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Effects of N supply on the accumulation of photosynthetic pigments and photoprotectors in *Gracilaria tenuistipitata* (Rhodophyta) cultured under UV radiation

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Abstract We have studied the effects of nitrate supply under photosynthetic active radiation (PAR) plus ultraviolet radiation (UVR) exposure on photosynthetic pigments (chlorophyll *a* and carotenoids), photoprotective UV screen mycosporine-like amino acids (MAAs), and photosynthetic parameters, including the maximum quantum yield (F_v/F_m) and electron transport rate (ETR) on the red agarophyte *Gracilaria tenuistipitata*. Apical tips of *G. tenuistipitata* were cultivated under ten different concentrations of NO_3^- for 7 days. It has been shown that *G. tenuistipitata* cultured under laboratory conditions has the ability to accumulate high amounts of MAAs following a nitrate concentration-dependent manner under PAR+UVR. Two MAAs were identified, shinorine and porphyra-334. The relative concentration of the first increased under high concentrations of nitrate, while the second one decreased. The presence of antheraxanthin is reported for the first time in this macroalgae, which also contains zeaxanthin, lutein, and β-carotene. The accumulation of pigments, photoprotective compounds, and photosynthetic parameters of *G. tenuistipitata* is directly related to N availability. All variables decreased under low N supplies and reached constant maximum values with supplements higher than 0.5 mM NO_3^-. Our results suggest a high potential to acclimation and photoprotection against stress factors (including high PAR and UVR) directly related to N availability for *G. tenuistipitata*.

Keywords Carotenoids · *Gracilaria tenuistipitata* · *In vivo* chlorophyll *a* fluorescence · N availability · Photoprotection · Photosynthesis

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Introduction

During the last decades, ultraviolet radiation (UVR) has increased over the Earth surface, and there are predictions for further increase, mainly in tropical zones (Hegglin and Sheperd 2009; Li et al. 2009). UVR has harmful effects on marine primary producers (Villafañe et al. 2003), but macroalgae present different photoprotection strategies (Bischof et al. 2006) which reduce UVR exposure or limit the amount of photodamage, i.e., accumulation of UV screen substances (e.g., mycosporine-like amino acids, MAAs) or the production of antioxidant compounds (ascorbate-glutathione, MAAs, and carotenoids).

Mycosporine-like amino acids are water-soluble N compounds that have in common a cyclohexenone or cyclohexenimine chromophore conjugated with the N substitute of an amino acid. They are widely distributed among freshwater and marine organisms (Korbee et al. 2006). These compounds have been broadly studied because of their photoprotection ability as UVR screen substances and their photostability (Conde et al. 2004, 2007). In fact, several studies pointed out that these compounds could play a UVR-protective role in algae, most of them based on a positive correlation between MAA

concentrations and ambient levels of UVR (Karsten et al. 1998; Neale et al. 1998; Yakovleva and Titlyanov 2001).

The accumulation of MAAs in red macroalgae is dependent not only on the light signal induction (for example, UVR), but also on N availability (Korbee-Peinado et al. 2004; Korbee et al. 2005; Huovinen et al. 2006; Figueroa et al. 2008). Furthermore, the accumulation of MAAs can be affected by other environmental variables such as salinity, temperature, and desiccation (Karsten et al. 2003; Jiang et al. 2008). High ammonium concentration was found to induce MAA synthesis and accumulation in *Porphyra* spp. (Korbee-Peinado et al. 2004; Korbee et al. 2005), *Grateloupia lanceola* (Huovinen et al. 2006), and *Asparagopsis armata* (Figueroa et al. 2008). Besides UV screen and antioxidant capacities, the accumulation of MAAs with the increase of ammonium availability suggests that these substances can function also as a reservoir of N (Korbee-Peinado et al. 2004; Korbee et al. 2005, 2006; De la Coba et al. 2009). The N of MAAs can be used when N sources are reduced (anticipating strategy), as it has been suggested for phycobiliproteins (Talarico and Maranzana 2000). Besides photoprotection mechanisms, the limitation of N affects other processes in macrophytes, including photosynthetic capacity (Pérez-Lloréns et al. 1996) and protein content (Vergara et al. 1995), and reduces cell size (García-Pichel 1994).

On the other hand, light-dependent conversion of violaxanthin to zeaxanthin, the so-called xanthophyll cycle, has been shown to serve as a major, short-term light acclimation mechanism in higher plants. Unlike in plants, the role of the xanthophyll cycle in algae is ambiguous, since its contribution to energy dissipation can vary significantly among species (Demmig-Adams and Adams 2006). Additionally, its presence in red macroalgae has been controversial. Initially, Brown and McLachlan (1982) found antheraxanthin, violaxanthin, and zeaxanthin in some red algae species, which was later confirmed by studies of Schubert et al. (2006) and Esteban et al. (2009). Only one study has been performed using *Gracilaria tenuistipitata*, detecting the presence of zeaxanthin, lutein, and β-carotene, which were analyzed under different radiation treatments (Carnicas et al. 1999). However, the presence of a thermal dissipating xanthophyll cycle was found only in *Gracilaria birdiae* (Ursi et al. 2003).

The positive effect of N availability in the xanthophyll cycle pigment synthesis was recently analyzed by Korbee et al. (2010) in a dinoflagellate, *Heterocapsa* sp. In this work, the authors concluded that not only MAA accumulation but also N availability is very important to determine the photoprotective capacity against UVR of *Heterocapsa* sp., considering its xanthophyll cycle (Korbee et al. 2010).

The agarophyte red macroalga *G. tenuistipitata* var. *liui* has been used for molecular (Hagopian et al. 2002, 2004) and physiological studies (e.g., Barufi et al. 2010; García-Sánchez et al. 1993; Lopes et al. 2002). Five MAAs were already found in this red macroalgae by Cardozo et al. (2006), but their regulation by N supply has not yet been investigated. Moreover, the studies cited above on the effects of N supply on MAA accumulation have been conducted only at three N conditions (absence and low and high concentrations), and no stationary or saturation responses were evaluated. Furthermore, the source of N was ammonium. The aim of the present work is to investigate the response to ten different nitrate concentrations of two photoprotection systems, i.e., the photoprotective N compounds MAAs and non-N molecules (xanthophyll pigments), in the red alga *G. tenuistipitata*.

Material and methods

The experiment was conducted with a tetrasporophytic individual of *G. tenuistipitata* var. *liui* Zhang and Xia. This organism was collected in Haikou, Hainan Island, China, by E.C. Oliveira in 1990. This material was isolated by J. Macchiavello and has been kept in unialgal culture in vitro (photoperiod of 14 h, 25°C and 30± 10 μmol photons $m^{-2}s^{-1}$), without UVR, in the *Gracilaria* germplasm bank at the "Edison J. de Paula" Laboratory, University of São Paulo, São Paulo, Brazil.

Gracilaria tenuistipitata was cultivated in methacrylate plastic vessels (UV-transparent Plexiglas, GS 2332) under optimal growth conditions (Macchiavello et al. 1998): filtered seawater diluted with distilled water to 20 psu, enriched with Von Stosch (VS) solution, which contains 0.5 mM NO_3^- (Edwards 1970, modified by Ursi and Plastino 2001). The photosynthetic active radiation (PAR) applied to the cultures was at 56 $W m^{-2}$ (i.e., approximately 260 μmol photons $m^{-2}s^{-1}$), 12:12-h light/dark cycle, and 25±1°C. Salinity and temperature were selected from previous studies (Haglund and Pedersén 1993; Israel et al. 1999; Macchiavello et al. 1998).

Experimental design

Apical portions (2 g of 3–5-cm length of branched algae for 1 L of enriched seawater) of *G. tenuistipitata* were treated under the conditions described above for 1 week, as a pre-acclimation period. After that, 2 g of fresh weight (FW) was placed into new vessels, maintained under the following experimental conditions during 1 week: algae were exposed to PAR+UVR and the seawater was enriched with VS containing different NO_3^- supplements. The inclusion of the UVR is due to the fact that previous results on the same species showed that MAA concentration increased only when the UVR was added to PAR. Ten different NO_3^- final

concentrations in the enriched seawater were evaluated: 0, 0.03, 0.075, 0.1, 0.25, 0.5, 0.75, 1, 1.5, and 2 mM of NO_3^-. The other VS components were kept the same in all treatments, and temperature, photoperiod, and salinity were the same used in the previous acclimation period. The control treatment was 0.5 mM NO_3^-, as this concentration was the optimum used in previous studies with the same species (Macchiavello et al. 1998), and is the nitrate usually supplied in laboratory conditions when the VS medium is used in other macroalgae (Ursi and Plastino 2001). The nitrate concentration measured in the natural environment of *G. tenuistipitata* was between 0.16 and 0.096 μM (Huang et al. 2003).

Photosynthetic active radiation (in the pre-acclimation and during the experimental period) was provided by two daylight fluorescent tubes (Phillips TL-D® 36W/54-765), and UVR of the experimental period was obtained using two Q-Panel® 340 tubes (Q-Panel Co., Ohio, USA). The total incident spectral irradiance used in these experiments was determined with the Sphere Optics multidiode spectroradiometer, model SMS-500 (Hoffman Sphere Optics LLC, USA). The methacrylate cylindrical vessels were covered by an Ultraphan 295 cutoff filter (Digefra GmbH, Germany) to remove wavelengths lower than 295 nm. Three types of radiation, PAR, UV-A, and UV-B, comprised the PAR+UVR treatments, containing wavelengths between 400 and 700, 315 and 400, and 280 and 315 nm, respectively. During the experimental period, PAR irradiance was 56 Wm^{-2}, and UVR irradiance was 8.55 Wm^{-2} (8.13 Wm^{-2} of UV-A and 0.42 Wm^{-2} of UV-B). The weighted irradiances for DNA damage and chloroplast photoinhibition were 0.01 and 1.28 Wm^{-2}, respectively (Jones and Kok 1966; Setlow 1974).

At the beginning and after 7 days of the experimental period, samples were taken for analyses of photosynthetic pigments (chlorophyll *a* and carotenoids) and MAAs. Photosynthetic parameters including maximum quantum yield (F_v/F_m) and electron transport rates (ETRs) were evaluated, as described below. Three samples were taken from each vessel.

Photosynthetic pigments

Chlorophyll *a* (Chl*a*) contents were determined spectrophotometrically, while carotenoids were obtained by high-performance liquid chromatography (HPLC). For the Chl*a* extraction, 20 mg of FW was inserted in 1 mL of dimethylformamide (DMF) in darkness and at 4°C for 24 h. After this period, the extracts were read in a spectrophotometer, and absorbance values to the equation of Wellburn (1994) were used to determine the total concentration.

Carotenoids were extracted from 0.1 g of FW with 1 mL of DMF during 24 h, as described above. After the extraction period, samples were filtered through 0.2 μm filters and inserted in the HPLC vessels, resulting in at least 700 μL of algal extract. Carotenoids were detected with injection of 65 μL of this extract in a Waters HPLC system, using a two-solvent gradient as the mobile phase, composed by the solutions A (distilled water + tetrabutyl ammonium 0.05 M and ammonium acetate 1 M + methanol) and B (acetone + methanol). The mobile-phase flow was 1 mL min^{-1}, and the total run was composed of the following steps: (1) stable 75% solution A+25% solution B (initial); (2) linear changed to 25% solution A+75% solution B (8 min); (3) isocratic flow (2 min); (4) convex changed to 10% solution A+90% solution B (8 min); (5) concave changed to pure solution B (5 min); (6) concave changed to 75% solution A+25% solution B (maintained in this condition up to the end of the run, completed at 40 min). The carotenoids were separated using a C18 5-μm column (Symmetry ® C18 of 5-μm 4.16×150 mm column T91671L 02). The pigment peaks were determined with a Waters Photodiode Array Detector at 350–380 nm. The different carotenoids were identified by comparing the absorbance peaks with commercial standards for zeaxanthin, antheraxanthin, β-carotene, and lutein (DHI Water and Environment, Denmark). The quantification followed standard curves of these known carotenoid concentrations versus peak absorbance area with five dilutions.

Mycosporine-like amino acids

The UV screen MAAs were determined according to methods of Karsten et al. (1998) and modified by Korbee-Peinado et al. (2004). The samples were dried in silica gel (10–20 mg of DW) and extracted in 1 mL of 20% aqueous methanol (*v*/*v*) for 2 h at 45°C, followed by sonication (2× for 5 min), ensuring complete extraction. Then the extract was separated by centrifugation (13,000×*g* at 4°C for 10 min), and 600 μL of supernatant was evaporated in a vacuum microcentrifuge. The dried extracts were re-suspended in 600 μL absolute methanol. The MAAs were detected by HPLC (Waters) using an isocratic run containing 2.5% aqueous methanol (*v*/*v*) plus 0.1% acetic acid (*v*/*v*) in bidistilled water as the mobile phase. The flow rate was 0.5 mL min^{-1} and each run took 20 min; 30 μL of the each sample was injected into a Sphereclone C8 column (Phenomenex, Germany) with a pre-column attached (5-mm packing; 250×4 mm I.D.). Mycosporine-like amino acids were detected with a Waters Photodiode Array Detector at 330 nm. Absorption spectra were recorded between 290 and 400 nm. The identification and quantification of the MAAs were performed according to Korbee-Peinado et al. (2004).

Photosynthetic activity as *in vivo* chlorophyll a fluorescence

The photosynthetic activity was evaluated as *in vivo* chlorophyll *a* fluorescence using a portable pulse modulation fluorometer Water-PAM (Walz, Germany). Samples were dark-acclimated for 10 min. This allowed measurement of the basal fluorescence (F_o), followed by a saturating pulse (approximately 9,000 μmol photons $m^{-2}s^{-1}$ for 0.6 s). After this pulse, the maximum fluorescence (F_m) value was obtained, and these two values were used to calculate the variable fluorescence (F_v). The maximum quantum yield of the photosystem II was calculated as F_v/F_m (Schreiber et al. 1986). This first parameter was followed by the light-curve settings of the Water-PAM. This consisted in the exposure of the apical tips of *G. tenuistipitata* to 12 increasing irradiances, and after 20 s of exposure to each, a saturating pulse was emitted. This allowed calculation of the effective quantum yield ($\Delta F/F_m'$) for each actinic irradiance, as $\Delta F/F_m' = (F_m' - F_t)/F_m'$. The values of $\Delta F/F_m'$ and the increasing actinic irradiances (E) were used to calculate the ETRs, applying the following formula: $ETR = \Delta F/F_m' * E * A * 0.15$, where A is the absorptance and 0.15 the fraction of chlorophyll associated to photosystem II according to Grzymski et al. (1997) and Figueroa et al. (2003). The absorptance was measured as described by Korbee et al. (2005). From the ETR versus irradiance curves measured for each treatment, maximum electron transport rate (ETR_{max}), photosynthetic efficiency (α_{ETR}), and the slope of photoinhibition (β) were obtained by fitting these curves to the formula of Platt et al. (1980). Three replicates were sampled from each vessel.

Statistics

One-way analysis of variance was performed, and the factor evaluated was the different NO_3^- concentrations. Where significant differences were detected, post hoc multiple comparisons were made using the Newman–Keuls test to identify differences for MAA concentration and photosynthetic pigments, as well as the photosynthetic parameters (F_v/F_m, ETR_{max}, α_{ETR}, and the β) at different concentrations of nitrate. Probability of type I error was 0.05. Pearson correlation analysis was done, with significance when $p<0.05$.

Results

The concentrations of photosynthetic pigments of *G. tenuistipitata* cultivated under increasing nitrate supply are shown in Figs. 1 and 2. The Chl*a* content under up to 0.1 mM NO_3^- was lower in comparison to those cultivated with NO_3^- concentrations higher than 0.25 mM (Fig. 1, $F=41.35$, $p<0.001$, $df=10$). After 1 week, the Chl*a* concentration of *G. tenuistipitata* cultivated under concentrations greater than 0.25 mM was similar to the initial value.

Four carotenoids were detected in *G. tenuistipitata*: zeaxanthin, antheraxanthin, lutein, and β-carotene. After exposure to PAR+UVR, there was little variation in the zeaxanthin content under different NO_3^- concentrations, including no NO_3^-, with values similar to the value observed at the beginning of the experiment (PAR-acclimated samples). Under 1 and 1.5 mM NO_3^-, *G. tenuistipitata* accumulated around 0.3 mg of zeaxanthin gDW^{-1}, i.e., the highest zeaxanthin amount ($F=10.47$, $p<0.05$, $df=10$) (Fig. 2). The other three carotenoids were influenced by the N supply after the 7-day experimental period (antheraxanthin, $F=34.96$, $p<0.05$, $df=10$; β-carotene, $F=7.14$, $p<0.05$, $df=10$; and lutein, $F=93.91$, $p<0.05$, $df=10$). The concentrations of antheraxanthin and lutein were low under supplements of NO_3^- lower than 0.1 mM. Antheraxanthin reached values of up to 0.016 mg per g of DW when supplied with higher N amounts (0.75 and 1 mM NO_3^-, Fig. 2). The lutein content was constant between 0.25 and 2 mM NO_3^-, and similar to the value observed before the experimental period. β-Carotene concentration increased with the N supply reaching maximal values under 0.5 mM NO_3^-.

Two types of MAAs were identified in *G. tenuistipitata*: shinorine and porphyra-334. The total MAA content was significantly affected by different supplements of nitrate ($F=116.28$, $p<0.05$, $df=10$). At the beginning of the experiment, *G. tenuistipitata* showed 0.36±0.04 mg total MAAs gDW^{-1} composed of 6.0±0.2% shinorine and 94.0±0.2% porphyra-334. After 7 days under PAR+UVR, MAA concentration was low under 0 and 0.03 mM NO_3^-. An increase was observed under 0.075 or 0.1 mM NO_3^-,

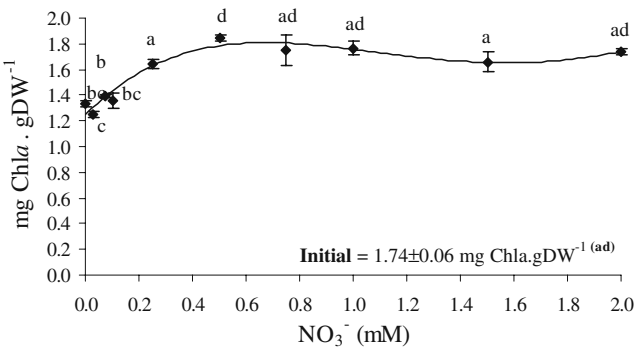

Fig. 1 Chl*a* concentration of *G. tenuistipitata* cultivated under ten nitrate concentrations (0, 0.03, 0.075, 0.1, 0.25, 0.5, 0.75, 1, 1.5, and 2 mM) and exposed to PAR+UVR after 7 days of experimentation, expressed as mg of Chl*a* by g of dry weight of alga (mg gDW^{-1}). Initial value is indicated in the figure. *Different letters* indicate significant differences observed with the Newman–Keuls a posteriori test ($p<0.05$). $n=3$. *Bars* indicate standard deviations

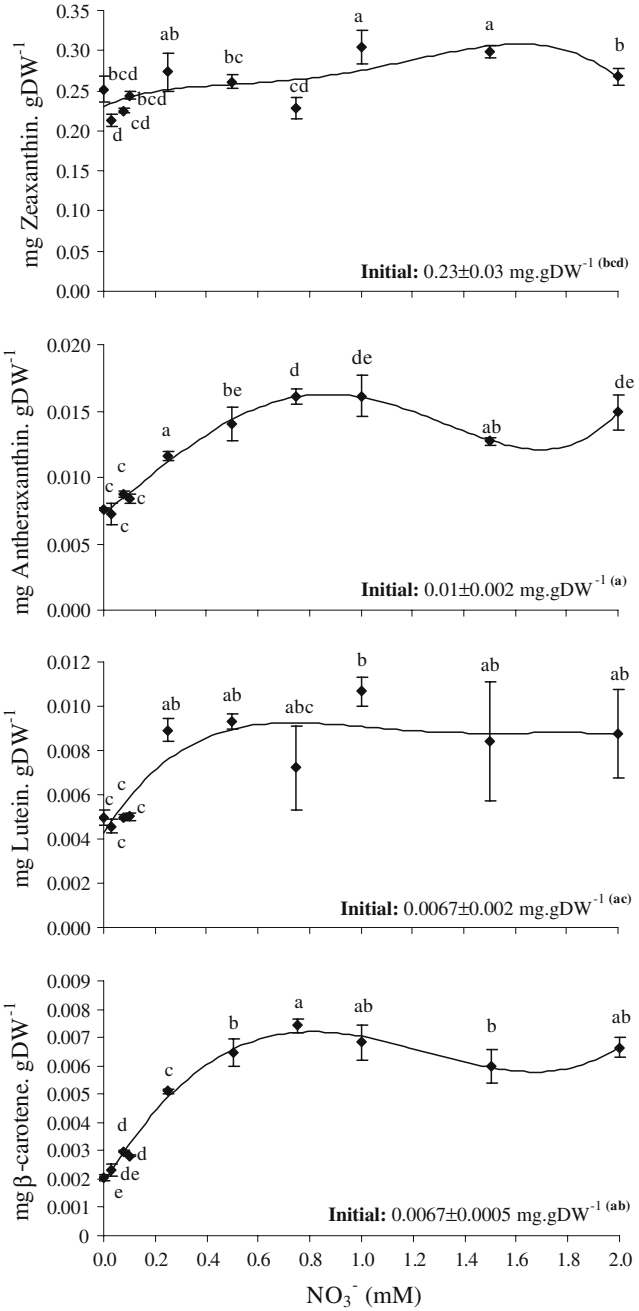

Fig. 2 Carotenoid concentrations (*zeaxanthin*, *antheraxanthin*, *lutein*, and *β-carotene*) of *G. tenuistipitata* cultivated under ten nitrate concentrations and exposed to PAR+UVR after 7 days of experimentation, expressed as mg of each carotenoid by g of dry weight of alga. Initial value is indicated in the figure. *Different letters* indicate significant differences observed with the Newman–Keuls a posteriori test ($p<0.05$). $n=3$. *Bars* indicate standard deviations

reaching values two times higher than those recorded at the beginning of the experiment. However, if supplied with at least 0.25 mM NO_3^-, the increment varied between 5.2 and 7.0 times. The highest amount of MAAs (2.52±0.07 mg gDW^{-1}) was recorded in samples supplied with 0.5 mM NO_3^-. Within the other N supplements, the total MAA amounts were maintained high, varying between 1.9 and 2.2 mg MAAs gDW^{-1} (Fig. 3). The percentage of shinorine at the end of the exposure time increased significantly ($F=136.02$, $p<0.05$, $df=10$) with the N concentration, meanwhile porphyra-334 decreased (Table 1). However, at 0.5 and 0.75 mM, shinorine represented the highest percentage (about 4.5%) and porphyra-334 the lowest (about 95.4%). The maximal difference between maximum and minimum values was of about 4.3% for both MAAs (Table 1).

The maximum quantum yield (F_v/F_m) was significantly affected by nitrate concentration, as these values increased with the rise of the N supply, reaching the lowest value of 0.26±0.03 (Fig. 4, $F=32.48$, $p<0.05$, $df=10$) at absence of nitrate. At 0.5 mM, F_v/F_m reached the maximum value (0.57±0.02), which was similar to the one recorded at the beginning of the experiment and to those recorded by the algae cultivated under concentrations above 0.75 mM NO_3^- (Fig. 4).

The parameters (ETR_{max}, α_{ETR}, and β) calculated after fitting the ETRs versus irradiance curves are shown in Table 2. These parameters were significantly affected by different nitrate treatments ($F=2.93$, $p<0.05$, $df=10$ for ETR_{max}; $F=18.04$, $p<0.05$, $df=10$ for α_{ETR}; and $F=5.30$, $p<0.05$, $df=10$ for β). ETR_{max} values decreased only in samples cultured under VS without NO_3^- supply (0 mM), while the α_{ETR} values decreased when *G. tenuistipitata* was treated with 0 to 0.1 mM NO_3^-. When the samples received at least 0.25 mM NO_3^-, the photosynthetic efficiency (α_{ETR}) was similar to the initial values and did not change under higher nitrate concentrations (Table 2). Above about 300 μmol photons $m^{-2}s^{-1}$, ETR decreased (photoinhibition) in algae cultured under all N supplies. The photoinhibition

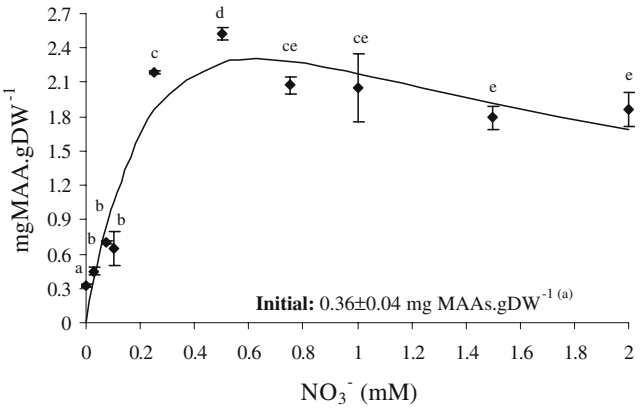

Fig. 3 MAA concentrations of *G. tenuistipitata* cultivated under ten nitrate concentrations and exposed to PAR+UVR after 7 days of experimentation, expressed as mg of MAAs by g of dry weight of alga. Initial value is indicated in the figure. *Different letters* indicate significant differences observed with the Newman–Keuls a posteriori test ($p<0.05$). $n=3$. *Bars* indicate standard deviations

Table 1 Percentages of mycosporine-like amino acid types (shinorine and porphyra-334) of *G. tenuistipitata* cultivated under ten nitrate concentrations and exposed to PAR+UVR after 7 days of experimentation

Nitrate concentration (mM)	MAA type	
	Shinorine	Porphyra-334
0	0.513±0.031 [a]	99.487±0.031 [a]
0.03	1.034±0.135 [b]	98.966±0.135 [b]
0.075	2.884±0.326 [d]	97.116±0.326 [d]
0.1	2.034±0.073 [c]	97.966±0.073 [c]
0.25	3.040±0.123 [d]	96.960±0.123 [d]
0.5	4.808±0.246 [f]	95.192±0.246 [f]
0.75	4.431±0.555 [f]	95.569±0.555 [f]
1	3.964±0.170 [e]	96.036±0.170 [e]
1.5	3.802±0.190 [e]	96.198±0.190 [e]
2	3.630±0.122 [e]	96.370±0.122 [e]

Different letters indicate significant differences observed with the Newman–Keuls a posteriori test ($p<0.05$). $n=3$

terms (β) varied between 0.002±0.0006 and 0.029±0.014 (Table 2).

These similar responses in the different variables evaluated in *G. tenuistipitata* cultivated with PAR+UVR and increasing N supplements are reflected in the correlation coefficients shown in Table 3. Most of the variables were significantly and positively correlated. The Chl*a* content was highly correlated with different parameters (α_{ETR} and F_v/F_m), as well as with the three carotenoids. β-Carotene content was strongly correlated with the F_v/F_m, α_{ETR}, and antheraxanthin content. Finally, total MAA concentration showed the highest correlation with antheraxanthin, lutein, and β-carotene amounts (Table 3).

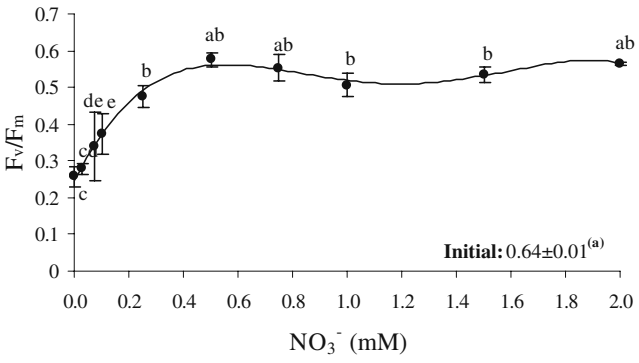

Fig. 4 Maximum quantum yield (F_v/F_m) estimated using *in vivo* chlorophyll *a* fluorescence of *G. tenuistipitata* cultivated under ten nitrate concentrations (0, 0.03, 0.075, 0.1, 0.25, 0.5, 0.75, 1, 1.5, and 2 mM) and exposed to PAR+UVR after 7 days of experimentation. Initial value is indicated in the figure. *Different letters* indicate significant differences observed with the Newman–Keuls a posteriori test ($p<0.05$). $n=3$. *Bars* indicate standard deviations

Discussion

In this study, the importance of N availability on the accumulation of photosynthetic pigments and photoprotectors, as MAAs and xanthophylls, in *G. tenuistipitata* grown under PAR+UVR has been shown. Ten different concentrations of nitrate were used from 0 to 2 mM. This species grows naturally in the coast of Hainan Island, where the concentration of nitrate is less than 0.16 μM (Huang et al. 2003). However, benthic macroalgae such as *G. tenuistipitata* are exposed to constant washing and renewal of seawater which supplies them with new nutrients (McLachlan 1982). In this study, the algae used seem to be acclimated to laboratory conditions, where they have been cultured with VS solution (0.5 mM NO_3^-) for years.

The photosynthetic parameters ETR_{max} and F_v/F_m decreased under low nitrate supply in *G. tenuistipitata*. This result was expected because more than half of the total N is allocated in the photosynthetic apparatus in higher plants (Makino and Osmond 1991). Additionally, an increase of pigments and MAAs (as N compounds) was found, and these results can be a consequence of the increase in photosynthetic activity under high N supply. The decrease of Chl*a* in N-deficient higher plants is accompanied by a decrease of rubisco concentration (Seemann et al. 1987). Thus, a part of the decrease in photosynthetic capacity occurring with N deficiency can be ascribed to the diminished amount of Calvin cycle enzymes (Sugiharto et al. 1990). In addition, considering N-deficient plants, the thermal dissipation may account for 64–73% of the light absorbed by PSII antenna, whereas in N-replete plants, it is in the range of 36–59% (Verhoeven et al. 1997). The fraction of light absorbed in PSII and used in photochemistry decreases with N deficiency. Taking into account that the fraction of light absorbed by PSII in red algae is low (0.15) (Grzymski et al. 1997; Figueroa et al. 2003), this value could diminish with N deficiency in *G. tenuistipitata* as in higher plants, and then, much higher decrease of ETR_{max} could be expected than the one estimated in this study.

In *G. tenuistipitata*, the maximum quantum yield of fluorescence (F_v/F_m) was positively correlated to all photosynthetic pigments (Chl*a* and carotenoids) and also to MAAs. According to the observed results, the photosynthetic efficiencies were lower under low availability of NO_3^-. However, the ETR_{max} was maintained at the same level as that in higher concentrations of NO_3^-. These results could be explained as low concentrations of nitrate (lower than 0.25 mM NO_3^-) favor a small antenna size, as it occurs in high-light conditions. This downregulation of PSII size is reported for higher plants (Ballottari et al. 2007), but few studies have been conducted with macroalgae (see Figueroa et al. 2003). The decreases of ETR_{max}

Table 2 Maximal electron transport rate (ETR_{max}), the slope of the ETR versus irradiance function (α_{ETR}), and the photoinhibition parameter (β) at the beginning (initial) and after 7 days of cultivation of *G. tenuistipitata* under ten nitrate concentrations and exposed to PAR+UVR

Time	Nitrate concentration (mM)	ETR_{max} (μmol electrons $m^{-2}s^{-1}$)	α_{ETR}	β
Initial	–	5.44±0.76 [a]	0.055±0.006 [a]	0.017±0.003 [ab]
7 days	0	2.86±0.28 [b]	0.023±0.002 [c]	0.008±0.004 [ab]
	0.03	3.64±1.08 [ab]	0.027±0.005 [bc]	0.002±0.001 [a]
	0.075	3.60±0.83 [ab]	0.029±0.004 [c]	0.009±0.003 [ab]
	0.1	3.87±1.20 [ab]	0.037±0.008 [b]	0.006±0.001 [ab]
	0.25	4.40±0.99 [ab]	0.046±0.004 [a]	0.009±0.007 [ab]
	0.5	4.78±0.61 [ab]	0.056±0.003 [a]	0.015±0.003 [ab]
	0.75	5.02±0.73 [a]	0.054±0.006 [a]	0.030±0.014 [c]
	1	4.32±0.27 [ab]	0.051±0.008 [a]	0.008±0.001 [ab]
	1.5	4.11±0.19 [ab]	0.048±0.002 [a]	0.012±0.010 [ab]
	2	4.58±0.17 [ab]	0.055±0.002 [a]	0.021±0.006 [bc]

Different letters indicate significant differences between values ($p<0.05$) for the parameter values ($n=3$)

and F_v/F_m in low-N-grown algae under PAR+UVR indicate photoinhibition. The susceptibility to photoinhibition is larger in plants grown with low N than in those grown with high N availability (Grassi et al. 2001). However, the positive effect of NO_3^- on ETR was only observed until about 300 μmol of photons $m^{-2}s^{-1}$. Above this irradiance, photoinhibition was not reduced in the presence of a high NO_3^-, probably by increasing respiration rates. Cabello-Pasini and Figueroa (2005) showed a decrease in the ETR/gross photosynthesis ratio in the green alga *Ulva rigida* cultivated under high nitrate supply. In the case of *Ulva rotundata* cultured under N-limited treatment, there was a lower capacity of acclimation to high irradiances, while when receiving high supply of ammonium, this alga was able to adjust its photosynthetic apparatus, increasing respiration rate and the photosynthetic capacity (Henley et al. 1991). In the case of *G. tenuistipitata* cultured above 0.25 mM nitrate, values of F_v/F_m and α_{ETR} were the same as those for initial samples.

Ultraviolet radiation can produce photoinhibition due to the accumulation of reactive oxygen species. However, UVR can stimulate the accumulation of MAAs, which act mainly as UV screen photoprotectors but are also antioxidant molecules (Korbee et al. 2005; De la Coba et al. 2009). Ultraviolet radiation is a light signal that stimulates the accumulation of MAAs in red algae (Karsten et al. 1998; Yakovleva and Titlyanov 2001). In fact, previous results for *G. tenuistipitata* (article in preparation) indicated the UV range of the spectrum to stimulate the synthesis of MAAs, as under only PAR, MAAs do not increase, independent of the concentration of N in the culture medium. The same occurred with the dinoflagellate *Heterocapsa* sp. cultured with two different concentrations of NO_3^- (Korbee et al. 2010). Thus, after the pre-acclimation period at 0.5 mM NO_3^- under PAR without UVR, MAA content in *G. tenuistipitata* was low, and this content increased after 1 week of exposure to UVR in the presence of N enrichment above 0.25 mM NO_3^-. Under the experimental conditions, a nitrate concentration-dependent manner was observed in the accumulation of MAAs in this macroalgae. Under low-N treatments (0 and 0.03 mM NO_3^-), the content of MAAs was only 0.5 mg gDW^{-1} and seems to be not enough to protect the algae against photoinhibition, in contrast to algae growing at high N

Table 3 Pearson correlation values obtained among dependent variables after treatment of *G. tenuistipitata* with PAR+UVR and ten nitrate supplements (0 to 2 mM)

Values range between −1 and 1. Italicized values are significantly correlated. Dependent variables: *Chla* (chlorophyll *a*), ETR_{max}, α, β, F_v/F_m, antheraxanthin, zeaxanthin, lutein, β-carotene, and MAAs. $n=33$

	ETR_{max}	α	β	F_v/F_m	Antherax.	Zeax.	Lutein	β-Carot.	MAAs
Chl *a*	*0.62*	*0.89*	*0.56*	*0.88*	*0.88*	*0.50*	*0.82*	*0.93*	*0.73*
ETR_{max}		*0.74*	*0.49*	*0.75*	*0.50*	0.16	*0.38*	*0.64*	0.34
α			*0.51*	*0.94*	*0.79*	*0.41*	*0.67*	*0.89*	*0.67*
β				*0.55*	*0.56*	−0.01	0.24	*0.62*	*0.34*
F_v/F_m					*0.75*	*0.35*	*0.60*	*0.90*	*0.58*
Antheraxanthin						*0.47*	*0.74*	*0.93*	*0.79*
Zeaxanthin							*0.77*	*0.39*	*0.52*
Lutein								*0.67*	*0.71*
β-carotene									*0.70*

supply with content of MAAs of 2.5 mg gDW^{-1}. Therefore, the antioxidant capacity of MAAs (Dunlap and Yamamoto 1995; De la Coba et al. 2009) and its dependence to N supply can explain the highest photoprotection under PAR +UVR in high N supply.

In other red algae, including three different *Porphyra* spp., *G. lanceola*, and *A. armata*, MAAs have been found to be related to N availability (Figueroa et al. 2008; Huovinen et al. 2006; Korbee-Peinado et al. 2004; Korbee et al. 2005). However, in all of these studies, the source of N used was ammonium, and only three different concentrations were tested, instead of the use of NO_3^- and ten different concentrations as was done for *G. tenuistipitata* in this study. *A. armata* cultivated in tanks also showed an increase of MAA content related to N availability (Figueroa et al. 2008). MAAs increased with the total ammonium (TAN) flux, but only until values lower than 0.1 mMh^{-1}, probably because of lower removal ability of the TAN rates in this species at higher fluxes of ammonium (Figueroa et al. 2008).

In this study, two types of MAAs were identified in *G. tenuistipitata*, shinorine and porphyra-334. The latest one was the majority, as recorded in other *Gracilaria* species (Huovinen et al. 2004). However, Cardozo et al. (2006) identified two other MAAs in *G. tenuistipitata*, asterina-330 and palythine. It is possible that the concentrations of the last two MAAs were very low (traces) and thus were undetected in our analysis. Both shinorine and porphyra-334 are composed by two atoms of N (Carreto et al. 2005). They varied in an N-dependent form, shinorine increased in relative concentration with N availability, and porphyra-334 decreased. The difference in the molecular structure of both MAAs is negligible, and in both cases they are synthesized directly from mycosporine-glycine (Carreto et al. 2005). Moreover, both MAAs have similar UV screen and antioxidant properties (Conde et al. 2004; De la Coba et al. 2009). Thus, it is not clear why they varied between treatments in our study.

Four different carotenoids were detected in this species, zeaxanthin, antheraxanthin, lutein, and β-carotene. Schubert et al. (2006) described the existence of three main groups of red algae according to their carotenoid composition. *G. tenuistipitata* belongs to the group in which zeaxanthin is the main xanthophyll. This group includes species in which the β-pathway is dominant or exclusive. Zeaxanthin, as the major carotenoid, has been previously described in other red algae such as *Galdieria sulphuraria* (Marquardt 1998), *Porphyridium cruentum* (Marquardt 1998; Schubert et al. 2006), and *Gracilaria gracilis* (Rmiki et al. 1996). The zeaxanthin-to-antheraxanthin epoxidation takes place by the action of zeaxanthin epoxidase. However, in most red algae with β-xanthophylls, biosynthesis stops at zeaxanthin (Marquardt and Hanelt 2004). Regarding the photosynthetic pigments (carotenoids and Chl*a*), similar patterns were observed in *G. tenuistipitata* cultured at ten different concentrations of nitrate, i.e., at lower nitrate supplements, there were lower pigments amounts, with the exception of zeaxanthin content. The zeaxanthin, the major carotenoid, did not show a clear nitrate concentration dependence. In the case of lutein, the maximum amount was reached at 0.25 mM NO_3^- whereas the maximal concentrations of Chl*a*, antheraxanthin, and β-carotene were reached under 0.5 mM NO_3^-.

Although xanthophyll cycle pigments are not N compounds, N availability seems to influence the accumulation of these pigments in *G. tenuistipitata*, mainly for antheraxanthin. As far as we know, this is the first time that xanthophyll pigments are related to N supply in macroalgae. This result could be explained by the large amount of carbonate skeletons available for the synthesis of carotenoids under high availability of N. However, more studies are needed in order to test specifically the effect of different nutrient conditions in those pigments. An increase in the concentrations of xanthophyll cycle pigments have been observed recently when increasing N availability for diatoms, dinoflagellates, and prymnesiophytes (Buma et al. 2000; Korbee et al. 2010; Van de Poll and Buma 2009), as well as in a natural phytoplankton community from temperate latitudes after exposure to UVR (Mohovic et al. 2006).

The presence of a functional xanthophyll cycle in Rhodophyta is controversial. The presence of zeaxanthin and antheraxanthin is common among red algae (Esteban et al. 2009). However, the variations between them were more likely due to differential rates of synthesis and degradation of xanthophylls than to the operation of a xanthophyll cycle, as analyzed for 13 red macroalgae collected in northern Spain (Esteban et al. 2009). Among them, no *Gracilaria* species was analyzed. However, three different xanthophylls (antheraxanthin, zeaxanthin, and violaxanthin) have been observed in different species of *Gracilaria* (Schubert et al. 2006), but only in *G. birdiae* was an active xanthophyll cycle found (Ursi et al. 2003). More experiments are needed in order to evaluate the role of the xanthophyll cycle in *G. tenuistipitata*, and they could include different UVR–PAR doses, as well as maintain *G. tenuistipitata* in dark conditions, to identify the possible activation or de-activation of this cycle.

In conclusion, this study has shown for the first time that *G. tenuistipitata* cultured under laboratory-controlled conditions has the ability to accumulate high amounts of MAAs (N compound), following a nitrate concentration-dependent manner, with the accumulation being saturated around 0.5 mM nitrate. However, other non-N-compounds, as carotenoids, show a similar pattern as that of MAAs. In fact, carotenoids, Chl*a*, and photosynthetic parameters

decreased under lower N supplies and recovered to values similar to the initial ones at high N availability. Our results suggest a high potential to acclimation and photoprotection against stress factors (including high PAR and UVR) directly related to N availability for the *G. tenuistipitata*.

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