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NH_4^+ enrichment and UV radiation interact to affect the photosynthesis and nitrogen uptake of *Gracilaria lemaneiformis* (Rhodophyta)

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ABSTRACT

Solar ultraviolet radiation (UVR, 280–400 nm) is known to inhibit the photosynthesis of macroalgae, whereas nitrogen availability may alter the sensitivity of the algae to UVR. Here, we show that UV-B (280–315 nm) significantly reduced the net photosynthetic rate of *Gracilaria lemaneiformis*. This inhibition was alleviated by enrichment with ammonia, which also caused a decrease in dark respiration. The presence of both UV-A (315–400 nm) and UV-B stimulated the accumulation of UV-absorbing compounds. However, this stimulation was not affected by enrichment with ammonia. The content of phycoerythrin (PE) was increased by the enrichment of ammonia only in the absence of UVR. Ammonia uptake and the activity of nitrate reductase were repressed by UVR. However, exposure to UVR had an insignificant effect on the rate of nitrate uptake. In conclusion, increased PE content associated with ammonia enrichment played a protective role against UVR in this alga, and UVR differentially affected the uptake of nitrate and ammonia.

1. Introduction

Marine macroalgae play a key role in the coastal carbon cycle, contributing to the coastal primary productivity and playing key roles in coastal fisheries. Solar UV radiation (UVR, 280–400 nm) is known to have harmful effects on physiological function in these algae (Gao and Xu, 2010; Häder et al., 2011). Although the Montreal protocol has been effective in slowing the thinning of the ozone layer, the cooling effect in the stratosphere accompanying global warming would stimulate the depletion of ozone (McKenzie et al., 2007). Therefore, differential increases in UV-B irradiance have been observed at different latitudes (Hegglin and Shepherd, 2009).

Solar UVR affects growth (Gao and Xu, 2008; Henry and Van Alstyne, 2004), photosynthesis and pigmentation (Aguilera et al., 1999; Davison et al., 2007; Schmidt et al., 2010a), uptakes of nutrients (Hessen et al., 1995; Viñegla et al., 2006), and even damages DNA (Atienzar et al., 2000; Pakker et al., 2000) and alters ultrastructure (such as chloroplasts and mitochondria) (Holzinger and Lütz, 2006; Schmidt et al., 2010b) in macroalgae. However, moderate levels of UV-A can stimulate the photosynthesis or growth of particular red algae under certain conditions (Gao and Xu, 2008; Xu and Gao, 2009).

The strategies against UVR that algae have acquired during evolution include: (1) the accumulation of UV-screening compounds; (2) photorepairing mechanisms; and (3) photoprotective mechanisms. Mycosporine-like amino acids (MAAs), known as important UV-absorbing compounds, are water-soluble, nitrogen-containing substances. Their accumulation remediates the UV-induced inhibition of algae (see the review by Oren and Gunde-Cimerman, 2007 and literature therein). Repair mechanisms include the resynthesis of damaged proteins (Häder et al., 2002) and the photo-repair of UV-damaged DNA molecules (Pakker et al., 2000). Photoprotective mechanisms are usually stimulated in the presence of UVR (Poppe et al., 2003; Roleda et al., 2010, 2009). These protective or repair capabilities are often affected by the availability of nutrients (Davison et al., 2007; Zheng and Gao, 2009). Enrichment with nitrate (Zheng and Gao, 2009) or ammonia (Korbee et al., 2005; Korbee-Peinado et al., 2004) stimulated the synthesis of MAAs, alleviating the UV-related inhibition of photosynthesis or growth. In another study, however, enrichment with ammonia did not enhance the accumulation of MAAs in the red alga *Grateloupia lanceola* if exposed to UVR (Huovinen et al., 2006).

Gracilaria lemaneiformis (Bory) has been commercially farmed for food and agar production and is playing a significant role in the remediation of the eutrophicated waters along the Chinese coast (Fei, 2004; Zhou et al., 2006). We hypothesized that this economically important red alga may respond to solar UVR differentially if supplied with additional ammonia. We tested this hypothesis under solar radiation with or without UVR in NH_4^+-enriched or ambient seawater.

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2. Materials and methods

2.1. Material and cultivation

Thalli of *G. lemaneiformis* Bory were collected from an area in which this alga is farmed at a depth of 0.5 m around Nanao Island (23.3°N, 116.6°E), Shantou, China during May 2009. The thalli were transported to the laboratory in an insulated cooler (5 °C) within 2 h. Representative individuals were selected, and branches (1 g total fresh weight (FW)) were taken from these thalli and cultured in quartz tubes containing 250 mL of autoclaved natural seawater enriched with 50 μM of phosphate to guarantee a phosphorus-replete state if grown under solar radiation (Xu and Gao, 2009). The biomass density in the culture vessels was maintained at approximately 4 g FW L^{-1} (equivalent to the medium density found in the farmed area) during the entire experiment by removing partial thalli every 48 h when the culture medium was renewed. The cultures were maintained in a water bath, through which seawater was circulated by a refrigerating circulator (CAP-3000, Tokyo Rikakikai Co. Ltd., Tokyo, Japan) to maintain the water temperature at 25 °C. The cultures were aerated with ambient air of 390 ppmv CO_2 at a rate of 0.6 L min^{-1}. The thalli within the tubes did not shade each other and were therefore exposed equally to solar radiation.

2.2. Enrichment of NH_4^+—N

Two N concentrations, 51.85 μM (L–N, natural seawater with 50.12 μM nitrate and 1.73 μM ammonia) and 151.85 μM (H–N, natural seawater enriched with 100 μM NH_4Cl), were prepared in the culture medium.

2.3. Solar radiation treatment and measurements

The thalli were exposed to three different solar radiation treatments: (1) photosynthetically active radiation (PAR, P treatment), quartz tubes wrapped with Ultraphan 395 film (UV Opak, Digefra, Munich, Germany), transmitting solar radiation above 395 nm; (2) PAR + UV-A (PA treatment), quartz tubes wrapped with Folex 320 (Montagefolie, No. 10155099, Folex, Dreieich, Germany), transmitting solar radiation above 320 nm; and (3) PAR + UV-A + UV-B (PAB treatment), quartz tubes wrapped with Ultraphan 295 (UV Opak, Digefra, Munich, Germany), transmitting solar radiation above 295 nm. The characteristics of transmission through these cut-off foils and the quartz tube are reported elsewhere (Zheng and Gao, 2009). The cut-off filters uniformly cut down 4% of the PAR in water due to their reflection (Gao et al., 2007). An Eldonet broadband filter radiometer (Eldonet XP; Real Time Computer, Möhrendorf, Germany) was used to monitor the incident solar radiation. This device has three channels for PAR (400–700 nm), UV-A (315–400 nm) and UV-B (280–315 nm) irradiance, respectively (Häder et al., 1999), and has been used worldwide (certificate No. 2006/BB14/1). It was calibrated regularly with support from the maker every year against a double monochromator spectroradiometer and a certified calibration lamp. The measured and exposed UV-A wavebands differed by approximately 5 nm. This difference resulted in approximately 2% less UV-A.

2.4. Measurement of photosynthetic rate

The photosynthetic oxygen evolution rate was determined using a Clark-type oxygen electrode (Chlorolab-3, Hansatech, Norfolk, UK) at 25 °C. PAR was provided by a halogen lamp and measured with a light meter (QRT1, Hansatech, Norfolk, UK). Different levels (0–600 μmol photons m^{-2} s^{-1}) of PAR were obtained by altering the distance between the light source and the electrode chamber. The dark respiration rate (R_d) was measured by covering the chamber with a black sheet of cloth. The apparent photosynthetic efficiency (α) and the PAR-saturated net photosynthetic rate (P_{max}) were estimated from the *P–E* curves (Henley, 1993).

2.5. Determination of UV-absorbing compounds (UVACs) and pigment contents

Approximately 0.1 g (FW) of thalli was ground and extracted in 10 mL absolute methanol at 4 °C in darkness for 24 h (Gao and Xu, 2008). The absorbance of the supernatant after centrifugation (5000*g*, 15 min, 4 °C) was measured from 250 to 750 nm using a scanning spectrophotometer (DU 530, Beckman Coulter, Fullerton, CA, USA). The total absorbance of the UVACs was estimated at 325 nm (Dunlap et al., 1995). Chl *a* concentration was calculated according to Wellburn (1994). Phycoerythrin (PE) and phycocyanin (PC) were extracted in 0.1 M phosphate buffer (pH 6.8) with 0.2 g FW of thalli (ground with quartz sand), and their contents were determined according to Siegelman and Kycia (1978).

2.6. Determination of nitrogen uptake rate

The uptake rate of nitrogen was determined by the clearance of nitrate or ammonia from the culture media over a given time interval and expressed by the following equation: nitrogen uptake rate = $(N_o - N_t) \times V \times W_o^{-1} \times 12^{-1}$, where N_o is the nitrogen concentration after medium renewal, N_t the concentration after 12 h of photoperiod, V the volume of the culture medium and W_o the fresh weight (FW) of the alga. The values of light and temperature were the same as those in the cultures. The concentrations of nitrate and ammonia were measured according to Jones (1984) and Slawyk and MacIsaac (1972), respectively.

2.7. Measurement of nitrate reductase (NR) activity

Nitrate reductase (NR) activities were assayed in the thalli grown under the different radiation or nutrient treatments according to Corzo and Niell (1991). As the activity of NR usually displays circadian periodicity with a maximum after several hours during the light period (Deng et al., 1991; Velasco et al., 1989), the measurement was performed during the local noon period (13:00). Approximately 0.2 g (FW) of thalli was incubated for 1 h at 30 °C in darkness in the reaction solution (10 mL), which contained 0.1 M phosphate buffer, 0.5 mM EDTA, 0.1% propanol (v/v), 0.01 mM glucose, and 50 mM KNO_3, with a pH value of 8.0. The mixture was prepared in an anaerobic state by sparging N_2 gas for 2 min before used. The concentration of nitrite produced was determined colorimetrically at 540 nm (Strickland and Parsons, 1972). The NR activity was expressed as μmol NO_2^- h^{-1} g^{-1} FW.

2.8. Data analysis

Inhibition caused by UVR was estimated as: $(G_P - G_U)/G_P \times 100$, where G_P and G_U, respectively, represent the rates for the thalli grown under PAR alone and under PAR + UV-A or PAR + UV-A + B. A one-way ANOVA and a Tukey test were used to analyze the differences among treatments. The significance level was set at 0.05.

3. Results

The *G. lemaneiformis* thalli grown at different N levels received daily solar doses that varied over the ranges of 1.40–7.14 MJ m^{-2} for PAR, 0.28–1.16 MJ m^{-2} for UV-A, and 0.01–0.04 MJ m^{-2} for UV-B, respectively (Fig. 1). The proportions of PAR, UV-A and

UV-B were approximately 197:33:1 on average. The noontime levels of PAR, UV-A and UV-B ranged from 119.17 to 349.56 W m^{-2}, 22.37 to 53.93 W m^{-2} and 0.81 to 1.85 W m^{-2}, respectively.

After acclimation to the experimental conditions for 16 days, photosynthetic O_2 evolution was examined in the thalli of *G. lemaneiformis* grown at the different nitrogen levels under different solar radiation with or without UVR (Table 1). The maximal net photosynthesis (P_{max}) of the thalli exposed to PAR + UV-A + B decreased by approximately 40% and 20% in the low and NH_4^+-enriched seawater, respectively, reflecting lower UVR-induced inhibition in the N-enriched seawater. The inhibition of P_{max} caused by UV-A was insignificant ($P > 0.05$) at the low- and at the high-N levels, indicating the specific harmful effects of UV-B (Fig. 2). The apparent photosynthetic efficiency (α) was not affected by the enrichment of NH_4^+—N or exposure to UVR ($P > 0.1$), except that a decrease was found in NH_4^+-enriched seawater with UVR ($P < 0.05$). Under all three radiation treatments, the dark respiration rate (R_d) was repressed by the enrichment of NH_4^+—N ($P < 0.05$). Additionally, exposure to UVR enhanced the R_d of the thalli grown in the low N seawater ($P < 0.05$), but such stimulation was not found in the thalli grown in NH_4^+-enriched seawater ($P > 0.1$).

The Chl *a* content increased with the NH_4^+ enrichment under all radiation treatments except for PAR + UV-A, for which insignificant changes were found between the low and high N levels ($P > 0.1$).

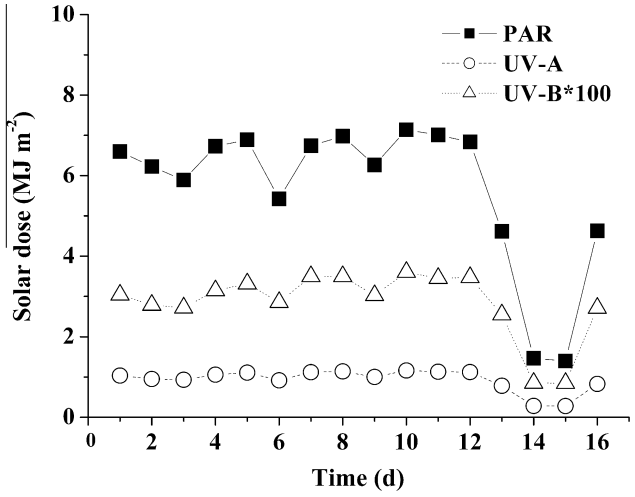

Fig. 1. Daily solar doses of PAR, UV-A and UV-B over the experimental period from May 5 to 20, 2009.

Table 1
Photosynthetic parameters derived from the *P–E* curves of *G. lemaneiformis* thalli grown under different nitrogen and solar radiation treatments.[a]

Treatments		P_{max} (μmol O_2 h^{-1} g^{-1} FW)	α (μmol O_2 h^{-1} g^{-1} FW)/(μmol photons m^{-2} s^{-1})	R_d (μmol O_2 h^{-1} g^{-1} FW)
L–N	P	47.26 ± 2.03^a	0.25 ± 0.02^a	−9.47 ± 0.23^a
	PA	50.63 ± 6.04^a	0.20 ± 0.02^a	−14.00 ± 0.69^b
	PAB	27.58 ± 3.87^b	0.23 ± 0.05^a	−12.40 ± 0.72^b
H–N	P	57.21 ± 4.17^c	0.30 ± 0.04^a	−7.47 ± 0.46^c
	PA	52.95 ± 1.90^{ac}	0.19 ± 0.03^b	−7.73 ± 0.23^c
	PAB	44.95 ± 1.31^d	0.18 ± 0.02^b	−8.53 ± 0.92^{ac}

[a] The thalli were cultured for 16 days in the ambient (L–N) or NH_4^+-enriched (H–N) seawater with or without UVR (P, PAR; PA, PAR + UV-A; PAB, PAR + UV-A + B). The *P–E* curves were measured under PAR alone. P_{max}, the maximal photosynthetic rate (μmol O_2 h^{-1} g^{-1} FW); α, the apparent photosynthetic efficiency (μmol O_2 h^{-1} g^{-1} FW)/(μmol photons m^{-2} s^{-1}); R_d, the dark respiration rate (μmol O_2 h^{-1} g^{-1} FW). Within each column of the data, different superscript letters or letter combinations indicate significant difference at $P = 0.05$. Data are means ± SD ($n = 3$).

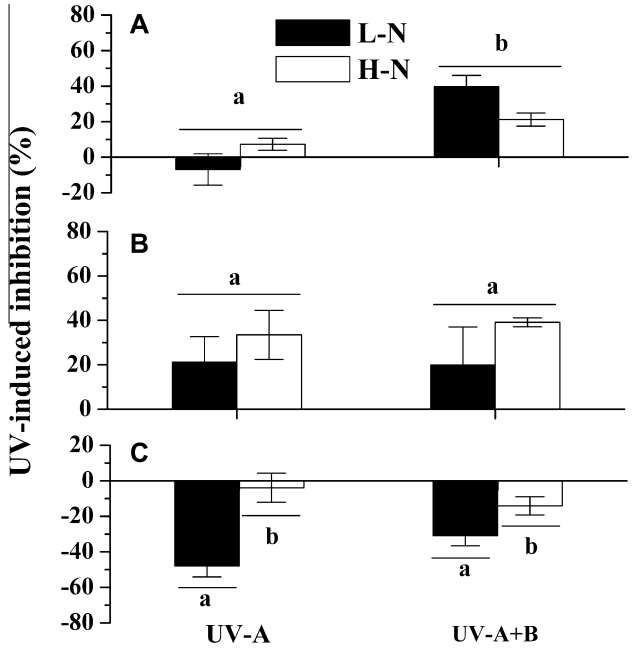

Fig. 2. UV-induced inhibition of the maximal photosynthetic rate (P_{max}, A), apparent photosynthetic efficiency (α, B) and dark respiration rate (R_d, C) in *G. lemaneiformis* thalli grown for 16 days in the ambient (L–N) or NH_4^+-enriched (H–N) seawater under different solar radiation treatments with or without UVR. Significant ($P < 0.05$) differences among the treatments are indicated by different lowercase letters. Vertical bars represent ± SD of the means ($n = 3$).

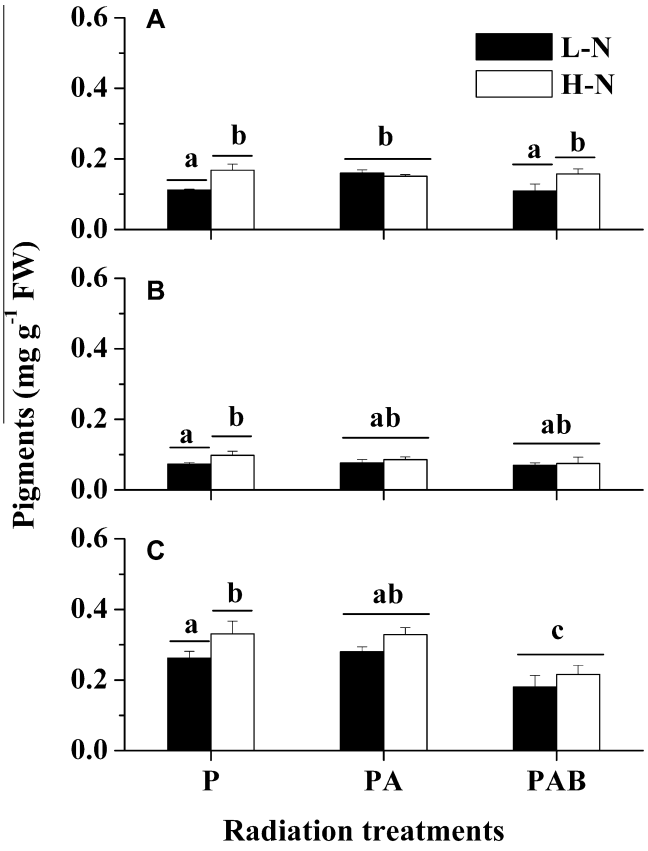

Fig. 3. Chlorophyll *a* (A), phycocyanin (PC, B) and phycoerythrin (PE, C) contents of *G. lemaneiformis* thalli grown for 16 days in the ambient (L–N) or NH_4^+-enriched (H–N) seawater under different solar radiation treatments with or without UVR. Significant ($P < 0.05$) differences between treatments are indicated by different lowercase letters. Vertical bars represent ± SD of the means ($n = 4$).

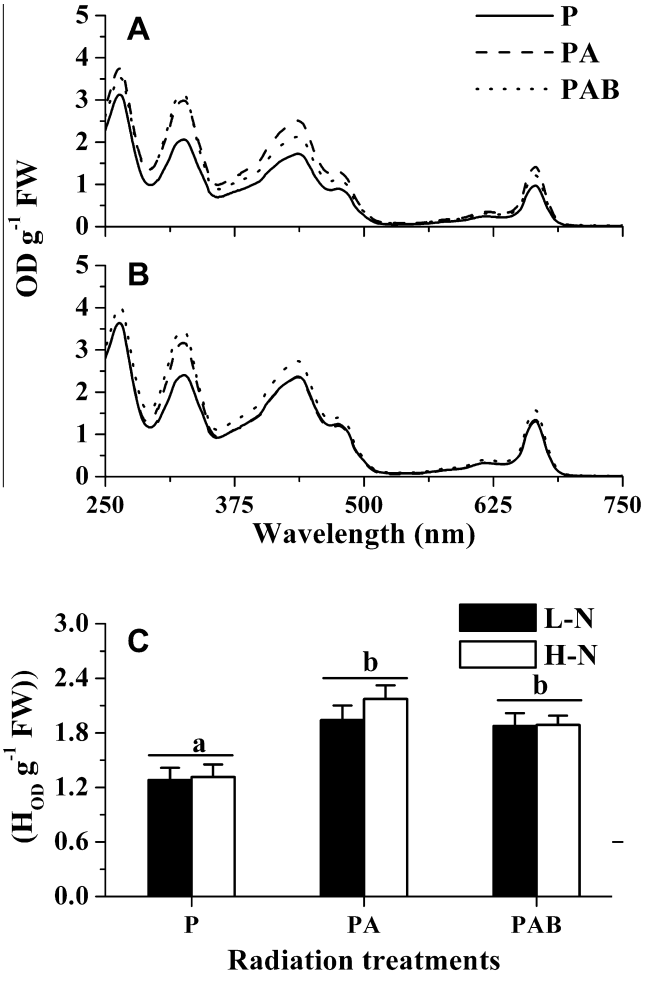

Fig. 4. Absorption spectra of the methanol extracts from the thalli (A, L–N; B, H–N) and absorptivity (C) of the UV-absorbing compounds (UVACs) of *G. lemaneiformis* thalli grown for 16 days in the ambient (L–N) or NH_4^+-enriched (H–N) seawater under different solar radiation treatments with or without UVR. Significant ($P < 0.05$) differences between treatments are indicated by different lowercase letters. Vertical bars in (C) represent ± SD of the means ($n = 4$).

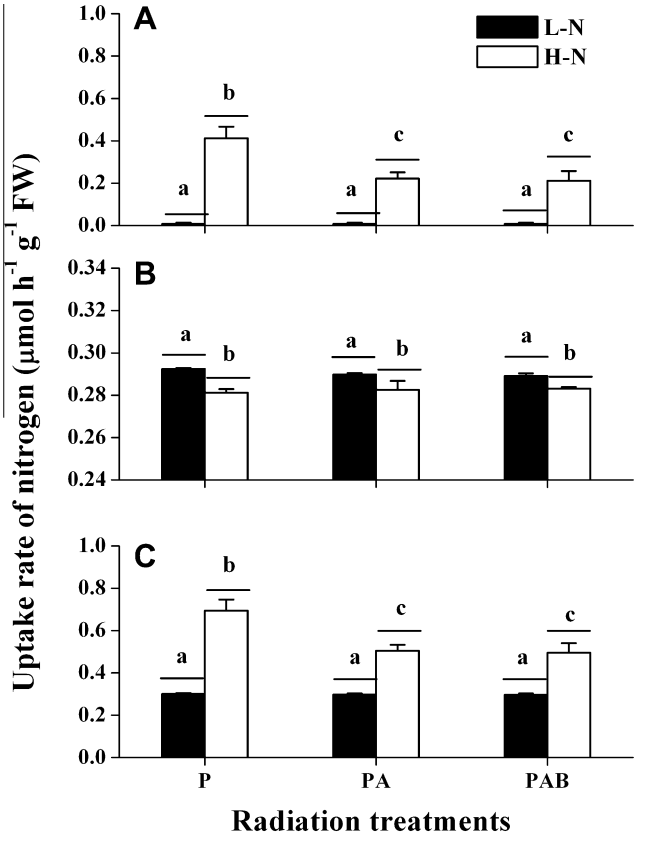

Fig. 5. Uptake rate of nitrogen (A, NH_4^+—N; B, NO_3^-—N; C, total N) of *G. lemaneiformis* thalli grown for 16 days in the ambient (L–N) or NH_4^+-enriched (H–N) seawater under different solar radiation treatments with or without UVR. Significant ($P < 0.05$) differences between treatments are indicated by different lowercase letters. Vertical bars represent ± SD of the means ($n = 4$).

UV-A significantly ($P < 0.001$, Fig. 3A) produced an increase in the Chl *a* content under the N-limited condition. In contrast, the effects of both UV-A and UV-B on the content of phycocyanin (PC) were insignificant ($P > 0.1$); however, UV-B significantly reduced the content of phycoerythrin (PE) ($P < 0.05$, Fig. 3B and C). The growth of the thalli under NH_4^+ enrichment increased the content of PC and PE ($P < 0.05$, Fig. 3B and C) in only the PAR-alone treatment.

As indicated by the absorption peak at 325 nm, UV-absorbing compounds (UVACs) accumulated more in thalli grown in the presence of UV-A or UV-A + B (Fig. 4). UVR significantly ($P < 0.01$) increased the absorptivity of UVACs in the thalli despite the nitrogen concentrations. However, no significant difference in the absorptivity was found between the PAR + UV-A and PAR + UV-A + B treatments ($P > 0.05$). Regardless of the radiation treatments, no increase in the absorptivity was found in the NH_4^+-enriched cultures ($P > 0.1$).

In contrast to the control (low N seawater), enrichment with NH_4^+ enhanced the total nitrogen uptake (Fig. 5). Exposure to UVR had no effect on the NO_3^-—N uptake by the thalli grown at both N levels ($P > 0.05$), but it significantly inhibited the NH_4^+—N uptake of the thalli grown in the NH_4^+-enriched seawater ($P < 0.001$, Figs. 5 and 6A), although no significant difference was observed between the PAR + UV-A and PAR + UV-A + B treatments ($P > 0.5$). This finding reflected the inhibition caused primarily by UV-A. Therefore, the uptake ratio of NH_4^+—N to NO_3^-—N in the thalli grown in the NH_4^+-enriched seawater was higher in the absence and lower in the presence of UVR, respectively (Fig. 6B). The NH_4^+ enrichment significantly decreased the activity of nitrate reductase (NR) in *G. lemaneiformis* ($P < 0.01$, Fig. 7). Despite the insignificant difference between the PAR + UV-A and PAR + UV-A + B treatments ($P > 0.1$), the presence of UVR inhibited the NR activity of the thalli grown at both N levels ($P < 0.05$). This result indicated that this inhibition was induced by UV-A.

4. Discussion

In this study, photosynthesis by *G. lemaneiformis* decreased significantly in the presence of UV-B, which induced a lower extent of inhibition in the thalli grown at the high ammonium level. The enrichment with ammonia had no effect on the content of UVACs. In the absence of UVR, the contents of PC and PE were enhanced by the enrichment of ammonia. However, this enhancement was not found in the presence of UVR. In addition, UVR inhibited the uptake rate of ammonia but not that of nitrate in *G. lemaneiformis*.

The presence of UV-B inhibited photosynthesis in many species of macroalgae (Davison et al., 2007; Figueroa and Gómez, 2001; Schmidt et al., 2010b), including *G. lemaneiformis*, the alga used in this study (Gao and Xu, 2008). In the present study, we found that enrichment with ammonia reduced the UV-B-related inhibition. Previously, enrichment with nitrate was shown to have relieved the UV-B-induced inhibition of the photosynthesis and enhanced the accumulation of MAAs in *G. lemaneiformis* (Zheng

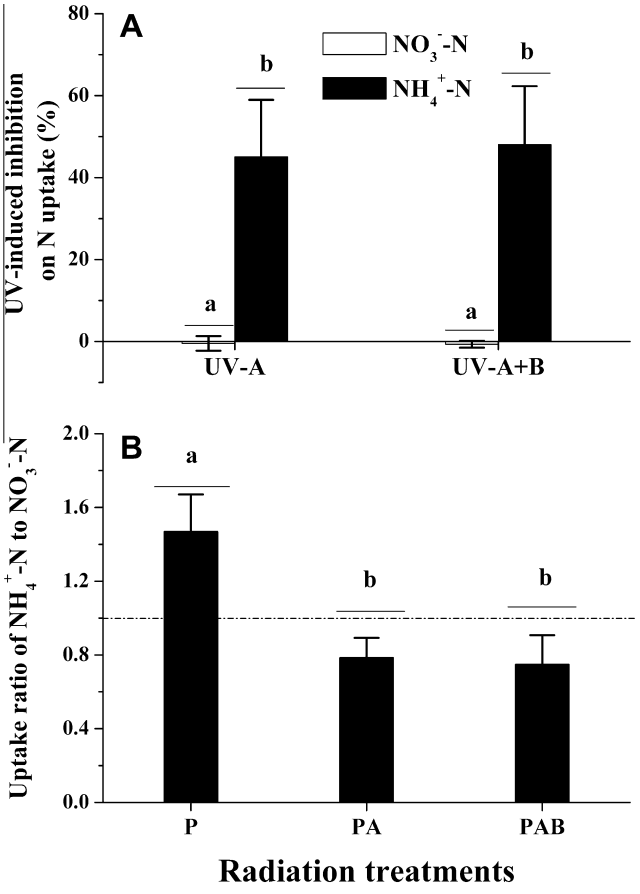

Fig. 6. UV-induced inhibition of N uptake (A) and uptake ratio of NH_4^+—N to NO_3^-—N (B) in *G. lemaneiformis* thalli grown for 16 days in NH_4^+-enriched seawater under different solar radiation treatments with or without UVR. Significant ($P < 0.05$) differences between treatments are indicated by different lowercase letters. Dashed line indicates a ratio equal to one. Vertical bars represent ± SD of the means ($n = 4$).

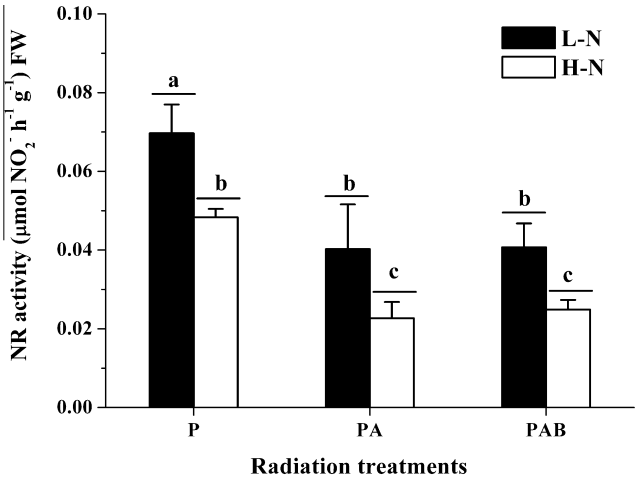

Fig. 7. Activity of nitrate reductase (NR) in *G. lemaneiformis* thalli grown for 16 days in the ambient (L–N) or NH_4^+-enriched (H–N) seawater under different solar radiation treatments with or without UVR. Significant ($P < 0.05$) differences between treatments are indicated by different lowercase letters. Vertical bars represent ± SD of the means ($n = 4$).

and Gao, 2009). In this study, the content of MAAs was not raised by NH_4^+ enrichment, and the associated lower level of photosynthetic inhibition must be related to other processes that NH_4^+ enrichment stimulated, such as repair mechanisms (Huovinen et al., 2006). The content of MAAs could increase if the algae are exposed to increased nitrate with reduced amounts of ammonia, due to the N metabolic pathway, in response to UV exposure. A greater restriction of nitrogen accessibility caused a higher accumulation of UVACs in the cyanobacterium *Nostoc punctiforme* (Fleming and Castenholz, 2008). However, Korbee-Peinado et al. (2004) showed that the content of MAAs was higher in *Porphyra columbina* grown at a high concentration of NH_4^+ with UV-B radiation than in cultures of this species grown at a low NH_4^+ level. In contrast to the sole nitrogen source of NH_4^+ used by the authors of the studies cited, both NO_3^- and NH_4^+ were provided as nitrogen sources for *G. lemaneiformis* in this study. Therefore, differences in the nitrogen sources and/or in the species used may explain the discrepancy between the results of the present study and the results of the previous studies cited. Additionally, the present study found that another absorption peak at 265 nm was higher in the presence of UVR. This peak could be the precursor of the MAAs associated with the peak occurring at 325 nm (Xu and Gao, 2010).

Repair mechanisms are one of the most important strategies protecting algae from UV-induced damage (Banaszak, 2003). Because these mechanisms need sufficient energy for the resynthesis of proteins and the repair of DNA, their activity produces enhanced respiration (Raven and Beardall, 1981; Xu and Gao, 2009). In this study, the respiration of *G. lemaneiformis* grown in the presence of UVR was enhanced under the low level of NH_4^+ (natural seawater) but not with enrichment by NH_4^+, which decreased the respiration regardless of the radiation treatments. Such a decrease, related to the assimilation of ammonia, could be attributed to a lower energetic cost for the use of ammonia than for the use of nitrate (Flores et al., 2005).

In the present study, the content of phycoerythrin (PE) in the *G. lemaneiformis* thalli grown in the absence of UVR was enhanced by NH_4^+ enrichment. PE is an important nitrogen-storage pool in red algae (Kursar and Alberte, 1983), and its synthesis depends on the availability of nitrogen (Korbee-Peinado et al., 2004). However, PE strongly absorbs UVR because of its protein characteristics and location on the thylakoid membrane (Aráoz and Häder, 1997). Therefore, its accumulation is prevented by its role in screening UV. Under certain conditions, PE can be photobleached by UVR (Davison et al., 2007; Schmidt et al., 2010b; Xu and Gao, 2009). The accumulation of PE in *G. lemaneiformis* grown in the NH_4^+-enriched seawater could have been used, in part, for protection against UVR.

Nitrate reductase activity was stimulated by exposure to UVR in a cyanobacterium, *Nostoc calcicola* (Kumar et al., 1996) and a brown alga, *Fucus spiralis* (Viñegla et al., 2006). Accordingly, nitrate uptake was enhanced. In the present study, however, the presence of UVR decreased NR activity in *G. lemaneiformis*. The impact of UVR on NR activity could be species-specific (Huovinen et al., 2007; Viñegla et al., 2006) and may depend on the level of UV exposure. The synthesis of periplasmic proteins in a diatom was stimulated under moderate UV doses but was hindered under higher levels of UV (Wu and Gao, 2009). NR is a blue-light and UV-A photoreceptor (Aparicio and Quiñones, 1991; Stöhr et al., 1995). High levels of UVR could damage its molecular structure and decrease its activity. In contrast, the uptake of nitrate was not affected by UVR. The absence of coupling between the uptake and reduction of nitrate has been reported in certain macroalgae (Corzo and Niell, 1991; Mercado et al., 1999). In contrast, UVR significantly decreased the uptake of ammonia by *G. lemaneiformis* if grown in the NH_4^+-enriched seawater. This result might be a direct effect of the downregulated ammonium pump or an indirect effect of the decrease in the availability of carbon skeletons for amino acid biosynthesis associated with decreased photosynthesis (Döhler et al., 1995). If ammonia and nitrate coexist, the uptake

rate of ammonia usually exceeds that of nitrate (Lomas and Glibert, 1999). However, the presence of UVR may damage the structure and the permeability of the cell membrane. It is therefore possible that UVR can cause changes in the incorporation of nitrate or ammonia (Murphy, 1983; Sobrino et al., 2004).

In coastal waters, ammonia often shows pulsed high levels due to temporary inputs from runoff or intensive aquaculture (Hopkins et al., 1995). The enrichment of ammonia or nitrate can relieve the photosynthetic damage caused by solar UVR. The enrichment of nitrate led to enhanced levels of UV-protective compounds (Zheng and Gao, 2009), whereas enriched ammonia increased the content of PE and also played a protective role in addition to energy capture as a key antenna pigment, as found in this study.

Acknowledgments

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