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Interactive effects of elevated CO_2 and nitrogen–phosphorus supply on the physiological properties of *Pyropia haitanensis* (Bangiales, Rhodophyta)

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Abstract The ongoing increases in atmospheric CO_2 concentrations and coastal eutrophication have affected the coastal environment and marine macroalgae. In this study, the interactive effects of CO_2 (390 and 1000 μL L^{-1}) and nutrient levels (nitrogen and phosphorus supplied simultaneously) on the physiological properties of the maricultured macroalga *Pyropia haitanensis* (Bangiales, Rhodophyta) were investigated. The results showed that elevated CO_2 significantly enhanced *P. haitanensis* growth and NO_3^- uptake, but lowered the pH compensation points, regardless of nutrient levels, and enhanced photosynthesis and apparent photosynthetic efficiency (α) when the nutrient levels were high. However, CO_2 had little effect on photosynthetic rates at low nutrient levels. At each nutrient level, CO_2 elevation lowered both the phycobiliproteins (PB) and soluble protein contents, but enhanced biomass accumulation. Chlorophyll *a* (Chl *a*) and carotenoid (Car) contents were markedly increased by high CO_2 concentrations at low nutrient levels. Increasing nutrient supply significantly enhanced growth, pH compensation points, and photosynthesis in *P. haitanensis* at each CO_2 level, but the differences of the effects between intermediate and high nutrient levels on this alga were not significant for photosynthesis, pigment content, and nutrient uptake, regardless of CO_2 levels. Our results suggest that the growth and physiological responses of *P. haitanensis* to CO_2 levels are largely dependent on the supplement of nutrients. However, the interactive effects of elevated CO_2 and nitrogen–phosphorus supplies on the physiological properties of *P. haitanensis* were limited through respective regulation by the CO_2 levels in the atmosphere and the nutrient concentrations in the seawater.

Keywords CO_2 · Nutrient · *Pyropia haitanensis* · Photosynthesis · Pigment · Global change

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Introduction

It is predicted that the atmospheric CO_2 concentration will continue to rise throughout this century (IPCC 2007), and great attention has been paid to how global climate change may affect the global environment. The current CO_2 concentration is reaching the highest it has been for the last 800,000 years (Lüthi et al. 2008). Increased CO_2 levels in the atmosphere not only cause global warming (Florides and Christodoulides 2009) but also cause a decrease in the marine surface pH because more atmospheric CO_2 is dissolved in the ocean (Caldeira and Wickett 2003).

Increasing atmospheric CO_2, together with seawater acidification, has the potential to greatly affect marine organisms (for a review, see Fabry et al. 2008). Marine macroalgae have a considerable biomass production and CO_2 bioremediation potential because the ocean is one of the largest carbon sinks of atmospheric CO_2 on the earth (Gao and McKinley 1994; Zhang et al. 2005). Furthermore, rising CO_2 levels have been shown to affect photosynthesis and other physiological processes in marine macroalgae (Zou and Gao 2002a; 2010; Wu et al. 2008).

Coastal eutrophication, caused by human activity, economic development, and overuse of the coastal environment, has become more and more serious (Fei 2004; Neori et al. 2004). Nutrients, such as nitrogen (N) and phosphorus (P), directly influence the biochemical composition of macroalgae, such as nitrogenous compounds such as Rubisco, a key enzyme in photosynthesis, which affects the storage of organic compounds (Lohman and Priscu 1992).

It is thought that the large scale cultivation of macroalgae in marine areas that are affected by eutrophication may act as a nutrient buffer and help lower N–P contamination risks, such as industrial and agricultural waste water, and/or the economic production of aquatic animals (Fei 2004; Troell et al. 2003). However, excessive nutrients from terrestrial sources cause anthropogenic eutrophication, which can result in the explosive proliferation of marine algae (Lin and Lin 2000; Yabe et al.2009). Moreover, under suitable light and temperature conditions, excessive nutrient levels may stimulate the coastal production of harmful algae and lead to expansion in toxic phytoplankton blooms (Paerl 1997).

Pyropia haitanensis (T.J. Chang and B.F. Zheng) N. Kikuchi and M. Myata (Bangiales, Rhodophyta) is a Chinese nori species that is artificially bred to be grown on a large scale in the South China Sea area (Tseng 1983). This species naturally grows in the mid intertidal zone and is greatly affected by fluctuating environmental condition, such as light, temperature, pH, and nutrient levels.

Previous research has shown that elevated CO_2 levels can enhance photosynthesis of emersed *P. haitanensis* (Zou and Gao 2002b). When the CO_2 concentration was between 60 and 1440 μL L^{-1}, increased CO_2 levels enhanced both intracellular and extracellular carbonic anhydrase (CA) (Zou and Gao 2004), by which the HCO_3^- is dehydrated to CO_2 which is then transported into algal cells (Zou et al. 2004). This suggested that CO_2 transport had increased, consequently leading to a rise in CO_2 assimilation by *P. haitanensis* (Zou and Gao 2004). Moreover, the nitrate reductase (NR) in *P. haitanensis* is positively regulated by seawater NO_3^- concentration and negatively regulated by seawater NH^{4+} (Xu et al. 2007). However, previous laboratory studies have shown that NR levels were not directly positively correlated with NO_3^- concentration in the culture medium (Xu et al. 2007). If the predicted rises in CO_2 and coastal eutrophication occur, then the physiological properties of *P. haitanensis* growing at elevated CO_2 and enriched nitrogen–phosphorus levels need to be investigated.

In this study, we cultured *P. haitanensis* in the laboratory at different levels of CO_2 and at different nutrient concentrations. During the experiments, CO_2 concentration was controlled at either 390 or 1000 μL L^{-1}, with low, medium, and high nitrogen–phosphorus supplies. The aim was to investigate how the elevated CO_2 and different nutrient concentration combinations influenced *P. haitanensis* and whether or not elevated CO_2 levels and N–P supply would have any long-term interactive effects of on this alga.

Materials and methods

Samples of *P. haitanensis* were collected from the mid intertidal rocky shore along the coast of Nan'ao Island, Shantou, China (23° 20′ N, 116° 55′ E). Collected algae were cleaned on-site and brought to laboratory under low temperature conditions. Healthy individuals were selected and rinsed again in sterile seawater. Then, they were pre-cultured in incubators in 5 L aquaria in filtered seawater (salinity 32‰) at a density of 0.5 g fresh weight per liter. The cultured thalli were continuously aerated using ambient air at a rate of about 9 L h^{-1} (i.e., a renewal rate of more than 90 % per hour) under 100 μmol photons m^{-2} s^{-1} illumination supplied by fluorescent tubes with a 12:12 (light/dark) photoperiod. Temperature was controlled at 18 °C.

The environmental CO_2 concentrations were respectively controlled at 390 μL L^{-1} (ambient air, AC) and 1000 μL L^{-1} (elevated CO_2, EC) in two CO_2 incubators, in which the CO_2 concentrations were automatically adjusted by controlling the inflow of ambient air and pure CO_2 gas, and each CO_2 incubator had three levels of nutrient gradients ($NaNO_3$ and NaH_2PO_4 supplied simultaneously): (i) low nutrient supplied (LN): three flasks contained filtered natural seawater (NO_3^- concentration≤40 μmol L^{-1}, PO_4^{3-} concentration <1 μmol L^{-1}) with no additional N and P; (ii) intermediate N–P supply (IN): three flasks contained filtered natural seawater with additional nitrogen (300 μmol L^{-1} NO_3^-) and phosphorus (15 μmol L^{-1} $H_2PO_4^-$); (iii) high nutrient supply (HN): three flasks contained filtered natural seawater with additional nitrogen (600 μmol L^{-1} NO_3^-) and phosphorus (30 μmol L^{-1} $H_2PO_4^-$). Final concentrations of 20 mmol L^{-1} Tris and 20 mmol L^{-1} maleate solution buffers were used in seawater culture (Sober 1974), and the pH in every tank was adjusted with 1 mol L^{-1} HCl or 1 mol L^{-1} NaOH to maintain constant pH on the total scale (AC: pH_{nbs}= 8.17; EC: pH_{nbs}=7.78). The measured concentrations of dissolved inorganic carbon (DIC), estimated using the CO_2SYS program (Lewis and Wallace 1998), were 2032.1 (±30.6) and 2208.5 (±38.3) μmol L^{-1} in the seawater aerated with ambient air and elevated CO_2, respectively. The culture medium was renewed every second day. The photosynthetic traits and biochemical criteria of each sample were determined after 10 days of culture, a period which could be enough for acclimation in marine macroalgae (Zou 2005; Zou et al. 2003; Mercado et al. 1999).

Growth rate estimation Changes in algal biomass (Fw) were measured at the end of culture to estimate growth. Mean

relative growth rate (RGR) was calculated using the formula: RGR (% day^{-1})=ln (W_t/W_0)/t×100, where W_0 is the initial Fw and W_t is the Fw after t days.

Photosynthetic oxygen evolution Approximately 0.06 g (Fw) of algae was utilized to obtain the net photosynthetic O_2 evolution rate (P_n) at different photon irradiances (P vs. E curve) using a Clark-type oxygen electrode (YSI Model5300, USA), with a water jacket connected to a cooling circulator for maintaining the temperature at 18 °C. Light (provided by a halogen lamp) intensity, which was measured with a spherical quantum sensor (SKP 200, ELE International, UK), was set at six levels from 0 to 600 μmol photos m^{-2} s^{-1} by altering the distance between the electrode chamber and the light source. The dark respiration rate (R_d) was measured by determining the dark O_2 consumption. The electrode chamber contained 8 mL culture solution for each growth treatment and was magnetically stirred. The samples of algae were allowed to acclimate to the electrode cuvette environment for 10 min before measurement. Photosynthetic rates are all expressed in μmol O_2 g^{-1}Fw h^{-1}. Photosynthetic parameters were calculated according to Henley (1993). The gross photosynthetic rate (P_g) was the sum of the maximum net photosynthetic O_2 evolution rate (P_m) and the respiration rate. Each treatment had three replications.

pH drift experiment The pH drift experiment of *P. haitanensis* was carried out according to Zou and Gao (2009) in order to estimate the pH compensation point of the alga grown under each treatment, indicating the ability to use HCO_3^-. Exactly 0.15 g (Fw) of alga sample was transferred into sealed glass bottle containing 20 mL natural seawater that was air-filled and stored about 1 h at 18 °C before use. The bottles were incubated at 18 °C with 100 μmol photons m^{-2} s^{-1} illumination, and pH values were determined at regular intervals until the value remained constant.

Pigment estimation Approximately 0.1 g (Fw) of alga was ground in 10 mL 100 % methanol and extracted at 4 °C in darkness for 24 h. This extract was centrifuged at 5000 rpm for 10 min and then used to determine the contents of chlorophyll *a* (Chl *a*) and carotenoid (Car) using an ultraviolet spectrophotometer (UV-1800, Shimadzu, Japan). Chl *a* and Car concentrations were estimated according to Porra (2002) and Parsons and Strickland (1963).

The phycobiliprotein (PB), phycoerythrin (PE), and phycocyanin (PC) contents were estimated by the equation proposed by Beer and Eshel (1985). The algal thallus was washed with deionized water and blotted dry. A sample of 0.2 g fresh weight (Fw) was homogenated in an ice bath with 5 mL phosphate buffer (0.1 mol L^{-1}, pH=6.8). The crude extract was centrifuged at 10,000 rpm for 20 min at 4 °C. The supernatant was transferred into centrifuge tube and the absorbances measured.

Soluble protein determination Content of soluble protein was determined by the Coomassie Brilliant Blue G-250 dye method according to Kochert (1978). Fw of 0.1 g was homogenized in mortar with 5 mL distilled water. The extract was centrifuged at 5000 rpm for 10 min before analysis. Bovine serum albumin (BSA) was used as standard.

NO_3^- uptake estimation Rates of NO_3^- uptake were estimated by determining the decrease in NO_3^- in 24 h in culture medium. NO_3^- concentrations were determined according to the method of Strickland and Parsons (1972). The NO_3^- uptake rate was calculated by the following equation: $\Delta NO_3^- = (N_0 - N_t) \times V/(W_0 \times t)$, where N_0 is the initial nitrate concentration, N_t is the nitrate concentration after t hours, V is the volume of the culture medium, and W_0 is the initial Fw of the alga. The NO_3^- uptake rate is expressed in μmol NO_3^- g^{-1} h^{-1}.

Statistical analyses Origin 8.0 (Origin Lab Corp, USA) was used for data processing and statistical analysis. One-way (ANOVA) and two-way analysis of variance (ANOV2A) and the Tukey test were used to analyze differences among treatments. All the data are expressed as means±SD ($n \geq 3$). A p value of 0.05 was considered as statistically significant.

Results

Relative growth rate The RGR and biomass accumulation of *P. haitanensis* growing under different CO_2 and nitrogen–phosphorus (N–P) supplied conditions are shown in Fig. 1. At each nutrient level, elevated CO_2 significantly enhanced the algal growth and biomass accumulation ($p<0.01$). Intermediate N–P supply significantly enhanced algal growth (AC: $F_{1,4}=81.56$, $p<0.01$; EC: $F_{1,4}=17.35$, $p=0.14$) and biomass accumulation (AC: $F_{1,4}=76.48$, $p<0.01$; EC: $F_{1,4}=18.85$, $p=0.12$) at both CO_2 levels, but there was no significant influence on algal growth (AC: $F_{1,4}=1.66$, $p=0.27$; EC: $F_{1,4}=1.68$, $p=0.26$) and biomass accumulation (AC: $F_{1,4}=1.68$, $p=0.26$; EC: $F_{1,4}=1.69$, $p=0.26$) between the treatments of intermediate and high nutrient levels at the two CO_2 levels.

Photosynthesis and respiration The P_n values in *P. haitanensis* at different irradiances (P–E curves) are shown in Fig. 2. Increased CO_2 substantially increased P_m under intermediate and high N–P supplies ($p<0.01$), but hardly enhanced the P_m under low nutrient treatment ($F_{1,4}=3.92$, $p=0.12$) (Table 1). Increased N–P supply significantly increased the P_m at each CO_2 concentration (Table 2, $p<0.01$), but there were no significant differences between the two increased nutrient levels at both CO_2 levels (AC: $F_{1,4}=0.64$, $p=0.47$; EC: $F_{1,4}=1.87$, $p=0.24$). It is worth noting that the

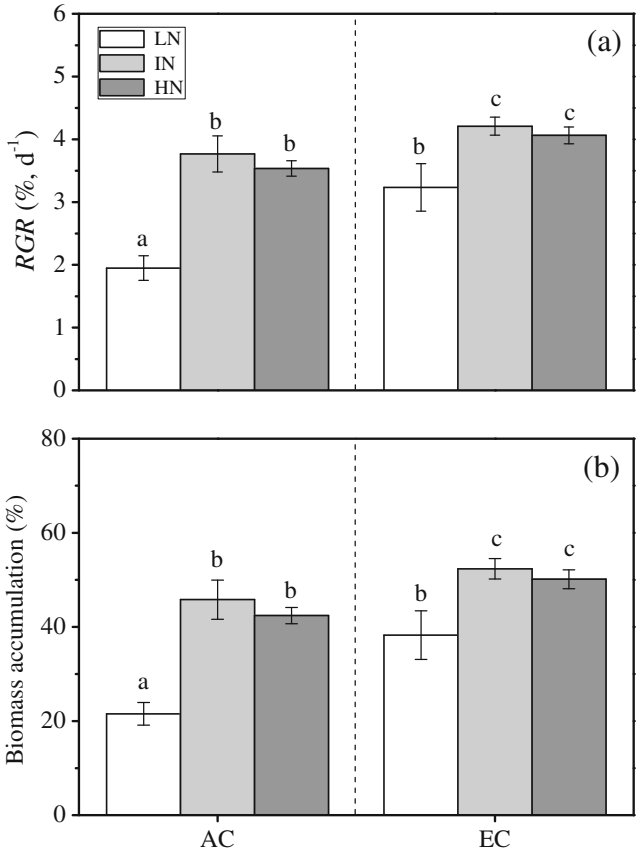

Fig. 1 Relative growth rate (*RGR*) (**a**) and biomass accumulation (**b**) of *Pyropia haitanensis* grown at different CO_2 concentrations (CO_2 level of ambient air, AC, ca. 390 μL L^{-1}; elevated CO_2, EC, 1, 500 μL L^{-1}) and different nitrogen–phosphorus levels. Significant differences among the treatments are indicated by *different lowercase letters* (the Tukey test, $p<0.05$). Values are means±SD ($n=3$)

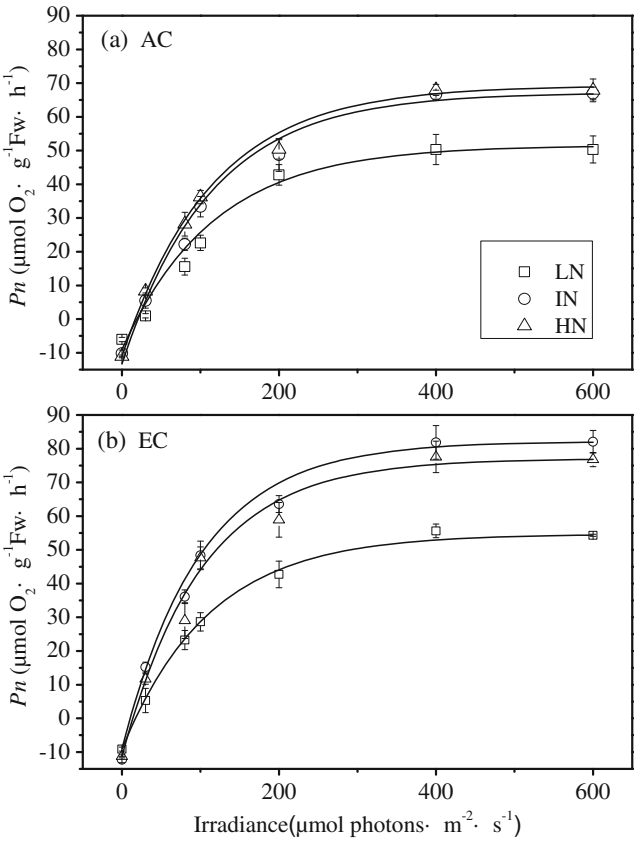

Fig. 2 Rates of photosynthetic O_2 evolution vs. irradiance (P–E) curves of *Pyropia haitanensis* at growth temperature (18 °C) for algae under different CO_2 level (*AC* ambient air, *EC* elevated CO_2 level) and nitrogen–phosphorus concentrations. Values are means±SD for triplicate samples

highest P_m values were observed at elevated CO_2 and intermediate supply treatments ($F_{5,12}=46.18$, $p<0.01$).

Dark respiration (R_d) increased with increased nutrient levels at ambient air (IN: $F_{1,4}=36.99$, $p<0.01$; HN: $F_{1,4}=141.20$, $p<0.01$), and significant enhancements were also observed at elevated CO_2 (IN: $F_{1,4}=24.73$, $p<0.01$; HN: $F_{1,4}=23.68$, $p<0.01$). It was noticed that R_d under low N–P level at ambient CO_2 had the lowest value among all the treatments ($F_{5,12}=29.73$, $p<0.01$). The culture conditions of CO_2 and nutrient levels significantly influenced the P_m and R_d in *P. haitanensis* (Table 2).

The apparent photosynthetic efficiency (α) values were significantly increased at intermediate and high N–P levels at both CO_2 levels ($p<0.01$), but no significant difference was observed in α values between intermediate and high nutrient levels, regardless of CO_2 concentration (AC: $F_{1,4}=0.33$, $p=0.60$; EC: $F_{1,4}=1.09$, $p=0.35$). Elevated CO_2 markedly increased α values regardless of nutrient level treatments ($p<0.01$).

Increasing values of the compensation irradiance point (E_c) and the irradiance saturation point (E_k) were observed at ambient air with N–P supply ($p<0.01$; Table 1). In the elevated CO_2 treatment, E_c (IN: $F_{1,4}=35.10$, $p<0.01$; HN: $F_{1,4}=18.09$, $p=0.01$) and E_k (IN: $F_{1,4}=191.42$, $p<0.01$; HN: $F_{1,4}=48.53$, $p<0.01$) also increased with intermediate and high nutrient supply, whereas at elevated CO_2 level, E_c and E_k values all decreased in algae cultured at the intermediate to high nutrient levels (E_c: $F_{1,4}=9.47$, $p=0.04$; E_k: $F_{1,4}=15.13$, $p=0.02$). The values of α, E_c, and E_k were all significantly influenced by CO_2 and nutrient supply conditions (Table 2).

As shown in Fig. 3, under low nutrient level, the value of R_d/P_g at ambient air condition was remarkably higher than that at high CO_2 level ($F_{1,4}=24.53$, $p<0.01$); however, it was substantially lower than that at elevated CO_2 under high nutrient supply ($F_{1,4}=645.67$, $p<0.01$). The R_d/P_g values were markedly increased with increased nutrient supplies with ambient air ($F_{1,4}=28.66$, $p<0.01$), but apparently declined at elevated CO_2 condition ($F_{1,4}=2.00$, $p=0.22$).

pH compensation point The pH compensation points were obtained during 8 h pH drift period (Fig. 4). At each CO_2 level, the pH compensation points under low N–P supply were all lower than at increased nutrient levels (AC: $F_{1,4}=64.76$,

Table 1 The photosynthetic parameters of P-E curves presented in Fig. 4 for *Pyropia haitanensis* grown at different CO_2 levels and nitrogen–phosphorus concentrations

	Air			Elevated CO_2		
	LN	IN	HN	LN	IN	HN
P_m	50.71±4.14a	67.33±1.63b	68.80±2.73b	55.82±1.68a	82.66±4.32c	78.36±3.33c
R_d	−6.04±0.61a	−10.16±1.00bce	−11.05±0.40bde	−9.12±0.62c	−12.27±0.91de	−11.25±0.44e
α	0.221±0.006a	0.251±0.008b	0.254±0.005b	0.236±0.004c	0.270±0.008d	0.278±0.010d
E_c	27.30±2.03a	40.40±3.21b	46.92±2.32c	33.71±1.82d	48.25±3.84c	40.50±2.07b
E_k	229.26±12.28a	267.92±2.25b	292.05±14.96c	206.64±10.20d	324.88±10.73e	282.09±15.74bc

Significant differences among the treatments are indicated by different lowercase letters (Tukey test, $p<0.05$). Values are means±SD ($n=3$)

P_m maximum net photosynthetic rate (μmol O_2 g^{-1} Fw h^{-1}), R_d dark respiration rate (μmol O_2 g^{-1} Fw h^{-1}), α apparent photosynthetic efficiency [(μmol O_2 g^{-1} Fw h^{-1})/(μmol photons m^{-2} s^{-1})], E_c compensation irradiance point (μmol photons m^{-2} s^{-1}), E_k irradiance saturation point (μmol photons m^{-2} s^{-1})

$p<0.01$; EC: $F_{1,4}=64.92$, $p<0.01$). Under each nutrient level, the pH compensation points at ambient air (Fig. 4a) were all higher than the values at elevated CO_2 (Fig. 4b) ($F_{2,5}=554.86$, $p<0.01$).

Pigment contents At each CO_2 level, increased nutrient supply significantly increased Chl *a* and Car contents in the algae ($p<0.01$) (Table 3). The Chl *a* and Car contents were increased by elevated CO_2 only under low nutrient level (Chl *a*: $F_{1,4}=25.42$, $p<0.01$; Car: $F_{1,4}=80.32$, $p<0.01$), while these contents were respectively increased by the additional nutrients (Chl *a*: $p<0.01$; Car: $p<0.01$).

Contents of PE in *P. haitanensis* significantly increased as the N–P supply increased at each CO_2 level ($p<0.01$), and the PC contents also increased with intermediate nutrient supply at both CO_2 levels ($p<0.01$) (Tables 3 and 4), while elevated CO_2 apparently lowered the PE and PC contents under each nutrient level, although the declines were not significant at intermediate nutrient supply (PE: $F_{1,4}=5.91$, $p=0.07$; PC: $F_{1,4}=5.78$, $p=0.07$). The culture conditions of CO_2 and nutrient levels exhibited significant influences on the pigment contents in *P. haitanensis* (Table 4).

Table 2 Results of two-way ANOVA for photosynthetic parameters derived from P–E curves (in Table 1) of *Pyropia haitanensis* grown at different CO_2 levels and nitrogen–phosphorus concentrations

Source	DF	MS	*F* value	*p* value
Pm				
CO_2 level	1	449.91	45.13	<0.01
Nutrient concentration	2	886.58	88.93	<0.01
CO_2 level×nutrient concentration	2	39.47	3.96	0.05
Rd				
CO_2 level	1	14.47	29.52	<0.01
Nutrient concentration	2	25.98	53.01	<0.01
CO_2 level×nutrient concentration	2	3.21	6.56	0.01
α				
CO_2 level	1	14.47	29.52	<0.01
Nutrient concentration	2	25.98	53.01	<0.01
CO_2 level×nutrient concentration	2	3.21	6.56	0.01
E_c				
CO_2 level	1	<0.01	33.17	<0.01
Nutrient concentration	2	<0.01	50.77	<0.01
CO_2 level×nutrient concentration	2	<0.01	0.61	0.56
E_k				
CO_2 level	1	30.79	4.38	0.06
Nutrient concentration	2	365.43	51.99	<0.01
CO_2 level×nutrient concentration	2	92.61	13.18	<0.01

NO_3^- uptake and protein contents As shown in Fig 5, the intermediate and high nutrient supplies significantly increased the ΔNO_3^- and the SP contents, regardless of CO_2 level ($p<0.01$). At each nutrient level, elevated CO_2 markedly increased the ΔNO_3^- ($p<0.01$), with a significant decline in the SP contents in the alga ($p<0.01$). At each CO_2 level, an

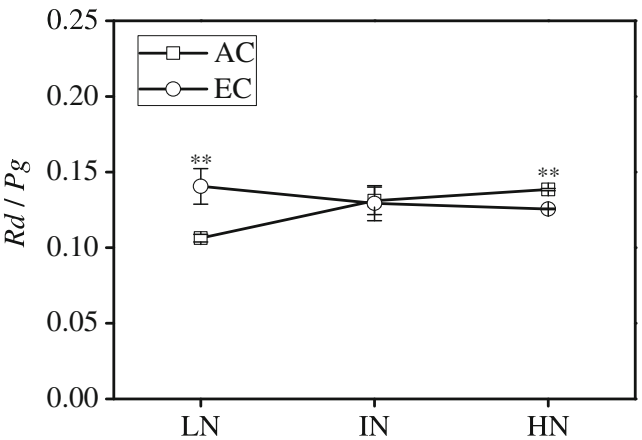

Fig. 3 Ratios of *Rd* to *Pg* of *Pyropia haitanensis* to different CO_2 levels (*AC* ambient air, *EC* elevated CO_2 level) and nutrient supplies. Values are means±SD ($n=3$). *Double asterisks*: Correlation is significant at the 0.01 level

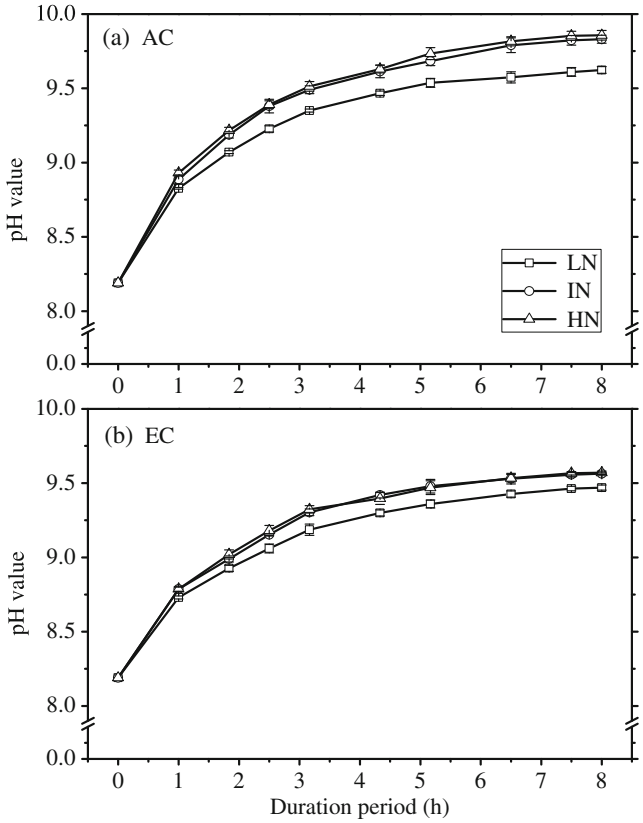

Fig. 4 The pH drifts of *Pyropia haitanensis* exposed to different CO_2 levels (*AC* ambient air, *EC* elevated CO_2 level) and different nitrogen–phosphorus supplemented conditions. Values are means±SD for triplicate samples

increase in nutrient supply from intermediate to high levels hardly influenced the ΔNO_3^- and the SP contents ($p>0.05$). The values of ΔNO_3^- in this alga exhibited a low correlation with SP contents (Fig. 5c).

Discussion

This study showed that *P. haitanensis* growth was significantly enhanced by elevated CO_2, and this increase was not related to nutrient supply. Similar findings have been reported for *Pyropia yezoensis* (Gao et al. 1991) and for *Hizikia fusiforme* (Zou 2005). Moreover, at each CO_2 concentration, increasing the nutrient supply also enhanced the growth and biomass accumulation by the alga. Elevated CO_2 also lowered the *P. haitanensis* pH compensation points, regardless of nutrient levels, which indicated that there had been a reduction in activities of the CO_2 concentrating mechanisms (CCMs). The growth response of the alga may partly depend on the presence of CCMs, which may have substantial energetic and metabolic costs (Israel and Hophy 2002), but may eventually contribute to algal biomass accumulation.

Under the low N–P supply treatment, the *P. haitanensis* photosynthetic rates were not markedly affected by CO_2 levels. In contrast, under the low N–P supply treatment, the *P. haitanensis* dark respiration, photosynthetic efficiency, and the compensation irradiance significantly increased when CO_2

Table 4 Results of two-way ANOVA for the results of the pigment contents in Table 3 in *Pyropia haitanensis* growing at different CO_2 levels and nitrogen–phosphorus concentrations

Source	DF	MS	*F* value	*p* value
Chl *a*				
CO_2 level	1	0.05	7.61	0.02
Nutrient concentration	2	0.16	26.53	<0.01
CO_2 level×nutrient concentration	2	0.02	2.47	0.13
Car				
CO_2 level	1	0.01	11.98	<0.01
Nutrient concentration	2	0.03	35.05	<0.01
CO_2 level×nutrient concentration	2	<0.01	5.40	0.02
PE				
CO_2 level	1	0.47	49.48	<0.01
Nutrient concentration	2	3.33	352.06	<0.01
CO_2 level×nutrient concentration	2	0.05	5.26	0.02
PC				
CO_2 level	1	0.15	23.33	<0.01
Nutrient concentration	2	0.71	111.52	<0.01
CO_2 level×nutrient concentration	2	<0.01	0.54	0.60

Table 3 Pigment contents in *Pyropia haitanensis* growing at different CO_2 levels and nitrogen–phosphorus concentrations

	Ambient air			Elevated CO_2		
	LN	IN	HN	LN	IN	HN
Chl *a*	0.96±0.05a	1.34±0.09b	1.32±0.11bc	1.17±0.05c	1.36±0.05b	1.39±0.10b
Car	0.41±0.01a	0.58±0.03b	0.54±0.03bc	0.51±0.01c	0.59±0.03b	0.57±0.03b
PE	1.80±0.07a	2.77±0.06b	3.07±0.11c	1.28±0.14d	2.61±0.10b	2.79±0.09b
PC	0.90±0.05a	1.49±0.12bd	1.43±0.04b	0.67±0.11c	1.30±0.07de	1.30±0.04e

Significant differences among the treatments are indicated by different lowercase letters (Tukey test, $p<0.05$). Values are means±SD ($n=3$)

Chl a chlorophyll *a* (mg g^{-1} Fw), *Car* carotenoid (mg g^{-1} Fw), *PE* phycoerythrin (mg g^{-1} Fw), *PC* phycocyanin (mg g^{-1} Fw)

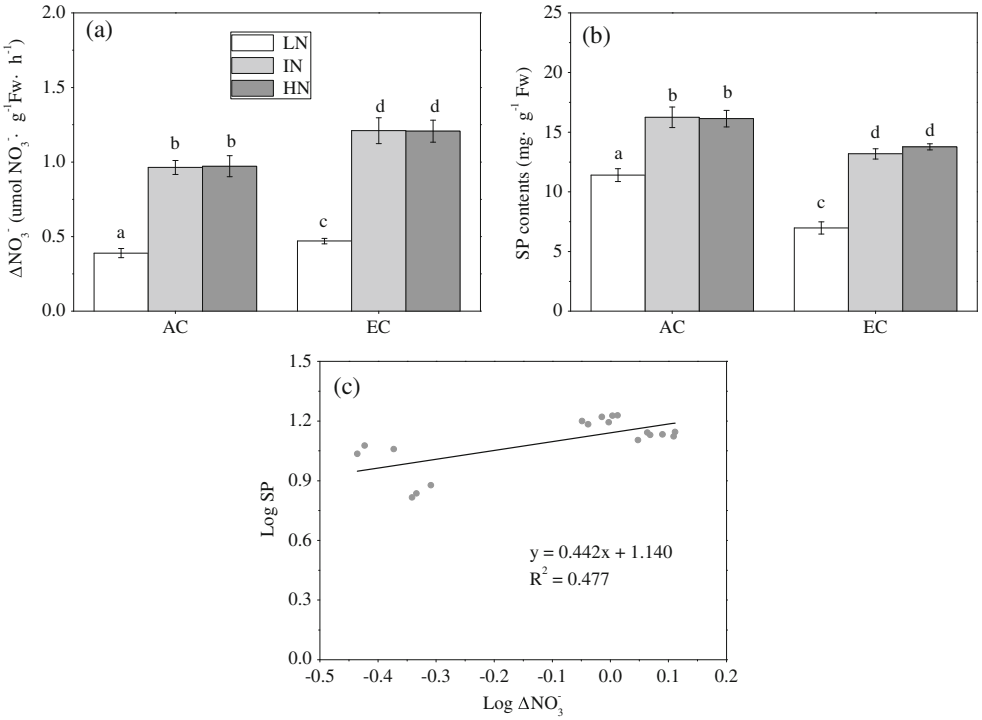

Fig. 5 Rates of NO_3^- uptake (ΔNO_3^-) (**a**) and soluble protein (SP) contents (**b**) and the relationships between log ΔNO_3^- and log SP (**c**) in *Pyropia haitanensis* grown at different CO_2 levels (*AC* ambient air, *EC* elevated CO_2 level) and different nitrogen–phosphorus concentrations. Significant differences among the treatments are indicated by *different lowercase letters* (the Tukey test, $p<0.05$). Linear regressions in (**c**) were used to test the correlation between the ΔNO_3^- and SP ($p<0.05$). Values are means±SD for triplicate samples

levels were high, but the algal irradiance saturation point was decreased by CO_2 elevation. The Chl *a* and Car contents in the alga also rose.

When nutrient supplies were increased, photosynthesis and the photosynthetic efficiency in *P. haitanensis* were markedly enhanced by elevated CO_2. The Chl *a* and Car contents in the algae at the intermediate and high nutrient levels were also increased by elevated CO_2. It has been reported that N enrichment could enhance photosynthesis rate and photosynthetic efficiency in algae (Dawes and Koch 1990; Crawford 1995; Chen et al. 2011). High nutrient supplies increased the contents of photosynthetic pigments and nitrogenous compounds, such as Rubisco, a key enzyme in photosynthesis, and finally enhanced photosynthesis. However, the PB contents, including PE and PC, declined under elevated CO_2 and low nutrient conditions, but increased when additional nutrients were supplied. It has also been reported that PE and PC contents in *Pyropia leucosticta* (Mercado et al. 1999) and in *Gracilaria lemaneiformis* (Zou and Gao 2009) cultured at high levels of inorganic carbon (Ci) also decreased.

The DIC in seawater increased under elevated CO_2 conditions, and the increased CO_2 levels enhanced both the intracellular and extracellular CA in *P. haitanensis* (Zou and Gao 2004). Thus, more nutrients and increased light were required to meet the demands of the enhanced photosynthesis activity caused by CO_2 elevation. Under the low nutrient treatment, *P. haitanensis* photosynthesis was depressed by the lack of N and P. Nitrogen plays an important role in photosynthesis and is an important component of many plant compounds, such as Rubisco, the key enzyme in photosynthesis (Dawes and Koch 1990; Crawford 1995), and phosphorus is required for various chloroplast functions, including ATP generation and photosynthetic protein and enzyme phosphorylation (Zer and Ohad 2003). Under the low nutrient level treatment, newly synthesized carbohydrates could not be used in protein synthesis and organic structure development due to the lack of N and P. This led to excessive carbohydrate accumulation, which probably depressed the relative expressions of photosynthetic enzyme genes that normally upregulated for the photosynthate production. Moreover, PB is used as an N reservoir in case N was needed for algal growth (Kursar and Alberte 1983; Zou and Gao 2009). This probably resulted in an increased allocation of energy to nutrient uptake and assimilation processes and ultimately affected dark respiration.

When nutrients levels were high, increased CO_2 levels enhanced photosynthesis and consequently led to a rise in photosynthetic pigment contents, which indicated more carbohydrates were being synthesized. Nitrogen enrichment can stimulate pigment synthesis by promoting nitrogen metabolism (Fujii et al. 2012), which competes for energy and electrons sinks with photosynthesis (Falkowski and Raven 2007). When N and P levels were high, the photosynthate in *P. haitanensis* at elevated CO_2 concentrations was functionally utilized for algal growth and protein synthesis, and when combined with sufficient Rubisco and phosphorylation intermediates, this led to photosynthesis enhancement in *P. haitanensis*.

It was noted that, under low nutrient level, the value of R_d/P_g at ambient air was remarkably higher than that at high CO_2

level. This result was inconsistent with the results of Zou et al. (2011), who found that the R_d/P_g in *Hizikia fusiformis* was not significantly influenced by CO_2 elevation. Under low nutrient level, CO_2 enrichment slightly enhanced the photosynthesis of *P. haitanensis*, and this stimulated the nutrient demands for algal growth. Thus, more energy was probably needed for nutrient uptake and active transport or utilization of the N reservoir (such as PB) (Kursar and Alberte 1983). Thus, dark respiration was enhanced by CO_2 elevation. However, the value of R_d/P_g at ambient air was significantly lower than that at elevated CO_2 under high nutrient supply, which was consistent with Zou et al. (2011). Under high nutrient level, a high CO_2 level decreases photorespiration in algae (Stitt and Krapp 1999; Zou et al. 2011), and CO_2 enrichment enhanced the nutrient uptake and assimilation, which would permit decreased investment of nitrogen in the nitrogen-intensive process of photosynthesis and photorespiration (Zou 2005).

Nitrogen metabolism in *P. haitanensis* was highly correlated with the NO_3^- concentration in the culture medium. At each CO_2 concentration, increased nutrient levels markedly enhanced NO_3^- uptake and soluble protein synthesis. Increased nutrient uptake at high nutrient level has also been reported in some other species of *Pyropia* (Pedersen et al. 2004) and in *Gracilaria lemaneiformis* (Xu et al. 2008). At each nutrient level, CO_2 elevation increased NO_3^- uptake, especially at the intermediate and high nutrient levels. It is possible that CO_2 enrichment of the culture medium increased both NO_3^- uptake and NR activity (Mercado et al. 1999; Zou 2005). However, the soluble protein contents in the algae fell when the CO_2 concentration was high. This is consistent with the results for some *Gracilaria* species (Andria et al. 1999; Xu et al. 2008). In this study, NO_3^- uptake by *P. haitanensis* had a low correlation with soluble protein contents. It could be that the enhanced algal growth caused by high CO_2 levels accelerated the assimilation and fixation of inorganic nitrogen or the NR activity was not directly related to NO_3^-–N concentration in the culture, although NO_3^- enhanced NR activity in *P. haitanensis* (Xu et al. 2007).

This research showed that increased nutrient supply greatly influenced the physiological properties of *P. haitanensis*, especially in combination with elevated CO_2. The interactive effect of elevated CO_2 and nutrient supply has also been shown in *G. lemaneiformis* (Xu et al. 2008). It also has been shown that the photosynthesis and growth of *Gracilaria gaditana* (Andria et al. 1999) were enhanced only under sufficient N nutrient condition. However, there must be an upper limit to the positive growth improvements brought about by increasing nutrient concentrations. The positive effects included rises in photosynthesis, pigment content, and nitrogen metabolism, but the differences in these improvements between the intermediate and high nutrient levels were not great. Moreover, excessive nutrient levels may result in the expansion of harmful blooms (Paerl 1997; Lin and Lin 2000; Yabe et al. 2009). Thus, a controlled fertilization strategy should be used during *P. haitanensis* mariculture. Additionally, as considered by Schanz and Juon (1983), N is the limiting factor for algal growth at NO_3^-–N/PO_4^{3-}–P ratios less than 10, while P is limiting at ratios greater than 20. In this study, the N/P ratios in three nutrient treatments were all greater than 20:1. It is worth to further investigate if P was a limited factor for *P. haitanensis* growth and photosynthesis.

In summary, the growth and physiological properties of *P. haitanensis* to elevated CO_2 levels were considerably affected by the nutrient concentrations in the seawater. When nutrient levels were insufficient, increasing the CO_2 concentration hardly had any effect on biomass accumulation and photosynthetic rate, whereas when nutrients were enriched, enhanced CO_2 levels significantly enhanced *P. haitanensis* photosynthesis and growth. However, the interactive effect of elevated CO_2 and nitrogen–phosphorus supplies on the physiological properties of *P. haitanensis* was limited because the processes were regulated by the CO_2 levels in the atmosphere and the nutrient concentrations in the seawater. Further studies need to explore whether *P. haitanensis* growth and photosynthesis would be further enhanced by the high nutrient level treatment combined with even higher CO_2 levels compared to the increase seen under the low and intermediate nutrient level treatments and the two CO_2 concentrations used in this study.

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