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Effects of elevated CO_2 on the photosynthesis and nitrate reductase activity of *Pyropia haitanensis* (Bangiales, Rhodophyta) grown at different nutrient levels*

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Abstract *Pyropia haitanensis*, a commercially important species, was cultured at two CO_2 concentrations (390×10^{-6} and 700×10^{-6} (parts per million)) and at low and high nutrient levels, to explore the effect of elevated CO_2 on the species under nutrient enrichment. Results show that in CO_2-enriched thalli, relative growth rate (RGR) was enhanced under nutrient enrichment. Elevated CO_2 decreased phycobiliprotein (PB) contents, but increased the contents of soluble carbohydrates. Nutrient enrichment increased the contents of chlorophyll *a* (Chl *a*) and PB, while soluble carbohydrate content decreased. CO_2 enrichment enhanced the relative maximum electronic transport rate and light saturation point. In nutrient-enriched thalli the activity of nitrate reductase (NRA) increased under elevated CO_2. An instantaneous pH change in seawater (from 8.1 to 9.6) resulted in reduction of NRA, and the thalli grown under both elevated CO_2 and nutrient enrichment exhibited less pronounced reduction than in algae grown at the ambient CO_2. The thermal optima of NRA under elevated CO_2 and/or nutrient enrichment shifted to a lower temperature (10–15°C) compared to that in ambient conditions (20°C). We propose that accelerated photosynthesis could result in growth increment. N assimilation remained high in acidified seawater and reflected increased temperature sensitivity in response to elevated CO_2 and eutrophication.

Keyword: *Pyropia haitanensis*; photosynthesis; nitrate assimilation; elevated CO_2; eutrophication

1 INTRODUCTION

The atmospheric CO_2 concentration has been rising, mainly because of the burning of fossil fuels, land use, and other anthropogenic activities. It is predicted that the future CO_2 concentration will be nearly double the ambient atmospheric CO_2 and reach 800×10^{-6} or even higher by 2100 (Doney et al., 2009). The oceans, which are considered as a CO_2 sink (Sabine and Feely, 2007), have absorbed about 2×10^{10} t of carbon per year, including large amounts of anthropogenic CO_2 (Sabine et al., 2004). The increase in CO_2 in the oceans will decrease the pH of seawater (Feely et al., 2004; Orr et al., 2005), which will result in ocean acidification (OA), more bicarbonate ions (HCO_3^-) and fewer carbonate ions (CO_3^{2-}), and thereby change in the speciation of inorganic carbon. Such changes have been expected to affect photosynthesis of macroalgae (Zou et al., 2009, 2011), the process of which relies on inorganic carbon (C_i), mainly HCO_3^-, to produce carbon substances for life activities.

Macroalgae play critical ecological roles in carbon cycles in coastal ecosystems. Some studies on calcifying macroalgae have shown negative responses to CO_2 enrichment in seawater (Martin et al., 2008; Diaz-Pulido et al., 2011), which are physically very different from non-calcifying ones. The non-calcareous taxa in particular play critical roles in ecosystem shifts, and may respond positively to future CO_2 enrichment (Connell and Russell, 2010). Zou et al. (2011) report that short- and long-term exposure to CO_2 enrichment both stimulated photosynthesis of

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Hizikia fusiformis. Likewise, maximum photosynthetic rates are enhanced significantly by high CO_2 treatments in the macroalga *Hypnea spinella* (Suárez-Álarez et al., 2012). Increased research into OA in recent years has indicated clearly that change in the saturation state of calcium carbonate ($CaCO_3$) particles has a considerable effect on the physiological response of calcifying macroalgae (Feely et al., 2004). However, for non-calcifying algae, physiological variation with respect to elevated CO_2 depends largely on utilization of the principal bulk source of C_i and carbon utilization mechanism (Raven et al., 2012). The ability to access the high HCO_3^- in seawater enhances carbon acquisition where CO_2 diffusion is severely limiting (Koch et al., 2013). For CO_2 preferred macroalgae, such as *Ulva lactuca*, the HCO_3^- uptake mechanism is important under conditions of low concentrations of C_i and high pH (Axelsson et al., 1999). Moreover, the photosynthesis of *Ulva lactuca* is clearly stimulated when the C_i dissolved in seawater is nearly saturated (Zou and Gao, 2002). Predictions have been preoccupied with the effects of OA on calcifying organisms (Connell and Russell, 2010), but focus should be turned to non-calcareous algae, especially marine macroalgae, to investigate whether the physiological metabolism of macroalgae could accelerate in response to climate change. Eutrophication in coastal oceans occurs mostly because of increasing anthropogenic land use and is considered to have serious effects on the physiological characteristics of macroalgae. In fact, maximum mean water column nitrate concentration across all Mediterranean estuaries ranged from 47 to 1 700 μmol/L in annual period (Kennison et al., 2013), and these were highly enriched with nitrogen. In China, the dissolved inorganic nitrogen (DIN) exceeded 14 μmol/L in the Yellow Sea over the year. The DIN concentrations of seawater in Haitou near Lianyungang were between 12.2 and 80.1 μmol/L during the year (Liu et al., 2013). Such eutrophication in nutrient-poor coastal ecosystems is anticipated to physiologically stimulate the photosynthesis and growth of marine algae (Zou et al., 2011). Moreover, increasing nitrogen availability in seawater is expected to affect the response to C_i availability in marine algae (Gordillo et al., 2001, 2003). At present, information on how photosynthesis and nutrient-related enzyme activity in algae will respond to elevated CO_2 under a different nutrient status is currently insufficient. Compared to the extensive information available for higher plants and microalgae, little is known about the metabolism of photosynthesis and nitrogen assimilation in marine macroalgae in response to elevated CO_2 and eutrophication in the ocean, even though there is an interactive effect. Exploring how these processes in marine macroalgae would be affected by elevated CO_2 in seawater resulting from increased atmospheric CO_2, and from eutrophication in coastal areas, is an important research subject.

Pyropia haitanensis (Bangiales, Rhodophyta) is a major cultivated species in South China found in the intertidal zones on coastlines and, because of its extensive use as a food delicacy, has very high economic value. It is interesting to explore how its metabolism could be affected by high CO_2 concentration and eutrophication on coastlines. In the present study, we cultured *P. haitanensis* under two different levels of CO_2 concentrations and nutrients, aiming to assess how photosynthesis and nitrate reductase activity respond to elevated CO_2 and eutrophication conditions. We hypothesized that the physiological metabolism in *P. haitanensis* would be improved because of increasing atmospheric CO_2 and coastal eutrophication, and that there would be interactive effects of the two factors on photosynthetic performance and nitrate reductase in this marine alga.

2 MATERIAL AND METHOD

2.1 Algal materials

Pyropia haitanensis was collected from Nanao Island, Guangdong Province, South China. Healthy thalli free of macroscopic epibiota and sediments were selected and returned to the laboratory in insulated plastic box (4°C) within 4 h. The alga was maintained in a glass aquarium tank containing filtered natural seawater (salinity 30) and light was provided by timer-controlled fluorescent tubes (100 μmol photons/(m^2·s) PAR=12D:12L scheme). Temperature in the incubator was kept at 18°C. Water motion was provided by aeration, and culture media were renewed every other day. Only healthy thalli were selected after being maintained for 2–3 days before the experiments.

2.2 Experiment design

We adopted fully factorial design with two levels of CO_2 concentration: ambient air levels (considered for this study to be 390×10^{-6} CO_2) and CO_2-enriched air (700×10^{-6} CO_2); and two levels of nutrient supply (shown in Table 1). Atmospheric CO_2 was generated

Table 1 Experiment design

	Air (390×10^{-6})		+CO_2 (700×10^{-6})	
	-NP	+NP	-NP	+NP
Treatments	Ambient	+NP	+CO_2	+NPCO_2
C_N	11.06	200.00	11.06	200.00
C_P	0.46	25.00	0.46	25.00

Air: ambient CO_2 level (390×10^{-6}); +CO_2: elevated CO_2 level (700×10^{-6}); -NP: non-nutrient enrichment; +NP: nutrient enrichment. Units of nitrate and phosphate concentrations (C_N, C_P) were μmol/L.

by air flow into Erlenmeyer flasks for the ambient CO_2 treatments. Elevated CO_2 (700×10^{-6}) was contained in a sealed steel cylinder. The cylinder was connected by a digital display to the incubator and the concentration of elevated CO_2 was displayed on a window on the incubator. When the experiments started, the initial flow rate of CO_2 was measured by a glass rotameter. The flow rate to each flask unit was about 0.5 L/min. Fluxes of gases (air and elevated CO_2) bubbled into cultures were then controlled by valves and remained as consistent as possible. The measured concentration of the total dissolved C_i, using the Total Organic Carbon Analyzer (TOC-5000A, Shimadzu, Japan), was 2.01 and 2.09 mmol/L. The pH values were 8.11–8.15 and 7.84–7.86 in seawater in equilibrium with ambient air and CO_2-enriched air, respectively. Experiments started when an aliquot of 4-g fresh weight (FW) algae were introduced into each of the Erlenmeyer flasks containing 2 L of filtered natural seawater. Twelve flasks were put into one chamber. Six flasks were programmed to supply 390×10^{-6} CO_2 (ambient CO_2 treatment, air) in aeration for cultivation, of which one-half contained nutrient-enriched seawater. Another six flasks were programmed to supply a CO_2 concentration of 700×10^{-6} (elevated CO_2 treatment, CO_2), of which three were filled with nutrient-enriched culture. The algae were permitted to shift gently, without tumbling through water stirred by the aeration. The culture media in each flask were renewed every 2 days. The light and temperature conditions for the algae were the same as described above. There were three replicates for each treatment, and after cultivating for 8–10 d, algae were harvested and used for the experimental assays.

2.3 Analytical method

2.3.1 Relative growth rate (RGR)

The mean growth rates were estimated every other day through the increased FW biomass during cultivation. Growth rates were calculated according to the exponential formula:

$$\mathrm{RGR}=\ln(W_t/W_0)\times t^{-1}\times100\%, \quad (1)$$

where W_0 refers to the initial FW (g), W_t is FW after t days and t is days of cultivation. Excess water on the surface of algae was blotted off gently before weighing. There were three replicates for all treatments.

2.3.2 Biochemical composition

Chlorophyll *a* (Chl *a*) and total carotenoids were extracted from fresh thalli in methanol. About 0.1 g FW thalli was ground with 10 mL methanol and submerged for 24 h at 4°C in a refrigerator and in total darkness. The concentrations were determined spectrophotometrically according to the method of Wellburn (1994).

An aliquot of 0.2 g FW thalli was ground with 8 mL phosphate buffer (0.1 mol/L, pH 6.8) in a homogenizer for 5 000×*g* with ice-cooling. The supernatant was used for the spectrophotometric measurement of phycobiliproteins (PB) according to Beer and Eshel (1985). The contents of phycoerythrin (PE) and phycocyanin (PC), the main components of PB, were then assayed.

For the extraction of soluble carbohydrates and soluble proteins, six fresh thalli from each culture were ground in a mortar with distilled water and extraction buffer (0.1 mol/L phosphate buffer, pH 6.8). Soluble carbohydrates (shown as sucrose equivalents) and soluble proteins were estimated from the supernatant.

2.3.3 Photosynthesis measurements

Photosynthetic rates of thalli grown under different treatments were measured in ambient and CO_2-enriched seawater. Here the ambient and CO_2-enriched seawater represented the seawater equilibrated with the ambient air (390×10^{-6}) and CO_2-enriched air (700×10^{-6}).

A biological oxygen monitor (YSI Model 5300, USA) was used in these experiments. The oxygen electrode was immersed in a temperature-controlled chamber at 18°C. First, algae (0.1 g FW) were cut into small segments with a sharp scalpel, and then incubated in seawater media for at least 1 h under 100 μmol photons/(m^2·s) and 18°C, in order to minimize the possible effect of cutting damage on photosynthetic assay. Next, algae were transferred to the O_2 electrode chamber with 8 mL reaction media. A halogen tungsten lamp was used as the light source.

The net photosynthetic rate as a function of light irradiance (P-I curve) was determined. Light irradiance varied between 0 and 800 μmol photons/(m^2·s), measured with a PAR quantum sensor (QSL2100, Biospherical Instruments Inc, San Diego, CA, USA). Incremental light irradiances were achieved through shortening the distance between light source and the electrode chamber. Photosynthetic O_2 evolution rates were normalized to Chl *a* of the algae samples. Dark respiration rate was assayed by covering the chamber with an opaque bag.

The apparent photosynthetic efficiency (α) was considered as the initial slope of the P-I curve. P_m was the maximum photosynthetic rate. R_d was the dark respiration rate. The light compensation point (E_c) and light saturation point (E_k) were calculated as R_d/α and P_m/α, respectively.

2.3.4 Chlorophyll fluorescence measurements

A JUNIOR-PAM chlorophyll fluorometer (Walz, Germany) was used for the measurement of chlorophyll fluorescence. Two different wavelengths of the LED (465 and 740 nm) were used as the light source and a photoelectric diode as a detector. Modulated fluorescence was excited by blue LED (wavelength of maximum emission: 465 nm), and two modulation frequencies (5 and 100 Hz) were used. Actinic light was excited by the same power LED as for modulated light. Photon flux densities at 1 mm distance from the tip of the 40 cm JUNIOR-PAM light guide were 25–1 500 μmol photons/(m^2·s), adjustable at 12 different levels. Saturating pulses were also excited by the same power LED as for modulated light. The default photon flux density of the saturating pulses was 8 000 μmol photons/(m^2·s) at 1 mm distance from the tip of the 40 cm JUNIOR-PAM light guide. The rapid light curves (RLCs) were made with the fluorescence response to eight increasing actinic irradiance levels from the range 0–820 μmol photons/(m^2·s), using the light curve option of the JUNIOR-PAM. *P. haitanensis* was placed at the end of the fiberoptic probe and four or more samples were used for each measurement of chlorophyll fluorescence. Before the measurement, samples were laid in darkness for 30 min. The maximum relative electron transport rate ($rETR_m$) was estimated according to Jassby and Platt (1976).

2.3.5 Nitrate reductase activity

Nitrate reductase activity (NRA) was measured using fresh materials directly from cultures according to the situ method by Corzo and Niell (1991). The method involved a buffer, a compound able to permeate the membrane, nitrate in excess and a source of reducing power. Thus the reaction mixture contained 0.1 mol/L potassium phosphate, 0.5 mmol/L EDTA, 0.1% propanol (v/v), 400 mmol/L nitrate and 10 μmol/L glucose, adjusting pH value to 8.1 with 0.1 mol/L HCl and 0.1 mol/L NaOH fresh preparation, in the final volume of 500 mL. The reaction mixture was used for NRA determination in the time range from 9:00 to 24:00. About 0.3 g FW seaweeds were added to each triangular flask that contained this reaction mixture, in a final assay media volume of 20 mL, flushed with N_2 for 2 min to remove O_2. Incubations lasted 30 min at 30°C. Nitrite concentration was determined colorimetrically at 543 nm. Our preliminary experiments showed that NRA was almost steady between 18:00 h and 20:00 h. Therefore, the algae for the following experiments were sampled and assayed for enzyme activity in this time series. As a result, daily fluctuations were eliminated from this NRA determination.

The impacts of pH and temperature on NRA were also determined. The reaction mixture detailed above needed to be adjusted to make the phosphate buffer with different pH gradients (from 6.6 to 9.6 with 0.5 pH unit interval between adjacent gradients). Freshly prepared HCl (1 mol/L) and NaOH (1 mol/L) solutions were used to obtain the pH of different reaction mixtures. In addition, the reaction mixtures with different temperatures (from 5 to 40°C with 5°C intervals between adjacent gradients), were put in one incubator with the corresponding temperature before the determination of NRA. Other measurements were the same as maintained above. Finally, the NRA was expressed as μmol/NO_2^-/g FW/h.

2.4 Statistics

Data presented were the mean of three independent experiments. A Levene test for equality of variances was used to check for homogeneity. A two-way ANOVA was used to analyze statistical differences in the data. When necessary, independent samples *t*-tests were used to reveal statistical groupings. All statistical analyses were operated using SPSS version 16.0 (SPSS Inc., Chicago, IL, USA). The significance level was set at $P<0.05$.

3 RESULT

3.1 Growth and biochemical components

In all laboratory culture treatments *Pyropia*

haitanensis thalli exhibited an increase of biomass (FW) (Fig.1). The mean RGR increased by 65.97% under elevated CO_2 and nutrient enrichment (+$NPCO_2$) ($F_{3,8}$=10.16, P=0.001) compared with the ambient treatment (i.e. ambient air + non-nutrient enrichment). Compared to the ambient treatment the RGR slightly increased under nutrient enrichment (P=0.046, Fisher's LSD test). However, the effect was less pronounced than the effect of elevated CO_2 on the RGR (t=2.80, df=4, P=0.049).

Chl *a* content increased significantly under nutrient enrichment ($F_{1,8}$=36.08, P=0.000) and remained constant at elevated CO_2 levels ($F_{1,8}$=0.076, P=0.789, Fig.2a). Phycoerythrin (PE) ($F_{1,8}$=169.10, P=0.000) and phycocyanin (PC) ($F_{1,8}$=50.251, P=0.000) contents increased remarkably under +NP and +$NPCO_2$ treatments, but decreased with elevated CO_2 (Fig.2c, d). The soluble carbohydrate content increased significantly ($F_{1,8}$=18.80, P=0.002) with elevated CO_2, but decreased slightly (P>0.05, Fig.3a) under +$NPCO_2$ conditions. Conversely, higher CO_2 concentrations decreased the content of soluble protein (t=3.18, df=4, P=0.033); a slight increase was observed under +NP conditions (Fig.3b).

3.2 Photosynthetic oxygen evolution and chlorophyll fluorescence measurements

Pyropia haitanensis exhibited a light-saturated photosynthetic rate (P_{max}) range from 22.72 to

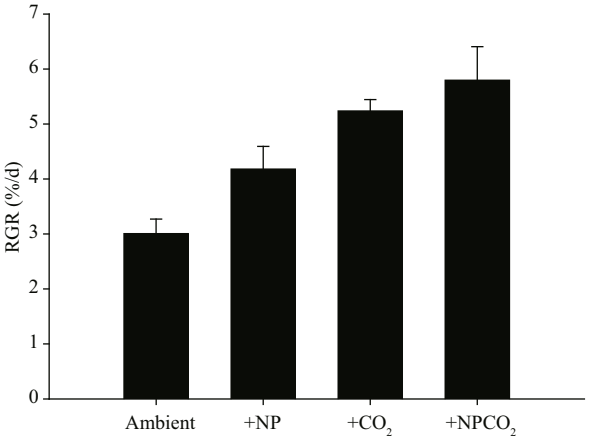

Fig.1 Relative growth rate (RGR) of *Pyropia haitanensis* cultured in ambient, elevated CO_2 (+CO_2), nutrient enrichment (+NP) and elevated CO_2 with nutrient enrichment (+$NPCO_2$) conditions

Values are means (±SD), n=3.

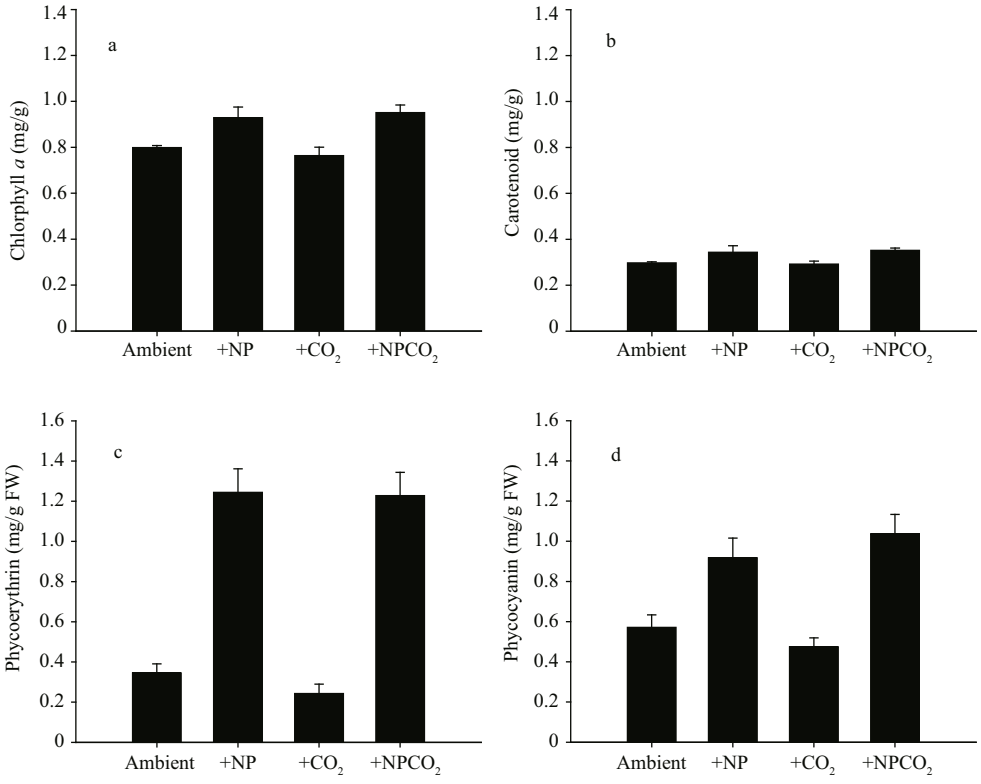

Fig.2 Pigment content of *Pyropia haitanensis* obtained under ambient, elevated CO_2 (+CO_2), nutrient enrichment (+NP) and elevated CO_2 with nutrient enrichment (+$NPCO_2$) conditions

a. chlorophyll *a*; b. total carotenoids; c. phycoerythrin (PE); d. phycocyanin (PC). Values are means (±SD), n=3.

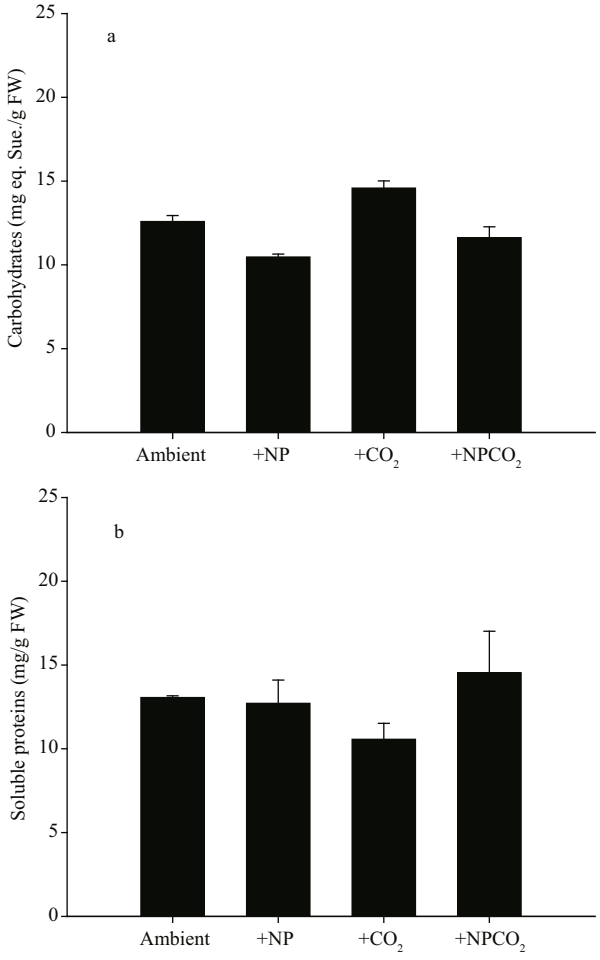

Fig.3 Biochemical characters of *Pyropia haitanensis* obtained under ambient, elevated CO_2 ($+CO_2$), nutrient enrichment (+NP) and elevated CO_2 with nutrient enrichment ($+NPCO_2$) conditions

a. carbohydrates; b. soluble protein. Values are means (±SD), n=3.

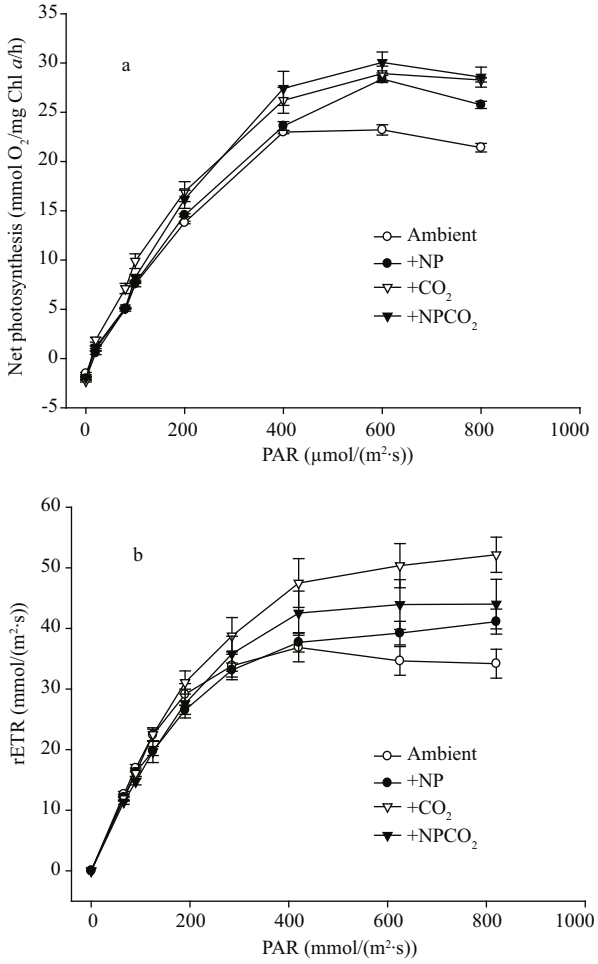

Fig.4 Net photosynthesis versus PAR curves (*P-I* curves)

a. *Pyropia haitanensis* grown in ambient, elevated CO_2 ($+CO_2$), nutrient enrichment (+NP) and elevated CO_2 with nutrient enrichment ($+NPCO_2$) conditions. Photosynthetic rates are measured at growth temperature (18°C). Values are means (±SD), n=3; b. relative electron transport rates (rETR) versus actinic irradiance (rapid light curves, *RLCs*) of *Pyropia haitanensis* grown at 18°C, at which the *RLCs* were measured. Data are means (±SD) (n=6).

32.99 μmol O_2/mg Chl *a*/h, with the highest value appearing in the $+NPCO_2$ treatments (P=0.000, LSD fisher test, Fig.4, Table 2). Both elevated CO_2 and nutrient enrichment significantly enhanced P_{max} compared with the ambient treatment (P<0.01, LSD fisher test). Additionally, nutrient enrichment had a positive effect on apparent photosynthetic rate (α), which showed a higher value at $+NPCO_2$ treatment (P=0.004). Under CO_2 enrichment, the dark respiration rate (R_d) increased significantly ($F_{1,8}$=18.09, P=0.003), the highest value of R_d exhibited under $+NPCO_2$ treatment. Likewise, E_c also increased with increasing CO_2 ($F_{1,8}$=18.21, P=0.003). Thalli under increased CO_2 conditions displayed higher $rETR_m$ than under the ambient treatment (P<0.01). The pattern of response for E_k was very similar to that for $rETR_m$ and it increased with CO_2 enrichment ($F_{1,8}$=8.95, P=0.017).

3.3 Nitrogen reductase activity (NRA)

NRA was measured in situ under four treatments. Both elevated CO_2 and nutrient enrichment treatments significantly increased the NRA of *P. haitanensis* (P<0.01). The highest NRA value was displayed under $+NPCO_2$ conditions ($F_{3,8}$=11.04, P=0.003, Fig.5, Table 3).

NRA was significantly affected by pH (from 6.6 to 9.6) in seawater media (Fig.6a). The results showed that the NRA was significantly higher under CO_2 enrichment and/or +NP treatment than in the ambient conditions in the pH series 7.1–9.6. Thalli under all treatments showed that the optimum value of pH for NRA was nearly 8.1. When pH>8.1, the NRA was decreased.

Table 2 Photosynthetic parameters for P_{max}, R_d, α, E_k, E_c, and rapid light curve coefficients for the relative electron transport rate ($rETR_m$) in *Pyropia haitanensis* grown in ambient, nutrient enrichment (+NP), elevated CO_2 (+CO_2) and elevated CO_2 with nutrient enrichment (+$NPCO_2$) conditions

	Ambient	+NP	+CO_2	+$NPCO_2$
P_{max}	22.7±0.7	28.1±0.7	29.7±0.8	33.0±2.0
α	0.09±0.00	0.10±0.01	0.10±0.00	0.12±0.01
R_d	-1.5±0.3	-1.4±0.2	-1.9±0.1	-2.3±0.4
E_k	256.9±11.9	273.4±31.2	311.3±12.0	282.2±19.8
E_c	16.6±2.7	13.2±2.1	20.0±0.9	19.8±1.9
$rETR_m$	36.2±2.4	42.1±4.8	49.8±1.7	47.4±2.5

Photosynthetic rates are normalized to chlorophyll *a* content of the algae samples. Data are the mean (±SD) (n=3). P_{max}: maximum net photosynthetic rate (μmol O_2/mg Chl *a*/h); R_d, dark respiration rate (μmol O_2/mg Chl *a*/h); α, photosynthetic efficiency (μmol O_2/mg Chl *a*/h); E_c, light compensation point (μmol photons/(m^2·s)); E_k, light saturation point (μmol photons/(m^2·s)); rETRm, μmol photons/(m^2·s).

Table 3 The effect of CO_2 and N concentration on nitrate reductase activity (NRA) of *Pyropia haitanensis*, as determined by two-way analysis of variance

	df	MS	*F*	*P*
NRA				
CO_2	1	1.76	31.15	0.001
Nitrogen concentration	1	3.72	65.77	0.000
CO_2×Nitrogen concentration	1	0.002	0.035	0.857

Table 4 The Q_{10} values (the rate increase caused by raising temperature 10°C) for nitrate reductase activity (NRA) in *Pyropia haitanensis* grown in ambient, nutrient enrichment (+NP), elevated CO_2 (+CO_2) and elevated CO_2 with nutrient enrichment (+$NPCO_2$) conditions

	Ambient	+NP	+CO_2	+$NPCO_2$
Q_{10}	1.2±0.1[1]	5.0±1.6[2]	4.3±0.6[2]	2.9±0.7[3]

Data are the mean (±SD) (n=3). [1] 5–20°C temperature range; [2] 5–10°C temperature range; [3] 5–15°C temperature range.

Temperature responses for NRA under all treatments are presented in Fig.6b. The results show that the NRA performance was strongly temperature dependent on either elevated CO_2 or nutrient enrichment. We propose that NRA activity in ambient conditions exhibited a broadening temperature optimum from 15 to 25°C. However, under elevated CO_2 and/or nutrient enrichment treatments, the

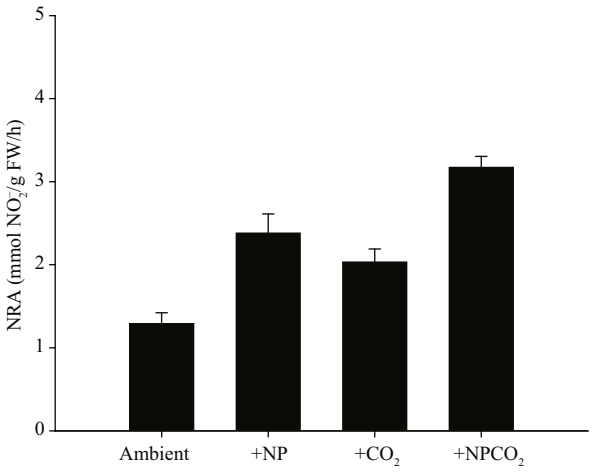

Fig.5 Nitrate reductase activity (NRA) of *Pyropia haitanensis* grown in ambient, elevated CO_2 (+CO_2), nutrient enrichment (+NP) and elevated CO_2 with nutrient enrichment (+$NPCO_2$) conditions

Data are means (±SD). n=3.

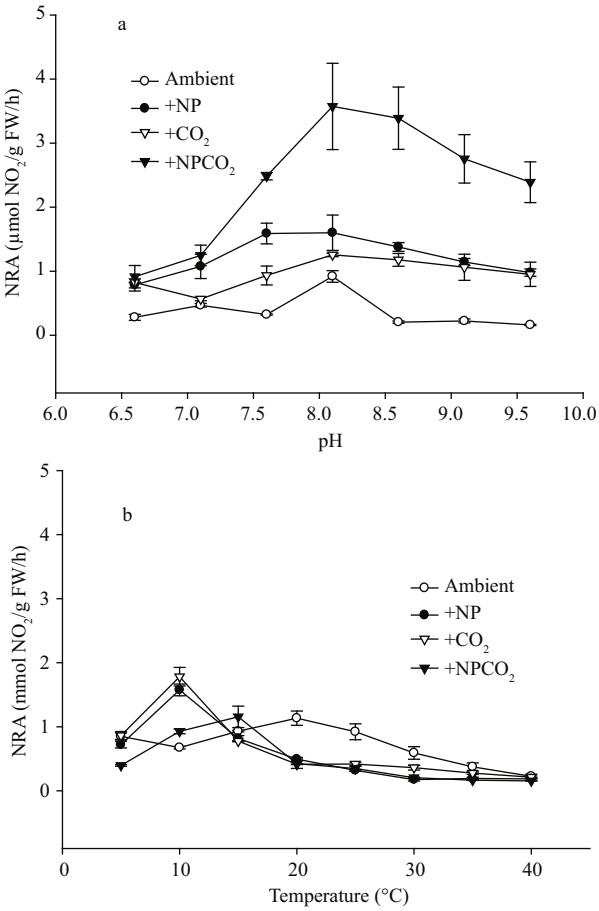

Fig.6 Nitrate reductase activity (NRA) of *Pyropia haitanensis* grown in ambient, elevated CO_2 (+CO_2), nutrient enrichment (+NP) and elevated CO_2 with nutrient enrichment (+$NPCO_2$) conditions

NRA was measured (a) in pH gradients from 6.6 to 9.1; and (b) at temperatures from 5 to 40°C. Values are means (±SD), n=3.

temperature optimum for NRA shifted to a lower temperature (10–15°C). The NRA at lower or higher temperatures (T<10°C, T>30°C) dropped rapidly. Under elevated CO_2 and/or nitrate enrichment conditions, the Q_{10} values of algal thalli increased greatly (Table 4, P<0.05). There was an obvious influence of temperature on the nitrogen assimilation by thalli.

4 DISCUSSION

The present results show that CO_2 enrichment enhanced the growth rate of *P. haitanensis*. Similar results were reported in *P. yezoensis* (Gao et al., 1991) and *Gracilaria lemaneiformis* (Zou et al., 2009). They demonstrate that the accelerated growth of seaweeds can be attributed either to accelerated photosynthesis (Kübler et al., 1999) or to the enhanced effect of CO_2 on nitrogen assimilation (Gordillo et al., 2001). Many seaweeds exhibit C_4-like photosynthetic rates gas exchange physiology, a CO_2-concentrating mechanism (CCM), the photosynthesis of which was hardly affected by O_2 and the photorespiration pathway (Raven et al., 2012). Moreover, the combination of two main environmental factors (e.g. high CO_2 and optimal temperature; high CO_2 and optimal irradiance) exhibited different interactive effects on the physiological performance of seaweeds (Gordillo et al., 2001; Zou et al., 2011; Sarker et al., 2013; Olischläger and Wiencke, 2013). The present results show that increased photosynthesis was consistent with elevated CO_2 in culture, and so the new biomass increase of *P. haitanensis* probably resulted from photosynthesis, suggesting that the thalli of this alga could benefit from ocean eutrophication. Additionally, both an unchanged and a decreased effect of high CO_2 on seaweeds were observed (Israel and Hophy, 2002; Boulus et al., 2007; Brown et al., 2014; Gutow et al., 2014). These results demonstrated that pCO_2 perturbation treatments depend on further environmental factors such as temperature, irradiance or nutrients. The response of thalli to elevated CO_2 is therefore species-specific.

Generally, nutrient enrichment has been considered to affect photosynthesis through its effects on pigment synthesis, nitrate uptake rate and Calvin cycle enzymes (Turpin, 1991; Huppe and Turpin, 1994). We showed higher PB content in *P. haitanensis* under nutrient enrichment. Moreover, under the same conditions, the higher $rETR_m$ and E_k with CO_2 elevation indicated a greater ability to transfer electrons and a greater energy investment in the physiological processes of *P. haitanensis* thalli to fulfill a higher photosynthetic rate. However, under non-nutrient enrichment, elevated CO_2 also resulted in an obvious increase in photosynthesis of this alga. Similar findings were reported in *Hizikia fusiformis* algae, whose photosynthetic rate was enhanced at higher C_i concentrations regardless of nitrogen levels (Zou et al., 2011). Therefore, elevated CO_2 demonstrated a predominant effect on enhanced photosynthesis of *P. haitanensis*; whatever nutrient levels were in place, the positive effect of CO_2 on gross photosynthesis was higher than dark respiration. Finally, the effect of elevated CO_2 on photosynthesis still agreed with the response in growth rate. Consequently, this algal thalli exhibited positive ecophysiological responses on elevated CO_2 and eutrophication.

Photosynthetic acclimation to elevated CO_2 in seawater has been demonstrated to influence the mechanism of inorganic carbon acquisition in seaweeds (Zou, 2005). It was considered that the maximum rate of photosynthesis is frequently related to Rubisco activity; per-cell decrease of the Rubisco content could result in a significant decline of the cellular photosynthetic capacity (Lapointe and Duke, 1984; Turpin, 1991). This point was verified by Gordillo et al. (2001). However, Andría et al. (1999) reported that a decrease of Rubisco content did not result in a decline of photosynthesis. In our study, the decrease in soluble protein content experienced at high CO_2 levels could well indicate a decrease in the main soluble protein, Rubisco. Conversely, the maximal photosynthetic rate of *P. haitanensis* still maintained a high level following exposure to elevated CO_2. The finding was inconsistent with previous studies (Gordillo et al., 2001), showing the heterogeneity of the photosynthetic acclimation to the high CO_2 levels reported by Zou (2005). The increase in soluble carbohydrate content repressed the transcription of genes related to photosynthetic pathways (Sheen, 1994) and eased an active photosynthesis. In addition, the decrease in soluble carbohydrates was considered to be partly consumption by respiration and partly because of transportation in the process of algal growth. Previous studies had proved that the growth of algae was closely related to their photosynthetic apparatus and metabolism with nutrient enrichment (Turpin, 1991; Jimenez del Rio et al., 1995; Gordillo et al., 2001, 2003). Additionally, the reduction in PB reflected the role of nitrogenous compounds as N reserves at

elevated CO_2 levels in *Hizikia fusiformis* (Zou et al., 2009), whereas the level of this pigment increases significantly with nutrient enrichment. As a result, we hypothesize that the present N level was limiting for photosynthesis of algae thalli when the atmospheric CO_2 level was doubled.

The nitrate assimilatory capacity of *P. haitanensis* was positively affected by both elevated CO_2 and nutrient enrichment during the day (measured between 18:00 h and 20:00 h), as revealed by the higher NRA. Such a stimulation of N assimilation was reported in *U. rigida* (Gordillo et al., 2001) and *Zostera noltii* (Alexandre et al., 2012). During the night (assayed at 24:00 h), thalli grown under CO_2 enrichment also showed higher capacity to assimilate nitrate (data not shown). As the deoxidation of nitrate required the provision of carbon skeletons from respiration pathways (Turpin et al., 1991) and a reducing agent, NAD(P)H (Corzo and Niell, 1991), the increased respiratory rate at elevated CO_2 could provide the means for the conversion from nitrate to nitrite and thereby stimulate the rate of nitrate assimilation. Moreover, with sufficient nutrient availability in culture, algal thalli exposed to elevated CO_2 reflected much higher diel periodicity of nitrate assimilation. Consequently, the processes of nitrogen assimilation and photosynthesis were considered to be integrally coupled. If levels of endogenous carbohydrate reserves diminished under nutrient enrichment, the assimilation of nitrate was dependent on the recent photosynthetic rate (Larsson and Larsson, 1987). As a result, the capacity to assimilate N remained at higher levels due to the increase in photosynthesis with eutrophication in cultures.

Interestingly, the acidified seawater and the sea surface temperature exhibited a prominent effect on N assimilation in *P. haitanensis*. As atmospheric CO_2 increased, the concentration of dissolved inorganic carbon (DIC) rose and OA declined. In our results, decreased pH in seawater repressed nitrate reductase compared to that in natural seawater. The increase of pH in media during photosynthesis also decreased its capacity to assimilate nitrate. However, algal thalli grown in conditions of eutrophication still possessed a strong capability to assimilate nitrate in response to pH changes. The activity of enzymes, including nitrate reductase (NR), is temperature sensitive, with optimum temperature for NRA in diverse algae measured in the range of 10–20°C (Gao et al., 2000; Berges et al., 2002). In this study, we measured NRA in the range 5–40°C when submerged. NRA of *P. haitanensis* showed different thermal optima at different treatments in response to an instantaneous change of seawater temperature. The thermal optima of NRA shifted to a lower temperature (10–15°C) at elevated CO_2 and/or eutrophication compared with that in ambient conditions (20°C), indicting an enhanced tolerance to lower seawater temperatures. At low temperatures a greater quantity of the enzyme may be required to achieve the same catalytic activity because the enzyme is working below its temperature optimum (Young et al., 2007). However, decreased NR enzyme activity below the optimal temperature could not be a component of cold acclimation. Based on increased Q_{10} values of algal thalli grown at elevated CO_2 or eutrophication and a temperature range at the collecting site, one would predict a decrease in NRA in response to lower temperatures. Consequently, the thermal variation in NRA is only partially due to temperature sensitivity.

5 CONCLUSION

This study showed that the growth rate and photosynthesis of *P. haitanensis* were significantly enhanced by both elevated CO_2 in the atmosphere and nutrient-enriched conditions in seawater. Nitrate assimilation was also improved with CO_2 enrichment and higher nutrient enrichment. Furthermore, the capacity to assimilate N was repressed in response to acidification and became temperature sensitive due to interactions between elevated CO_2 and nutrient enrichment. Our results suggest that mariculture in coastal systems of this economically important seaweed species, *P. haitanensis*, would benefit from the interaction of increased N availability and increased atmospheric CO_2 levels.

References

Alexandre A J, Silva P, Buapet M, Björk R S. 2012. Effects of CO_2 enrichment on photosynthesis, growth, and nitrogen metabolism of the seagrass *Zostera noltii*. *Ecology and Evolution*, **2**: 2 620-2 630.

Andría J R, Brun F G, Pérez-Llorénsand J L, Vergara J J. 2001. Acclimation responses of *Gracilaria* sp. (Rhodophyta) and *Enteromorpha intestinalis* (Chlorophyta) to changes in the external inorganic carbon concentration. *Bot. Mar.*, **44**: 361-370.

Andría J R, Vergara J J, Perez-Llorens J L. 1999. Biochemical responses and photosynthetic performance of *Gracilaria* sp. (Rhodophyta) from Cadiz, Spain, cultured under different inorganic carbon and nitrogen levels. *Eur. J. Phycol.*, **34**: 497-504.

Axelsson L, Larsson C, Ryberg H. 1999. Affinity, capacity and oxygen sensitivity of two different mechanisms for bicarbonate utilization in *Ulva lactuca* L. (Chlorophyta). *Plant Cell Environ*., **22**: 969-978.

Beer S, Eshe A. 1985. Determining phycoerythrin and phycocyanin concentrations in aqueous crude extracts of red algae. *Aust*. *J*. *Mar*. *Fresh*., **36**: 785-792.

Berges J A, Varela D E, Harrison P J. 2002. Effects of temperature on growth rate, cell composition and nitrogen metabolism in the marine diatom *Thalassiosira pseudonana* (Bacillariophyceae). *Mar*. *Ecol*. *Prog*. *Ser*., **225**: 139-146.

Boulus A, Spaneir E, Friedlander M. 2007. Effect of outdoor conditions on growth rate and chemical composition of *Gelidium crinale* in culture. *J*. *Appl*. *Phycol*., **19**: 471-478.

Brown M B. 2014. The effect of rising ocean temperature and pCO_2 on the physiology and growth of giant kelp, *Macrocystis pyrifera*, and grazing by *Purple urchins*, Strongylocentrotus purpuratus. http://hdl.handle.net/10211.3/115527.

Chauvin A, Vianne D, Cuet P. 2011. Is the response of coral calcification to seawater acidification related to nutrient loading? *Coral Reefs*, **30**: 911-923.

Christa C, White A. 1999. Rapid light curves: a new fluorescence method to assess the state of the photosynthetic apparatus. *Photosynth*. *Res*., **59**: 63-72.

Connell S D, Russell B D. 2010. The direct effects of increasing CO_2 and temperature on non-calcifying organisms: increasing the potential for phase shifts in kelp forests. *P*. *Roy*. *Soc*. *B-Biol*. *Sci*., **277**: 1 409-1 415.

Corzo A, Niell F X. 1991. Determination of nitrate reductase activity in *Ulva rigida* C. Agardh by the in situ method. *Exp*. *Mar*. *Biol*. *Ecol*., **146**: 181-191.

Diaz-Pulido G, Gouezo M, Tilbrook B, Dove S, Anthony K R. 2011. High CO_2 enhances the competitive strength of seaweeds over corals. *Ecological Letters*, **14**: 156-162.

Doney S C, Fabry V J, Feely R A, Kleypas J A. 2009. Ocean acidification: the other CO_2 problem. *Annu*. *Rev*. *Ma*. *Sci*., **1**: 169-192.

Feely R A, Sabine C L, Lee K, Berelson W, Kleypas J, Fabry V J, Millero F J. 2004. Impact of anthropogenic CO_2 on the $CaCO_3$ system in the oceans. *Science*, **305**: 362-366.

Figueroe F L, Conde-Álvarez R, Gómez I. 2003. Relations between electron transport rates determined by pulse amplitude modulated chlorophyll fluorescence and oxygen evolution in macroalgae under different light conditions. *Photosynth*. *Res*., **75**: 259-275.

Gao K, Aruga Y, Asada K, Ishihara T, Akano T, Kiyohara M. 1991. Enhanced growth of the red alga *Pyropia yezoensis* Ueda in high CO_2 concentrations. *J*. *Appl*. *Phycol*., **3**: 356-362.

Gao Y, Smith G J, Alberte R S. 2000. Temperature dependence of nitrate reductase activity in marine phytoplankton: biochemical analysis and ecological implications. *J*. *Phycol*., **36**: 304-313.

Gardner W S, Wynne D S, Dunstan W M. 1976. Simplified procedure for the manual analysis of nitrate in seawater. *Mar*. *Chem*., **4**: 393-396.

Gordillo F J L, Figueroa F L, Niell F X. 2003. Photon- and carbon-use efficiency in *Ulva rigida* at different CO_2 and N levels. *Planta*, **218**: 315-322.

Gordillo F J L, Niell F X, Figueroa F T. 2001. Non-photosynthetic enhancement of growth by high CO_2 level in the nitrophilic seaweed *Ulva rigida* C. Agardh (Chlorophyta). *Planta*., **213**: 64-70.

Gutow L, Rahman M M, Bartl K, Saborowski R, Bartsch I, Wiencke C. 2014. Ocean acidification affects growth but not nutritional quality of the seaweed *Fucus vesiculosus* (Phaeophyceae, Fucales). *J*. *Exp*. *Mar*. *Biol*. *Ecol*., **453**: 84-90.

Harrison P J. 1988. Determining phosphate uptake rates of phytoplankton. *In*: Lobban C S ed. Experimental Phycology: A Laboratory Manual. New York Cambridge Univ. Press. p.186-195.

Hofmann L C, Yildiz G, Hanelt D, Bischof K. 2012. Physiological responses of the calcifying rhodophyte *Corallina officinalis* (L.) to future CO_2 levels. *Mar*. *Biol*., **159**: 783-792.

Huppe H C, Turpin D H. 1994. Integration of carbon and nitrogen metabolism in plant and algal cells. *Annu. Rev*. *Plant Biol*., **45**: 577-607.

Israel A, Hophy M. 2002. Growth, photosynthetic properties and Rubisco activities and amounts of marine macroalgae grown under current and elevated seawater CO_2 concentration. *Glob*. *Chang*. *Biol*., **8**: 831-840.

Jassby A T, Platt T. 1976. Mathematical formulation of the relationship between photosynthesis and light for phytoplankton. *Oceanography*, **21**: 540-547.

Jimenez del R, Ramazanov M, Reina Z G G. 1995. Effect of nitrogen supply on photosynthesis and carbonic anhydrase activity in the green seaweeds *Ulva rigida* (Chlorophyta). *Mar*. *Biol*., **123**: 687-691.

Kennison R L, Fong P. 2013. Extreme eutrophication in shallow estuaries and lagoons of California is driven by a unique combination of local watershed modifications that trump variability associated with wet and dry seasons. *Estuaries and Coasts*, http://dx.doi.org/10.1007/s12237-013-9687-z.

Koch M, Bowes G, Ross C, Zhang X H. 2013. Climate change and ocean acidification effects on seagrasses and marine macroalgae. *Global*. *Change*. *Biol*., **19**: 103-132.

Kübler J E, Johnston A M, Raven J A. 1999. The effects reduced and elevated CO_2 and O_2 on the seaweed *Lomentaria articulata*. *Plant Cell Environ*., **22**: 1 303-1 310.

Lapointe B E, Duke C S. 1984. Biochemical strategies for growth of *Gracilaria tikuahiae* (Rhodophyta) in relation to light intensity and nitrogen availability. *J*. *Phycol*., **20**: 488-495.

Larsson C M, Larsson M. 1987. Regulation of nitrate utilization in green algae. *In*: Ullrich W W ed. Inorganic Nitrogen Metabolism. Springer-Verlag, New York. p.203-207.

Liu D Y, Keesing J K, He P M, Wang Z L, Shi Y J, Wang Y J.

2013. The world's largest macroalgal bloom in the Yellow Sea, China: formation and implications. *Estuarine, Coastal and Shelf Science*, **129**: 2-10.

Ma Z, Gao K. 2010. Spiral breakage and photo-inhibition of *Arthrospira platensis* (Cyanophyta) caused by accumulation of reactive oxygen species under solar radiation. *Environ. Exp. Bot.*, **68**: 208-213.

Martin S, Rodolfo-Metalpa R, Ransome E, Rowley S, Buia M C, Gattuso J P, Hall-Spencer J. 2008. Effects of naturally acidified seawater on seagrass calcareous epibionts. *Biological Letters*, **4**: 689-692.

Olischläger M, Wiencke C. 2013. Ocean acidification alleviates low-temperature effects on growth and photosynthesis of the red alga *Neosiphonia harveyi* (Rhodophyta). *J. Exp. Bot.*, **18**: 5 587-5 597.

Orr J C, Fabry V J, Aumont O et al. 2005. Anthropogenic ocean acidification over the twenty-first century and its impact on calcifying organisms. *Nature*, **437**: 681-686.

Parry M A, Andralojc P J, Scales J C, Salvucc M E, Carmo-SilvaA E, Alonso H, Whitney S M. 2013. Rubisco activity and regulation as targets for crop improvement. *J. Exp. Bot.*, **64**: 717-730.

Raven J A, Giordano M, Beardall J, Maberly S C. 2012. Algal evolution in relation to atmospheric CO_2: carboxylases, carbon-concentrating mechanisms and carbon oxidation cycles. *Philosophical Transactions of the Royal Society B: Biol. Sci.*, **367**: 493-507.

Sabine C L, Feely R A. 2007. The oceanic sink for carbon dioxide. *In*: Reay D et al ed. Greenhouse Gas Sinks. CABI Publishing, Oxfordshire. p.31-39.

Sabine G L, Feely R A, Gruber N et al. 2004. The oceanic sink for anthropogenic CO_2. *Science*, **305**: 367-371.

Sarker M Y, Bartsch I, Olischläger M, Gutow L, Wiencke C. 2013. Combined effects of CO_2, temperature, irradiance and time on the physiological performance of *Chondrus crispus* (Rhodophyta). *Bot. Mar.*, **56**: 63-74.

Schreiber U, Hormann H, Neubauer C, Klughammer C. 1995. Assessment of photosystem II photochemical quantum yield by chlorophyll fluorescence quenching analysis. *Funct. Plant. Biol.*, **22**: 209-220.

Sheen J. 1994. Feedback control of gene expression. *Photosynth. Res.*, **39**: 427-438.

Suárez-Álvarez S, Gómez-Pinchetti J L, García-Reina G. 2012. Effect of increased CO_2 levels on growth, photosynthesis, ammonium uptake and cell composition in the macroalga *Hypnea spinella* (Gigartinales, Rhodophyta). *J. Appl. Phycol.*, **24**: 815-823.

Turpin D. 1991. Effect of inorganic availability on algal photosynthesis and carbon metabolism. *J. Phycol.*, **27**: 14-20.

Wellbum A R. 1994. The spectral determination of chlorophylls *a* and *b*, as well as total carotenoids, using various solvents with spectrophotometers of different resolution. *Plant Physiol.*, **144**: 307-313.

Young E, Dring M J, Savidge G, Birkett D A, Berges J. 2007. Seasonal variations in nitrate reductase activity and internal N pools in intertidal brown algae are correlated with ambient nitrate concentrations. *Plant Cell Environ.*, **30**: 764-774.

Yu G. 2000. Temperature dependence of nitrate reductase activity in marine phytoplankton: biochemical analysis and ecological implications. *J. Phycol.*, **36**: 304-313.

Zou D H. 2005. Effects of elevated atmospheric CO_2 on growth, photosynthesis and nitrogen metabolism in the economic brown seaweed, *Hizikia fusiforme* (Sargassaceae, Phaeophyta). *Aquaculture*, **250**: 726-735.

Zou D H, Gao K S. 2002. Photosynthetic responses to inorganic carbon in *Ulva lactuca* under aquatic and aerial states. *Acta Bot. Sin.*, **44**: 1 291-1 296.

Zou D H, Gao K S, Xia J R. 2003. Photosynthetic utilization of inorganic carbon in the economic brown alga, *Hizikia fusiforme* (Sargassaceae) from the South China Sea. *J. Phycol.*, **39**: 1 095-1 100.

Zou D H, Gao K S. 2009. Effects of elevated CO_2 on the red seaweed *Gracilaria lemaneiformis* (Gigartinales, Rhodophyta) grown at different irradiance levels. *Phycologia*, **48**: 510-517.

Zou D H, Gao K S, Luo H J. 2011. Short- and long- term effects of elevated CO_2 on photosynthesis and respiration in the marine macroalgae *Hizikia Fusiformis* (Sargassaceae, Phaeophyta) grown at low and high N supplies. *J. Phycol.*, **47**: 87-97.