle/solid line)<sup>a</sup> or (▲) high light (closed triangle/dashed line)<sup>b</sup> at 12 °C in: (A) control group; (B) 0.05 μM DCMU-treated group; or (C) 8 μM DBMIB-treated group. Values represent mean ± S.E. ( $n\geq 3$ ). \*not included in the fitting curve. <sup>a</sup>LL; ( $54.3\pm 4.8$  μmol photon  $\text{m}^{-2} \text{s}^{-1}$ ). <sup>b</sup>HL; ( $505\pm 34$  μmol photon  $\text{m}^{-2} \text{s}^{-1}$ ).

other simultaneous treatments with photosynthesis' inhibitors and low temperatures severely abolished the availability of the PQ pool in the red alga *K. alvarezii*. All these combined effects on algal PQ pools were also well fitted by exponential decay curves: (i) DCMU/LL/12 °C:  $y_0=63.1\pm 14.0$ ;  $A_1=128.8\pm 25.1$ ;  $k=2.4\pm 1.1$ ;  $R^2=0.898$ ; (ii) DCMU/HL/12 °C:  $y_0=0.0\pm 0.0$ ;  $A_1=212.6\pm 0.3$ ;  $k=2.7\pm 0.1$ ;  $R^2=0.999$ ; (iii) DBMIB/LL/12 °C:  $y_0=0.0\pm 0.0$ ;  $A_1=126.9\pm 18.7$ ;  $k=0.57\pm 0.34$ ;  $R^2=0.796$ ; and (iv) DBMIB/HL/12 °C:  $i_0=0.0\pm 0.0$ ;  $A_1=195.2\pm 4.2$ ;  $k=0.64\pm 0.10$ ;  $R^2=0.998$ .

The kinetic study of chlorophyll fluorescence in the red alga *K. alvarezii* showed that most of the harmful conditions imposed on the algal samples resulted in lower but stabilized PQ pool levels after 24 h, except for the inhibitor-free/26 °C/HL samples (dashed line, Fig. 1A). In order to evaluate hypothetical correlations between redox imbalances in chloroplasts and overall antioxidant responses in algal tissues, the stabilized values of the PQ pool availability obtained after 24 h (including that of inhibitor-free/26 °C/HL samples) were plotted against the specific activities of the antioxidant enzymes superoxide dismutase (total SOD; Fig. 3A), ascorbate peroxidase (APX; Fig. 3C), and catalase (CAT; Fig. 3D), which were measured in total homogenates of algal tissues. The PQ pool availability in experimental samples was also correlated with total  $\text{H}_2\text{O}_2$  release in seawater (Fig. 3B) and with parameters of oxidative lesions in proteins (protein thiol groups; Fig. 3E) and lipids (conjugated dienes, CD; Fig. 3F). Although total SOD activity showed a moderate correlation with the PQ pool availability ( $R=0.689$ , Fig. 3A), CAT and APX – two of the most effective  $\text{H}_2\text{O}_2$ -scavenging enzymes in photosynthetic organisms – were not statistically correlated with it (Fig. 3C and D). Paradoxically, the  $\text{H}_2\text{O}_2$  concentration in seawater was moderately proportional to the PQ pool availability, with a correlation coefficient of  $R=0.673$  (Fig. 3B). Under the experimental conditions applied here, the highest correlation coefficients were obtained when comparing the estimated PQ pool availability with indexes of oxidative lesions in algal tissues: protein thiol content ( $R=0.869$ ; Fig. 3E) and conjugated diene content in the lipid fraction of algal homogenates ( $R=0.864$ ; Fig. 3F).

#### 4. Discussion

Membrane fluidity and intermolecular collisions are well-established thermo-dependent events that can affect the redox stoichiometry in the thylakoids, especially in reactions triggered by diffusible electron carriers such as PQ and plastocyanin [28]. The chilling-induced decrease of PQ mobility might enhance electron leakage from the thylakoid membrane under moderate or high light intensities, based on the same principles that explain overproduction of superoxide radical ( $\text{O}_2^{\bullet-}$ ) in response to changes in the fluidity of inner mitochondrial membranes: (i) hindrance of proportional electron flow in the membranes due to massive reducing power input in the electron transport system [29]; (ii) high concentrations of dissolved  $\text{O}_2$  in the hydrophobic core of the lipid bilayer [30]; and (iii) oxidation of hydroquinol ( $\text{QH}_2$ )

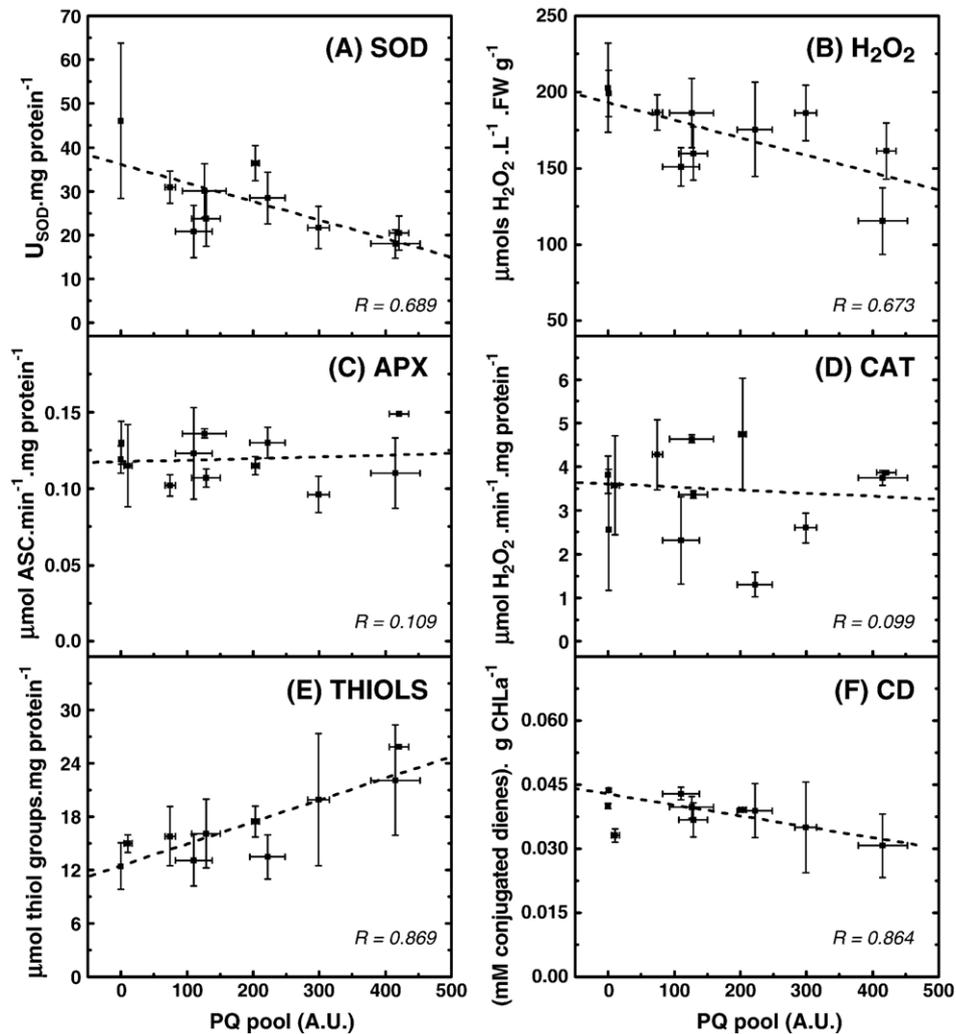


Fig. 3. Linear regressions between (A) total SOD activity<sup>a</sup>; (B) total H<sub>2</sub>O<sub>2</sub> release into seawater<sup>b</sup>; (C) ascorbate peroxidase activity<sup>c</sup>; (D) catalase activity<sup>d</sup>; (E) protein thiol content<sup>e</sup>; (F) conjugated diene content<sup>f</sup>, and plastoquinone pool availability (after 24 h) in *Kappaphycus alvarezii* cultures exposed to stressful oxidative conditions (unspecified in the plots) imposed by treatment with 0.05 μM DCMU or 8 μM DBMIB under different temperature (26 °C or 12 °C) and light conditions (45 μmol photons m<sup>-2</sup> s<sup>-1</sup> or 505 μmol photons m<sup>-2</sup> s<sup>-1</sup>, LL and HL, respectively). Values represent (mean  $\bar{y} \pm$  S.E.; mean  $\bar{x} \pm$  S.E.) of three experiments. Values in the right bottom of each plot represent the correlation index of the linear analysis. <sup>a</sup>U<sub>SOD</sub> mg protein<sup>-1</sup>; <sup>b</sup>μmols of H<sub>2</sub>O<sub>2</sub> L<sup>-1</sup> of seawater FW g<sup>-1</sup>; <sup>c</sup>μmol ascorbate min<sup>-1</sup> mg protein<sup>-1</sup>; <sup>d</sup>μmol H<sub>2</sub>O<sub>2</sub> min<sup>-1</sup> mg protein<sup>-1</sup>; <sup>e</sup>μmol thiol groups. mg protein<sup>-1</sup>; <sup>f</sup>mM conjugated dienes. <sup>g</sup>CHL a<sup>-1</sup>.

involving two single-electron transfer reactions (i.e., one at a time) and generation of, respectively, semiquinone (QH<sup>•</sup>), and then the quinone (Q), comprising the recognized slowest step in electron transfer at both mitochondrial and the thylakoid electron transport chains [31]. Thus, under experimental conditions leading to gradual PQH<sub>2</sub> accumulation and less diffusion within membranes, electrons could leak from the thylakoid membrane to increase the rate of O<sub>2</sub><sup>•-</sup> formation. The overall effect is that the photosynthetic apparatus of aquatic autotrophs is supposed to acclimate to changes in temperature in a fashion comparable to that of photoacclimation [32], as both ROS-mediated processes [33,34] are effectively signaled by the redox state of the PQ pool [35].

Although both HL and chilling stresses (as isolated stimuli) resulted in a significant reduction of the PQ pool availability in the red alga *K. alvarezii* within 24 h of treatment, each adverse experimental condition led to different kinetic profiles. The HL-effect on the PQ pool availability followed a Gaussian-function,

which could be interpreted as a phenomenon controlled by a large number of small additive and independent factors (dashed line in Fig. 1A). The HL-dependent Gaussian function indicates that a maximum PQ pool availability was obtained after 8 h of HL exposure, which could be interpreted as a temporary improvement in photosynthesizing activity due to the increase in light intensity (505 ± 34 μmol photon m<sup>-2</sup> s<sup>-1</sup>). In contrast, an exponential function better fitted the PQ pool kinetics in the LL/12 °C samples (solid line in Fig. 2A). Regarding heat conduction theory, the temperature equilibrium between an object (or organism) and the medium accordingly follows exponential decay [36]. A limited number of studies have applied kinetic models to elucidate the ROS-mediated inhibition of photosystems upon chilling stress, most of them focusing on the PSI [29,37,38].

Among all unfavorable conditions imposed on *K. alvarezii* cells, the combined effect of HL/12 °C, DCMU/LL/12 °C, DCMU/HL/26 °C, DBMIB/LL/26 °C and DBMIB/HL/26 °C

resulted in similar levels of PQ pool availability within 24 h, with an average value of  $(66.1 \pm 27.3)$  A.U. Hypothetically, the stabilization of the PQ pool under adverse conditions could be understood as resulting from the acclimation process, either thermo- or photo-responsive. It is worth noting that most of the experimental conditions imposed in the study did not lead to substantial variations in the CHL a content of *K. alvarezii* cells, except in the HL/12 °C group, in which a strong ROS-invoked photobleaching effect is suggested (35% less CHL a content; Table 1). In fact, the risky combination of HL and low temperatures has been reported to enhance chilling injury, an event that can persist even after restoration of the optimal growth temperature [39,40]. As for natural cultures of the red alga *K. alvarezii*, an optimum photosynthetic rate was obtained between 22 to 25 °C, but no acclimation was reported for incubations at 18 °C through either gradual or abrupt transfers [41].

The use of the xenobiotics DCMU and DBMIB – site-specific inhibitors of the thylakoid electron transport system – allows the redox state of the PQ pool to be artificially modified. The inhibitory chemistry of DCMU in thylakoid membranes is well documented, since it prevents electron transfers between  $Q_A^{\bullet-}$  and  $Q_B$  and, thus, keeping the PQ pool oxidized [42]. DBMIB has a more complex interaction with the thylakoid electron transfer system, since inconsistent results have shown that: (i) DBMIB can bind at the  $Q_B$  site of the PS II reaction center, acting as a DCMU-type inhibitor [43]; (ii) DBMIB can accept electrons from the PQ pool and be reduced in thylakoids under light conditions [44]; and (iii) cytochrome  $b_6/f$  (intermediate electron carrier between PSII and PSI) can accept one electron from DBMIB, which, as a bound semiquinone, prevents the reoxidation of other reduced PQ molecules (PQH<sub>2</sub>) [45]. Furthermore, all the DBMIB treatments in *K. alvarezii* cultures resulted in comparatively abrupt reductions of the PQ pool availability (within 2 h or less), which could be partially explained by the multiple factorial inhibitory effect of DBMIB on thylakoids. The harmful effect of DBMIB was synergistically enhanced by concomitant exposure to chilling conditions, possibly by restricting PQ mobility within the hydrophobic core of thylakoids. The PQ pool availability was completely eliminated in both DBMIB/LL/12 °C and DBMIB/HL/12 °C groups (Fig. 2C), while it was kept at a stabilized plateau in both DBMIB/LL/26 °C and DBMIB/HL/26 °C groups (Fig. 1C).

Several authors have reported the elimination of  $O_2^{\bullet-}$  production in plant and algal thylakoids upon high-dose administration of DCMU or DBMIB [28,29]. Nonetheless, drug pharmacokinetics and other factors as concentration, incubation time, cellular membrane composition (in terms of xenobiotic absorption throughout the cuticle tissue), temperature, and biotransformation reactions could be quoted as plausible events leading to primary ROS overproduction in plant/algal cells under chemical stress. Corroborating this fact, a supposed “effect lag phase” of 2 h was evident when monitoring PQ pool availability in DCMU/LL/26 °C group (Fig. 1B). The lack of the lag phase in other experimental groups could be explained by the synergistic effect of combined HL, chilling and

chemical stresses leading to a more immediate effect on the PQ pool of treated *K. alvarezii* cultures (Fig. 2B). Taking herbicide pharmacokinetics into account, a lag of up to 5 h was also observed before a  $10^{-5}$  M DCMU dose started to affect  $O_2^{\bullet-}$  production in the ichthyotoxic alga *Chattonella marina* [46]. Indeed, the ranges of DCMU and DBMIB concentrations typically used in photosynthesis studies are, in general from 1 to 20  $\mu$ M [28], compared to those applied in our study (0.05  $\mu$ M DCMU and 8  $\mu$ M DBMIB). Accordingly, DBMIB concentrations in our study rapidly diminished the PQ pool availability (Figs. 1C and 2C).

As a matter of fact, the single or combined light, chilling and DCMU/DBMIB chemical stresses applied here imposed distinct chloroplast-targeted oxidative challenges on the algal samples, which were clearly sensitized by significant variations in the PQ pool availability. Different levels of site-specific antioxidant adaptations apparently counterbalanced chloroplast redox imbalances provoked by single or combined stress conditions. The oxidative stress parameters were measured in each experimental group of treated algae and then correlated with the plateaus of PQ pool availability obtained after 24 h of experiment (endpoints in Figs. 1 and 2). Comparisons of antioxidant enzyme activities – generally superoxide dismutase (SOD), catalase (CAT) and ascorbate peroxidase (APX) – with oxidative injury biomarkers are often assumed to be valid tools for investigating the efficiency of adaptive responses in photoautotrophs under adverse conditions [32].

Hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) is proposed as a crucial chloroplast-originated redox signal for providing efficient photo- and thermoacclimation in plant and algal tissues [33,47,48]. On the other hand, excessive amounts of H<sub>2</sub>O<sub>2</sub> severely inactivate housekeeping hemeoproteins [49]. In higher plants, H<sub>2</sub>O<sub>2</sub> generated in chloroplasts is primarily scavenged by stromal APX [33], whereas CAT efficiently removes remnant H<sub>2</sub>O<sub>2</sub> molecules within peroxisomes [50]. Both CAT and APX are strongly responsive to H<sub>2</sub>O<sub>2</sub> accumulation in tissues of higher plants [51]. Conversely, red algae from the genus *Kappaphycus* and *Euclima* release H<sub>2</sub>O<sub>2</sub> into seawater as a primary event in preventing harmful peroxide accumulation within algal tissues under oxidative conditions [10,11,52]. In previous studies, *K. alvarezii* cultures treated with clofibrate – an induction factor of peroxisomal ROS-generating oxidases – also released massive amounts of H<sub>2</sub>O<sub>2</sub> into seawater in a short period of time, with maximum levels attained after almost 2 h of clofibrate addition [12]. Although total SOD was immediately responsive (almost 2.5-fold higher than the control after 4 h), significant increases in both APX and CAT were only observed after 72 h. In the study presented here, the apparent correlation observed between PQ pool and H<sub>2</sub>O<sub>2</sub> concentrations in seawater ( $R^2=0.673$ ) suggests that large amounts of H<sub>2</sub>O<sub>2</sub> were indeed produced inside chloroplasts of *K. alvarezii* under HL, chilling and chemical stresses. Nevertheless, the direct diffusion of H<sub>2</sub>O<sub>2</sub> throughout algal cellular membranes to seawater – a feasible mechanism for submerged seaweeds – prevented the immediate induction of the H<sub>2</sub>O<sub>2</sub>-responsive antioxidant enzymes CAT and APX in

the red alga *K. alvarezii*. Congruent with this fact, no significant correlations between PQ pool availability and CAT or APX activities were observed in *K. alvarezii* cells under light, chilling or chemical stresses (Fig. 2B and C). On the other hand, total SOD activities were still moderately responsive to redox variations in the PQ pool ( $R=0.689$ ). An intriguing point is that studies involving leaf discs of higher plants submerged in DCMU solutions showed chlorophyll fluorescence results analogous to those presented here with DCMU-treated red algae, although the authors did not report this fact with respect to  $H_2O_2$  diffusion from the leaves [42].

Thus, high PQ pool availability is seen here as an index of balanced electron flow through the thylakoid membrane, which associates efficient photon energy absorption (at least in PSII) with the generation rate of  $O_2^{\cdot-}$  and other ROS in chloroplasts. Corroborating this theory, the  $H_2O_2$  concentration in seawater, total SOD activity and conjugated diene content (as an index of lipoperoxidation) were exacerbated in *K. alvarezii* groups displaying lower PQ pool availability, which suggests that algal samples were increasingly exposed to oxidative conditions as the PQ pool became less efficient in accepting electrons from PSII. Lower levels of protein thiol groups in *K. alvarezii* samples ( $R=0.869$ ) also confirm this hypothesis, since thiol groups are reported to form unspecific S–S cross-linked bonds under oxidative stress [53].

The paradoxical  $H_2O_2$  release into seawater – which prevented efficient CAT and APX induction – could be interpreted as a physiological strategy of the red alga *K. alvarezii* to cope with the rapid and high scale production of toxic  $H_2O_2$  within algal tissues at the onset of the harmful stimulus. Based on  $H_2O_2$  toxicity, peroxide removal (even in a late onset mode) is presumably crucial in affecting algal susceptibility under permanent or long-term unfavorable (environmental) conditions [54]. Elegant studies of  $H_2O_2$  photoproduction using cyanobacteria immobilized in inert agar- or alginate-matrices in a continuous-flow system (allowing for the removal of peroxide as it formed) showed that, after an abrupt increase of  $H_2O_2$  concentration in the reaction cell (within 1 h), constantly  $H_2O_2$ -producing cultures of *Anacystis nidulans* were maintained viable for several additional hours under HL stress, with the concomitant establishment of a  $H_2O_2$  plateau in the reaction cell [55]. Unfortunately, no measurements of CAT and APX were evaluated in that study to check antioxidant adaptations under those circumstances. With respect to open field cultures or the natural habitats of *K. alvarezii*, it is tempting to suggest that gradual (but chronic)  $H_2O_2$  accumulation in the surrounding environment may be responsible for adequate delayed responses from CAT and APX ensuring long-term adaptation of algal cultures, as we observed in our previous study with clofibrate [12]. In addition, this work provides biochemical evidences of the reportedly high susceptibility of the red alga *K. alvarezii* under adverse environmental conditions, especially chilling stress [5], which should be taken into account in relocation programs of carrageenophyte cultures worldwide [3].

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