Physiological response of a golden tide alga (*Sargassum muticum*) to the interaction of ocean acidification and phosphorus enrichment

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Abstract

The development of golden tides would be influenced by global change factors, such as ocean acidification and eutrophication, but the related studies are very scarce. In this study, we cultured a golden tide alga, *Sargasssum muticum*, at two levels of pCO_2 (400, 1000 μatm) and phosphate (0.5 μM, 40 μM) conditions to investigate the interactive effects of elevated pCO_2 and phosphate on physiological properties of the thalli. The higher pCO_2 level and phosphate (P) level alone increased the relative growth rate by 41% and 48%, net photosynthetic rate by 46% and 55%, soluble carbohydrates by 33% and 62% respectively whilst the combination of these two levels did not promote growth or soluble carbohydrates further. The higher levels of pCO_2 and P alone also enhanced the nitrate uptake rate by 68% and 36%, nitrate reductase activity by 89% and 39%, and soluble protein by 19% and 15% respectively. The nitrate uptake rate and soluble protein was further enhanced although the nitrate reductase activity was reduced when the higher levels of pCO_2 and P worked together. The higher pCO_2 level and higher P level alone did not affect the dark respiration rate of thalli but they together increased it by 32% compared to the condition of the lower pCO_2 and lower P. The mute effect of the higher level of pCO_2 and higher P on growth, soluble carbohydrates, combined with the promoting effect of it on soluble protein and dark respiration, suggests more energy was drawn from carbon assimilation to nitrogen assimilation at the condition of higher pCO_2 and higher P, probably to act against the higher pCO_2 caused acid-base perturbation via synthesizing H^+ transport-related protein. Our results indicate ocean acidification and eutrophication may not boost the gold tides events synergistically although each of them alone has a promoting effect.

Key words: carbohydrates, growth, photosynthesis, protein, respiration, *Sargassum muticum*

1. Introduction

Sargassum C. Agardh (1820) is the most species-rich genus in the Phaeophyta and has a global distribution (Mattio and Payri, 2011). The species of this genus constitutes an important part of the marine flora and is considered as a valuable and

unique habitat for a number of highly adapted marine animal species (Laffoley et al., 2011). Some species of *Sargassum* are economically important, being used as animal fodder, manure in agriculture, as well as alginates production (Ashok-Kumar et al., 2012; Fenoradosoa et al., 2010; González-López et al., 2012). On the other hand, *Sargassum* is an aggressive genus and it can rapidly spread and invade new areas (Sfriso and Facca, 2013). The invasion of *Sargassum* would accordingly compete with indigenous species for nutrients and light and lead to the alteration of macroalgal community structure (Rueness, 1989; Stæhr et al., 2000). For instance, the increased abundance of *S. muticum* in Limfjorden (Denmark) between 1990 and 1997 led to decreased cover of several indigenous species belonging to the genera of *Codium*, *Fucus*, and *Laminaria*, and thus reduced species richness and diversity of the macroalgal community (Stæhr et al., 2000). Recently, the species of *Sargassum* inundate the coasts along Gulf of Mexico, West African, Caribbean, and Brazil in unprecedented biomass, termed as golden tides (Schell et al., 2015; Smetacek and Zingone, 2013). Apart from the negative effect on aesthetics and tourism, the occurrence of golden tides could kill the fish within the algal mass, mainly due to hypoxia or anoxia in the waters caused by decomposition of *Sargassum* thalli (Cruzrivera et al., 2015). In addition, the dense *Sargassum* accumulation could clog fishing nets and impede the passage of boats, leading to food shortages for local people who live on artisanal fisheries (Smetacek and Zingone, 2013). The occurrence of golden tides has been linked to higher nutrient levels in the seawaters (Lapointe, 1995; Smetacek and Zingone, 2013). The distribution pattern and biomass of *Sargassum* spp. are environment (temperature, light, nutrients, etc.)-dependent (Ang, 2006; Sfriso and Facca, 2013).

Due to burning fossil fuels and changes to land use, the atmospheric concentrations of carbon dioxide have increased to the level of 401.72 ppm in July 2016 (http://www.esrl.noaa.gov/gmd/ccgg/trends/global.html), which is unprecedentedly high in at least the last 800,000 years (IPCC, 2013). When CO_2 dissolves in seawater it forms carbonic acid and as more CO_2 is taken up by the ocean's surface, the pH decreases, moving towards a less alkaline and therefore more

acidic state, termed ocean acidification. The mean surface ocean pH has already decreased by 0.1 units since the beginning of the industrial era, corresponding to a 26% increase in hydrogen ion concentration (IPCC, 2013). By 2100, concentrations of CO_2 (aq) and HCO_3^- are predicted to increase by 192% and 14%, respectively, and CO_3^{2-} to decrease by 56%, with a concomitant decline in pH to 7.65 (Raven et al., 2005). Increased CO_2 could exert positive, neutral, or negative on physiological properties of macroalgae (Ji et al., 2016; Wu et al., 2008). In terms of *Sargassum* species, increased CO_2 (800 ppm) enhanced photosynthetic rate (based on CO_2 uptake) in *S. muticum* (Longphuirt et al., 2014). On the other side, the same level of increased CO_2 (750 ppm) did not affect growth, Rubisco's maximal activity, affinity for CO_2 or quantity in *S. vulgare* (Alvaro and Mazal, 2002). Furthermore, increased CO_2 (750 ppm) significantly decreased net photosynthetic rate and light saturation point of *S. henslowianum* (Chen and Zou, 2014).

Apart from ocean acidification, eutrophication is another environmental challenge. Eutrophication can occur naturally in lakes via transferring nutrients from the sediment to water by living or decomposing macrophytes, resuspension, diffusion, and bioturbation (Carpenter, 1981). However, anthropogenic activities have accelerated the rate and extent of eutrophication (Carpenter et al., 1998). Inevitable urbanization of a growing human population, increased use of coastal areas, and rising fertilizer use for agricultural intensification has led to accelerated nutrient inputs from land-water to coastal waters (Smith et al., 1999). These changes in nutrient availability result in eutrophication, an increasing threat for coastal ecosystems (Bricker et al., 2008). One consequence of eutrophication is that it can lead to algal bloom, such as green tides and golden tides (Smetacek and Zingone, 2013). There are relatively intensive studies regarding the effect of nutrients on physiological properties of *Sargassum* species (Hwang et al., 2004; Incera et al., 2009; Lapointe, 1995; Liu and Tan, 2014; Nakahara, 1990). Enrichment of nutrients usually can enhance the growth and photosynthetic parameters of *Sargassum*. For instance, the growth rate of *S. baccularia* almost doubled when nutrients increased from 3 μM ammonium plus 0.3 μM phosphate to 5 μM ammonium plus 0.5 μM phosphate

(Schaffelke and Klumpp, 1998) and the photosynthetic rates of *S. fluitans* and *S. natans* were also two-fold higher with 0.2 mM PO_3^- enrichment compared to the control (Lapointe, 1986). Furthermore, some studies have demonstrated that macroalgae experience more phosphorus limit instead of nitrogen limit (Lapointe, 1986; Lapointe et al., 1987, 1992; Littler et al., 1991). For instance, nitrogen enrichment did not affect growth rates of *S. fluitans* or *S. natans* whilst phosphorus enrichment increased them from 0.03–0.04 (control) to 0.05–0.08 doublings d^{-1} (Lapointe, 1986).

Neither ocean acidification nor eutrophication is proceeding in isolation; rather they occur simultaneously, particularly in coastal areas. The interactive effects of two factors may be completely different, or be of greater magnitude, compared to effects of any single stressor. To the best of our knowledge, no studies have been reported in regard to the interactive effects of ocean acidification and eutrophication on *Sargassum*. In this study, we chose the species *S. muticum* to investigate its responses to interaction of ocean acidification and eutrophication. *S. muticum* is an invasive macroalga and commonly habitats on rocky shores (Karlsson and Loo, 1999). It originates from Japan and was introduced to the northern Pacific coast of the United States in the early 20th century (Scagel, 1956), and was also introduced to Europe along with the imported Japanese oyster in the late 1960s (Jones and Farnham., 1973). Nowadays, its distribution is worldwide due to the introduction and the subsequent rapid expansion (Cheang et al., 2010). Our study would supply insight into how ocean acidification and eutrophication affect the physiological properties of *S. muticum* and thus the evolvement of golden tides.

2. Materials and methods

2.1. Sample collection and experiment design

S. muticum was collected from lower intertidal rocks on the coast of Lidao, Rongcheng, China (37°15′N, 122°35′E). The samples were transported to the laboratory in an insulated polystyrene cooler (4–6 ℃) within 3 hours. Healthy thalli were selected and rinsed with sterile seawater to remove sediments, epiphytes and small grazers. The thalli were maintained in an intelligent illumination incubator

(MGC-250P, Yiheng Technical Co. Ltd., Shanghai, China) for 24 hours before the experiment. The temperature in the incubator was set as 20°C with a 12h: 12h (light/dark) photoperiod of 150 μmol photons m^{-2} s^{-1} photosynthetically active radiation (PAR). After the maintenance, a two-way factorial experiment was set up to investigate the interactive effects of pCO_2 and phosphate on *S. muticum.* The thalli were placed in 3 L flasks with 2 L sterile seawater (one thallus per flask) and cultured at fully crossed two pCO_2 (400 μatm, LC; 1000 μatm, HC) and two phosphate (0.5 μM, LP; 40 μM, HP) levels with continuous aeration for 13 days. Phosphorus was selected as a nutrient variable since some findings have displayed that phosphorus, rather than nitrogen, is the primary limiting nutrient for macroalgae (Lapointe, 1986; Lapointe et al., 1987, 1992; Littler et al., 1991). The 400 μatm pCO_2 and 0.5 μM phosphate are the conditions of natural seawater. The 400 μatm pCO_2 was achieved by bubbling ambient air and 1000 μatm pCO_2 was obtained through a CO_2 plant chamber (HP1000 G-D, Wuhan Ruihua Instrument & Equipment Ltd, China) with the variation of CO_2 less than 5%. The higher P level (40 μM) was achieved by adding NaH_2PO_4 to natural seawater and the nitrate concentration was set as 200 μM for all treatments to avoid N limit. The media were refreshed every day.

2.2. Carbonate chemistry parameters

The seawater pH was recorded with a pH meter (pH 700, Eutech Instruments, Singapore) and total alkalinity (TA) was measured by titrations. The salinity of seawater was 29. Other carbonate system parameters, which were not directly measured, were calculated via CO2SYS (Pierrot et al., 2006), using the equilibrium constants of K_1 and K_2 for carbonic acid dissociation (Roy et al., 1993).

2.3. Measurement of growth

The growth of *S. muticum* was determined by weighing fresh thalli. The thalli of *S. muticum* were blotted gently with tissue paper to remove water on the surface of the thalli before weighing. The relative growth rate (RGR) was estimated as follows: $RGR = (\ln W_t - \ln W_0) / t \times 100$, where W_0 is the initial fresh weight (FW) and W_t is the weight after t days culture.

2.4. Determination of photosynthesis and respiration

The net photosynthetic rate of thalli was measured by a Clark-type oxygen electrode (Chlorolab-3, Hansatech, Norfolk, UK) at the end of the experiment. Approximately 0.1 g of fresh weight algae harvested from the culture flask was transferred to the oxygen electrode cuvette with 8 ml sterilized media, and the media were stirred during measurement. The irradiance and temperature conditions were set as the same as that in the growth incubators. The increase of oxygen content in seawater within five minutes was defined as net photosynthetic rate and the decrease of oxygen content in seawater in darkness within ten minutes was defined as respiration rate. Net photosynthetic rate (NPR) and respiration rate were presented as μmol O_2 g^{-1} FW h^{-1}.

Photosynthetic rates at different dissolved inorganic carbon (DIC) levels were measured under saturating irradiance of 600 μmol photons m^{-2} s^{-1} at the end of the experiment. The various DIC concentrations (0–13.2 mM) were obtained by adding different amounts of $NaHCO_3$ to the Tris buffered DIC-free seawater. DIC was removed from the natural seawater by reducing pH to approximately 4.0 with the addition of 1.0 M HCl, and then sparging for 2 h with pure N_2 gas (99.999%). Finally, Tris buffer (25mM) was added and the pH was adjusted to 8.1 with freshly prepared 1 M NaOH and 1 M HCl. The parameters, maximum photosynthetic rate (V_{max}) and the half saturation constant ($K_{0.5}$, i.e., the DIC concentration required to give half of Ci-saturated maximum rate of photosynthetic O_2 evolution), were calculated from the Michaelis-Menten kinetics equation (Caemmerer and Farquhar, 1981):

$V = V_{max} \times [S] / (K_{0.5} + [S])$, where [S] is the DIC concentration.

2.5. Assessment of photosynthetic pigments

Approximately 100 mg of fresh weight thalli from each culture condition at the end of the experiment was ground thoroughly in 2 ml 80% acetone and placed in darkness for 12 hours. Then the homogenate was centrifuged for 10 minutes at 5, 000 *g* and the supernatant was used to determine Chl *a* content spectrophotometrically according to the equation of Lichtenthaler (1987).

2.6. Measurement of nitrate uptake rate

The nitrate uptake rate (NUR) of thalli was estimated from the decrease of NO_3^-

concentration in the culture medium over a given time interval (12 hours) during light period using the following equation: NUR = $(N_0 - N_t) \times V / W / 12$, where N_0 is the initial concentration of NO_3^-, N_t is the concentration after 12 hours, V is the volume of the culture medium, and W is the fresh weight of the thalli in culture. NO_3^- concentration in the seawater was measured according to Strickland and Parsons (1972).

2.7. Estimate of nitrate reductase activity

Nitrate reductase activity of thalli was assayed according to modified in situ method of Corzo and Niell (1991). The measurement was conducted during the local noon period (13:00) since the activity of nitrate reductase usually displays circadian periodicity a maximum during the light period and a minimum in darkness (Deng et al., 1991; Velasco and Whitaker, 1989). Approximately 0.3 g (FW) of thalli from each culture condition was incubated for 1 h at 20°C in darkness in the reaction solution (10 mL), which contained 0.1 M phosphate buffer, 0.1% propanol (v/v), 50 mM KNO_3, 0.01 mM glucose, and 0.5 mM EDTA, with a pH of 8.0. The mixture was flushed with pure N_2 gas (99.999%) for 2 minutes to obtain an anaerobic state before the incubation. The concentration of nitrite produced was determined colorimetrically at 540 nm (Zou, 2005). The NR activity was expressed as μmol NO_2^- g^{-1} FW h^{-1}.

2.8. Analysis of biochemical composition

About 0.2 g of FW thalli from each culture condition at the end of the experiment were ground in a mortar with distilled water and soluble carbohydrates were extracted in a water bath of 80°C for 30 min. After being centrifuged for 10 minutes at 5, 000 *g*, supernatant was volumed to 25 ml with distilled water, and soluble carbohydrates content was determined by phenol-sulfuric acid method (Kochert, 1978).

Approximately 0.2 g of FW thalli from each culture condition at the end of the experiment were ground in a mortar with extraction buffer (0.1 mol L^{-1} phosphate buffer, pH 6.8) and then centrifuged for 10 minutes at 5, 000 *g*. Soluble protein was estimated from the supernatant using the Bradford (1976) assay with bovine serum albumin as a standard.

2.9. Data Analysis

Results were expressed as means of replicates ± standard deviation. Data were analyzed using the software SPSS v.21. The data under every treatment conformed to a normal distribution (Shapiro-Wilk, $P > 0.05$) and the variances could be considered equal (Levene's test, $P > 0.05$). Two-way ANOVA was conducted to assess the effects of pCO_2 and P on carbonate parameters, relative growth rate, net photosynthesis rate, V_{max}, $K_{0.5}$, Chl *a*, nitrate uptake rate, nitrate reductase activity, soluble carbohydrates, soluble protein, and dark respiration rate. Tukey HSD was conducted for *post hoc* investigation. A confidence interval of 95% was set for all tests.

3. Results

The effects of ocean acidification and P enrichment on seawater carbonate parameters were detected first (Table 1). Two-way ANOVA analysis ($P = 0.05$) showed that pCO_2 had a main effect on all parameters except TA whilst P did not affect any parameter. *Post hoc* Tukey HSD comparison ($P = 0.05$) showed that elevated pCO_2 decreased pH by 0.31 unit at both LP and HP, CO_3^{2-} by 45% (LP) and 45% (HP), but increased DIC by 10% (LP) and 9% (HP), HCO_3^- by 14% (LP) and 14% (HP), and CO_2 by 139% (LP) and 134% (HP).

The growth of *S. muticum* cultured at different pCO_2 and P conditions was recorded (Fig. 1). pCO_2 and P had an interactive effect on the relative growth rate of *S. muticum* (ANOVA, F = 5.776, df = 1, 8, $P = 0.043$) and each factor had a main effect (ANOVA, F = 19.145, df = 1, 8, $P = 0.002$ for pCO_2; ANOVA, F = 30.592, df = 1, 8, $P = 0.001$ for P). *Post hoc* Tukey HSD comparison ($P = 0.05$) showed that the higher levels of pCO_2 and higher P alone increased the relative growth rate by 41% and 48% respectively, compared to the relative growth rate (3.1 ± 0.4%) at the condition of lower pCO_2 and lower P. The combination of the higher pCO_2 and higher P levels did not enhance the relative growth rate as much as the sum of the higher pCO_2 alone plus the higher P alone, with an increase of 59.66%. Although the higher P level increased the relative growth rate at the condition of lower pCO_2, it did not affect the relative growth rate at the condition of higher pCO_2.

In terms of the net photosynthetic rate (Fig. 2), both pCO_2 (ANOVA, F = 26.556,

df = 1, 8, P = 0.001) and P had main effects (ANOVA, F = 38.963, df = 1, 8, P < 0.001) on it. *Post hoc* Tukey HSD comparison (P = 0.05) showed the higher pCO_2 level increased the net photosynthetic rate by 46% and 24% at the conditions of lower P and higher P respectively. The higher P level increased the net photosynthetic rate by 55% and 31% at the conditions of lower pCO_2 and higher pCO_2 respectively. The difference in the net photosynthetic rate between LCHP and HCLP was statistically insignificant.

The carbon-saturating maximum photosynthetic rate (V_{max}) and the half saturation constant ($K_{0.5}$), obtained from the photosynthesis versus DIC curves (Fig. 3), are shown in Table 2. The pCO_2 and P had an interactive effect on V_{max} of *S. muticum* (ANOVA, F = 10.095, df = 1, 8, P = 0.013) and each factor had a main effect (ANOVA, F = 31.402, df = 1, 8, P = 0.001 for pCO_2; ANOVA, F = 105.116, df = 1, 8, P < 0.001 for P). *Post hoc* Tukey HSD comparison (P = 0.05) showed the higher pCO_2 level increased the V_{max} by 42% at the condition of lower P while the increase at the condition of higher P was statistically insignificant. The higher P level increased the V_{max} at the conditions of both lower pCO_2 (65%) and higher pCO_2 (24%), with the larger promoting effect at the condition of lower pCO_2.

pCO_2 and P interacted on the $K_{0.5}$ of *S. muticum* (ANOVA, F = 5.928, df = 1, 8, P = 0.041) and each factor had a main effect (ANOVA, F = 14.713, df = 1, 8, P = 0.005 for pCO_2; ANOVA, F = 20.857, df = 1, 8, P = 0.002 for P). *Post hoc* Tukey HSD comparison (P = 0.05) showed the higher pCO_2 level increased the $K_{0.5}$ by 98% at the condition of lower P but did not affect it at the condition of higher P. In contrast, the higher P level decreased the $K_{0.5}$ by 55% at the condition of higher pCO_2 and the negative effect of the higher P level at the condition of lower pCO_2 was insignificant.

The contents of photosynthetic pigment Chl *a* under various treatments were also estimated (Fig. 4). pCO_2 and P had an interactive effect on the Chl *a* content (ANOVA, F = 8.184, df = 1, 8, P = 0.021), P had a main effect (ANOVA, F = 22.828, df = 1, 8, P = 0.001), while pCO_2 did not affect it (ANOVA, F = 0.676, df = 1, 8, P = 0.435). *Post hoc* Tukey HSD comparison (P = 0.05) showed the higher P level increased the Chl *a* content from 0.17 ± 0.00 to 0.25 ± 0.02 mg g^{-1} FW at the

condition of lower pCO_2 whereas the difference in the Chl *a* content between HCLP (0.21 ± 0.02 mg g^{-1} FW) and HCHP (0.23 ± 0.02 mg g^{-1} FW) was not statistically significant.

To assess the effects of ocean acidification and P enrichment on the nitrogen assimilation in *S. muticum*, nitrate uptake rate under various pCO_2 and P treatments was investigated first (Fig. 5). Both pCO_2 (ANOVA, F = 139.916, df = 1, 8, $P < 0.001$) and P (ANOVA, F = 43.923, df = 1, 8, $P < 0.001$) had main effects on the nitrate uptake rate of *S. muticum*. The nitrate uptake rates at the conditions of lower pCO_2 were 0.18 ± 0.01(LP) and 0.25 ± 0.03 μmol NO_3^- g^{-1} FW h^{-1} (HP) respectively. *Post hoc* Tukey HSD comparison ($P = 0.05$) showed the higher pCO_2 level increased the nitrate uptake rate to 0.31 ± 0.02 μmol NO_3^- g^{-1} FW h^{-1} at the condition of lower P and to 0.39 ± 0.01 μmol NO_3^- g^{-1} FW h^{-1} at the condition of higher P, compared to those at the conditions of lower pCO_2. The higher P level also increased the nitrate uptake rate by 36% at the condition of lower pCO_2 and by 28% at the condition of higher pCO_2, compared to those at the conditions of lower P.

Apart from nitrate uptake, the nitrate reductase activity (NRA) of *S. muticum* under various pCO_2 and P treatments was also detected (Fig. 6). pCO_2 and P interacted on NRA of *S. muticum* (ANOVA, F = 28.435, df = 1, 8, $P = 0.001$) and pCO_2 had a main effect (ANOVA, F = 59.038, df = 1, 8, $P < 0.001$). The NRA at the conditions of lower pCO_2 were 0.10 ± 0.01 (LP) and 0.14 ± 0.02 μmol NO_2^- g^{-1} FW h^{-1} (HP) respectively, and the higher pCO_2 level increased it to 0.19 ± 0.00 μmol NO_2^- g^{-1} FW h^{-1} at the condition of lower P and to 0.15 ± 0.02 μmol NO_2^- g^{-1} FW h^{-1} at the condition of higher P. The higher P level increased the NRA by 39% at the condition of lower pCO_2, however, it decreased NRA by 18% at the condition of higher pCO_2.

The soluble carbohydrates (Fig. 7a) and protein (Fig. 7b) were estimated to understand the effects of ocean acidification and P enrichment on the products of carbon and nitrogen assimilation in *S. muticum*. pCO_2 and P had an interactive effect on the soluble carbohydrates (ANOVA, F = 18.294, df = 1, 8, $P = 0.003$) and P had a main effect (ANOVA, F = 23.129, df = 1, 8, $P = 0.001$). The higher P level increased the soluble carbohydrates from 25.40 ± 1.66 to 41.10 ± 1.74 mg g^{-1} FW at the

condition of lower pCO_2 but did not alter it at the condition of higher pCO_2. The higher pCO_2 level increased the soluble carbohydrates to 33.72 ± 3.31 mg g^{-1} FW at the condition of lower P while the decrease of soluble carbohydrates caused by the higher pCO_2 level was not statistically significant at the condition of higher P.

Both pCO_2 (ANOVA, F = 106.663, df = 1, 8, $P < 0.001$) and P (ANOVA, F = 75.003, df = 1, 8, $P < 0.001$) had main effects on the soluble protein of *S. muticum* and the interactive effect of the two factors was not detected (ANOVA, F = 4.961, df = 1, 8, $P = 0.057$). The soluble protein contents at the conditions of lower pCO_2 were 8.49 ± 0.49 (LP) and 9.77 ± 0.14 mg g^{-1} FW (HP) respectively. The higher pCO_2 level increased it to 10.11 ± 0.16 mg g^{-1} FW at the condition of lower P and to 12.28 ± 0.44 mg g^{-1} FW at the condition of higher P. The higher P level also increased the soluble protein contents by 15% at the condition of lower pCO_2 and by 21% at condition of higher pCO_2.

Finally, the effects of ocean acidification and P enrichment on the dark respiration rate of *S. muticum* were investigated (Fig. 8). pCO_2 and P had an interactive effect on the dark respiration rate (ANOVA, F = 19.584, df = 1, 8, $P = 0.002$) and each factor had a main effect (ANOVA, F = 6.428, df = 1, 8, $P = 0.035$ for pCO_2; ANOVA, F = 6.754, df = 1, 8, $P = 0.032$ for P). The higher pCO_2 level increased the dark respiration rate from 14.21 ± 1.94 to 21.24 ± 1.28 μmol O_2 g^{-1} FW h^{-1} at the condition of higher P but did not affect it at the condition of lower P. Likewise, The higher P level increased the respiration rate from 14.15 ± 0.65 to 21.24 ± 1.28 μmol O_2 g^{-1} FW h^{-1} at the condition of higher pCO_2 but did not change it at the condition of lower pCO_2.

4. Discussion

4.1. Effects of pCO_2 *and P on carbon assimilation*

The higher pCO_2 level increased the net photosynthetic rate in *S. muticum* at the condition of lower P in the present study. Although the dissolved inorganic carbon in seawater is around 2 mM, the dominant form is HCO_3^-, with CO_2 typically accounting for less than 1% (Dickson, 2010). In addition, CO_2 in seawater diffuses ~8, 000 times slower than in air (Gao and Campbell, 2014). Furthermore, the marine macroalgae

have high $K_{0.5}$ values (40–70 μM CO_2) for Rubisco, the carbon assimilating enzyme (Ji et al., 2016). The evidence above indicates that the CO_2 in seawater should be carbon limited for marine macroalgae. The promoting effect of elevated CO_2 on photosynthesis was also reported in other macroalgae species, such as green algae *Ulva linza* (Gao et al., 1999), red algae *Pyropia haitanensis* (Zou and Gao, 2002), and brown algae *Petalonia binghamiae* (Gao and Kunshan, 2010). Meanwhile, the higher pCO_2 level increased $K_{0.5}$ of *S. muticum* at the condition of lower P in the present study, which indicates the plant grown at the condition of higher pCO_2 reduced its photosynthetic affinity for DIC. This phenomenon is commonly found in both microalgae and macroalgae (Gao and Campbell, 2014; Ji et al., 2016; Wu et al., 2008) and is considered as a sign of down-regulated CCMs at high CO_2 conditions (Gao and Campbell, 2014). But this decrease of photosynthetic affinity for DIC did not lead to reduced photosynthesis in *S. muticum* compared to that at the lower pCO_2 in the present study, mainly because of increased CO_2 availability for Rubisco and depressed photorespiration at the elevated ratio of CO_2 to O_2, which has been confirmed in red seaweed *Lomentaria articulate* (Kübler et al., 1999).

The higher P level also increased the net photosynthetic rate of *S. muticum* in the present study, which can be partially explained by the decreased $K_{0.5}$ at the condition of higher P. The decreased $K_{0.5}$ is an indication of increased photosynthetic carbon-use capability. Phosphorus is a key macronutrient component for organisms and high levels of P availability is not only essential for chloroplast DNA and RNA synthesis (Vered and Shlomit, 2008), but is required for various chloroplast functions, referring to phosphorylation of photosynthetic proteins , synthesis of phospholipids and generation of ATP (Zer and Ohad, 2003). Therefore, High P levels could speed up the transport of Ci from media to the site of Rubisco by supplying necessary energy. In addition, P enrichment can increase both activity and amount of Rubisco (Lauer et al., 1989). Meanwhile, phosphorus, with low concentration in seawater, is generally considered to be limiting for marine primary producer (Elser et al., 2007; Howarth, 1988; Müller and Mitrovic, 2015). Therefore, adding extra phosphorus to natural seawater can stimulate photosynthesis of algae. For instance, the midday (12:00)

photosynthetic rates increased from 1.3 to 2.3 mg C g^{-1} DW h^{-1} for *S. natans*, from 0.9 to 2.1 mg C g^{-1} DW h^{-1} for *S. fluitans* when 0.2 mM P was added (Lapointe, 1986). In the present study, the addition of 40 μmol P also resulted in nearly two-fold increase of the net photosynthetic rate and V_{max}, which suggests the significant importance of P in photosynthesis of this alga. In addition, the higher P level promoted the synthesis of Chl *a* at the condition of lower pCO_2, which may also contribute to the increased net photosynthetic rate in *S. muticum* at the condition of higher P. Although P is not the component constituting Chl *a*, higher P supply may stimulate the content of Chl *a* synthesis-related enzymes and thus the production of Chl *a*. The positive effect of P on Chl *a* was also reported in *S. thunbergii* (Nakahara, 1990). On the other hand, the higher P level did not increase the Chl *a* content at the condition of higher pCO_2 in the present study. The possible reason is that there is more ATP available at the condition of higher pCO_2 due to the down-regulation of CCMs and thus there is no need to synthesize more Chl *a* to capture more light for cells as excessive energy can lead to the harm to photosynthesis and growth of algae (Gao et al., 2012; Xu and Gao, 2012).

4.2. Effect of pCO_2 *and P on nitrogen assimilation*

The higher pCO_2 level noticeably enhanced the nitrate uptake rate in *S. muticum* regardless of P concentration in the present study. This could be attributed to the increased nitrate reductase activity (NRA) at the condition of higher pCO_2. The enhanced NRA at the conditions of high CO_2 was also reported in *U. rigida* (Gordillo et al., 2001), *Hizikia fusiforme* (Zou, 2005), *P. haitanensis* (Liu and Zou, 2015), *Corallina officinalis* (Hofmann et al., 2013), as well as the higher plants *Plantago major* (Fonseca et al., 1997), tomato (Yelle et al., 1987), etc. Taken together, these findings indicate that the response of NRA in plants to elevated CO_2 may be homogeneous.

The higher P level also enhanced the nitrate uptake in *S. muticum* regardless of pCO_2 level, which can be partially due to the increased NRA at the condition of higher P. This is very evident at the condition of lower pCO_2. However, the higher P level decreased the NRA at the condition of higher pCO_2, which did not lead to reduced nitrate uptake. This indicates there should be other mechanisms to account

for the promoting effect of the higher P level on the nitrate uptake. One possible mechanism is that the higher P level can increase the availability of ATP that is required for the active uptake of nitrate across the plasma membrane. The phenomenon that ATP concentration increases with P level has been found in higher plants (Olivera et al., 2004; Rychter et al., 2006). Apart from *S. muticum*, the positive effect of higher P level on nitrate uptake was also reported in red macroalgae *Gracilaria lemaneiformis* (Xu et al., 2010) and higher plant *Phaseolus vulgaris* (Gniazdowska and Rychter, 2000). The increased nitrate uptake, NRA and soluble protein at the condition of higher P in the present study suggest that high P availability promoted nitrogen assimilation in *S. muticum*. It is worth noting that the nitrate uptake rates were commonly higher than the corresponding reduction rates of NO_3^- to nitrite NO_2^- by nitrate reductase in the present study, which might be due to the intercellular nitrate storage (Collos, 1982; Lartigue and Sherman, 2005) and the underestimation of RNA measured by the in situ assay (Lartigue and Sherman, 2002).The higher P level increased the nitrate uptake rate and soluble protein at the conditions of both lower pCO_2 and higher pCO_2 but it only increased the NRA in *S. muticum* at the condition of lower pCO_2 in the present study. Surprisingly, it decreased the NRA at the condition of higher pCO_2. The reason for that may be not onefold but must be related to interaction of pCO_2 and P. High pCO_2, on one hand, could enhance photosynthetic carbon fixation and thus growth by supplying sufficient CO_2. On the other hand, it also results in the decrease of pH and increase of seawater acidity, which can disturb the acid-base balance on cell surface of algae (Flynn et al., 2012). Algae may accordingly allocate additional energy to act against the acid–base perturbation in some way. This hypothesis is supported by increased respiration at the condition of higher pCO_2 and higher P in the present study. The increased soluble protein and decreased NRA at the condition of higher pCO_2 and higher P suggest some H^+ transport-related protein, such as plasma membrane H^+-ATPase, might be synthesized to counteract the acid–base perturbation caused by increased pCO_2 and H^+. The additional production of H^+ transport-related protein like plasma membrane H^+-ATPase could competitively decrease the synthesis of nitrate reductase. This

hypothesis needs further experimental evidence to stand even though it could explain the results in the present study.

4.3. Connection between carbon and nitrogen assimilation

The increased net photosynthetic rate at the condition of higher pCO_2 and higher P did not result in higher soluble carbohydrates compared to the condition of higher pCO_2 and lower P. The additional ATP produced by photosynthetic electron transport at the condition of higher pCO_2 and higher P may be drawn to nitrogen assimilation as more soluble protein was synthesized at the condition of higher pCO_2 and higher P. The additional energy allocation to protein synthesis, possibly H^+ transport-related protein to maintain the balance of acid-base, hindered the increase of growth, which may be the reason that the higher P increased the net photosynthetic rate but not the growth rate at the condition of higher pCO_2. Although synthesized protein can also contribute to the increase of thalli weight, it is not as energy-effective as carbohydrates (Norici et al., 2011; Raven, 1982). It seems that *S. muticum* tends to maintain a steady state in vivo even if it can sacrifice growth to some extent, considering that regulation of intracellular acid-base balance is crucial for organismal homoeostasis (Flynn et al., 2012; Smith and Raven, 1979). The increased respiration at HC was also demonstrated in *G. lemaneiformis* (Xu et al., 2010) and *U. prolifera* (Xu and Gao, 2012). The respiration at the condition of higher pCO_2 and lower P did not increase compared to at the condition of lower pCO_2 and lower P in the present study, suggesting the action against acid-base perturbation did not commence. The acid-base perturbation at the condition of higher pCO_2 and lower P may lead to the decreased photosynthetic rate compared to that at the condition of lower pCO_2 and lower P.

5. Conclusion

Our study, for the first time, demonstrates the combined effects of elevated pCO_2 and P enrichment on the physiological traits of a golden alga, *S. muticum.* It suggests current ocean environment is both CO_2 and P limited for the photosynthesis and grow of *S. muticum*. Therefore, future ocean acidification and eutrophication may promote the growth of *S. muticum* and thus occurrence of gold tide events. Meanwhile, *S.*

muticum tends to maintain homoeostasis taking advantage of phosphate enrichment, at the cost of growth. Accordingly, the combination of ocean acidification and eutrophication may not boost gold tides further compared to ocean acidification or eutrophication alone.

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Table 1. Parameters of the seawater carbonate system at different CO_2 and phosphate conditions. Measurements and estimation of the parameters are described in Materials and Methods. Data are the means ± SD (n = 3). LCLP, the low pCO_2 and low P condition, LCHP, the low pCO_2 and high P condition, HCLP, the high pCO_2 and low P condition, HCHP, the high pCO_2 and P condition, DIC = dissolved inorganic carbon, TA = total alkalinity. Different superscript letters indicate significant differences in one parameter between treatments ($P < 0.05$).

Treatment	pH	pCO_2 (μatm)	HCO_3^- (μmol kg^{-1})	CO_3^{2-} (μmol kg^{-1})	CO_2 (μmol kg^{-1})	DIC (μmol kg^{-1})	TA (μmol kg^{-1})
LCLP	8.07±0.02^b	426.9±31.1^a	2000.2±51.7^a	200.9±5.8^b	14.2±1.0^a	2215.3±49.7^a	2475.2±44.2
LCHP	8.07±0.02^b	423.9±21.1^a	1987.6±10.9^a	199.8±11.4^b	14.1±0.7^a	2201.5±19.3^a	2504.7±33.8
HCLP	7.76±0.02^a	1017.2±83.2^b	2282.5±27.6^b	110.0±10.0^a	34.0±2.9^b	2426.5±32.5^b	2541.5±44.2
HCHP	7.76±0.02^a	992.2±44.9^b	2261.8±35.9^b	110.5±5.9^a	33.1±1.5^b	2405.4±39.4^b	2563.6±44.2

710 **Table 2.** The carbon-saturating maximum photosynthetic rate (V_{max}, μmol O_2 g^{-1} FW
711 h^{-1}) and half saturation constant ($K_{0.5}$, mM) for *S. muticum* cultured under different
712 pCO_2 and P conditions for 13 days. Different superscript letters indicate significant
713 differences in one parameter between treatments ($P < 0.05$).

	LCLP	LCHP	HCLP	HCHP
V_{max}	57.00 ± 2.88^{a}	93.99 ± 0.98^{c}	81.18 ± 5.94^{b}	100.67 ± 6.81^{c}
$K_{0.5}$	0.21 ± 0.02^{a}	0.14 ± 0.05^{a}	0.42 ± 0.08^{b}	0.19 ± 0.05^{a}

Fig. 1. Relative growth rate (RGR) of *S. muticum* grown at different pCO_2 and P conditions for 13 days. Data are the means ± SD (n = 3). LCLP, the low pCO_2 and low P condition, LCHP, the low pCO_2 and high P condition, HCLP, the high pCO_2 and low P condition, HCHP, the high pCO_2 and high P condition. Different letters above error bars indicate significant differences between treatments ($P < 0.05$).

Fig. 2. Net photosynthetic rate (RGR) of *S. muticum* after being grown at different pCO_2 and P conditions for 13 days. Data are the means ± SD (n = 3). LCLP, the low pCO_2 and low P condition, LCHP, the low pCO_2 and high P condition, HCLP, the high pCO_2 and low P condition, HCHP, the high pCO_2 and high P condition. Different letters above error bars indicate significant differences between treatments ($P < 0.05$).

Fig. 3. The photosynthesis versus DIC curves of *S. muticum* after being cultured under pCO_2 and P conditions for 13 days. Data are the means ± SD (n = 3). LCLP, the low pCO_2 and low P condition, LCHP, the low pCO_2 and high P condition, HCLP, the high pCO_2 and low P condition, HCHP, the high pCO_2 and high P condition. DIC = dissolved inorganic carbon.

Fig. 4. Chl *a* content of *S. muticum* after being grown at different pCO_2 and P conditions for 13 days. Data are the means ± SD (n = 3). LCLP, the low pCO_2 and low P condition, LCHP, the low pCO_2 and high P condition, HCLP, the high pCO_2 and low P condition, HCHP, the high pCO_2 and high P condition. Different letters above error bars indicate significant differences between treatments ($P < 0.05$).

Fig. 5. Nitrate uptake rate of *S. muticum* after being grown at different pCO_2 and P conditions for 13 days. Data are the means ± SD (n = 3). LCLP, the low pCO_2 and low P condition, LCHP, the low pCO_2 and high P condition, HCLP, the high pCO_2 and low P condition, HCHP, the high pCO_2 and high P condition. Different letters above error bars indicate significant differences between treatments ($P < 0.05$).

Fig. 6. Nitrate reductase activity (NRA) of *S. muticum* after being grown at different pCO_2 and P conditions for 13 days. Data are the means ± SD (n = 3). LCLP, the low pCO_2 and low P condition, LCHP, the low pCO_2 and high P condition, HCLP, the high pCO_2 and low P condition, HCHP, the high pCO_2 and high P condition. Different letters above error bars indicate significant differences between treatments ($P < 0.05$).

Fig. 7. The contents of soluble carbohydrates (a) and protein (b) of *S. muticum* after being grown at different pCO_2 and P conditions for 13 days. Data are the means ± SD (n = 3). LCLP, the low pCO_2 and low P condition, LCHP, the low pCO_2 and high P condition, HCLP, the high pCO_2 and low P condition, HCHP, the high pCO_2 and high

P condition. Different letters above error bars indicate significant differences between treatments ($P < 0.05$).

Fig. 8. Dark respiration rate of *S. muticum* after being grown at different pCO_2 and P conditions for 13 days. Data are the means ± SD (n = 3). LCLP, the low pCO_2 and low P condition, LCHP, the low pCO_2 and high P condition, HCLP, the high pCO_2 and low P condition, HCHP, the high pCO_2 and high P condition. Different letters above error bars indicate significant differences between treatments ($P < 0.05$).

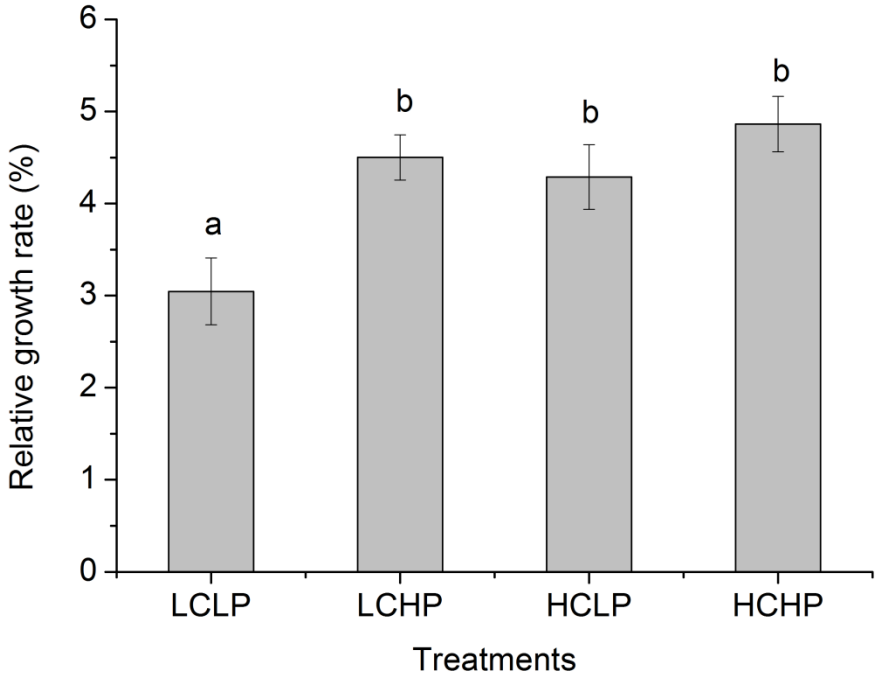
Relative growth rate (%)
0
1
2
3
4
5
6
a
b
b
b
LCLP
LCHP
HCLP
HCHP
Treatments

Fig. 1

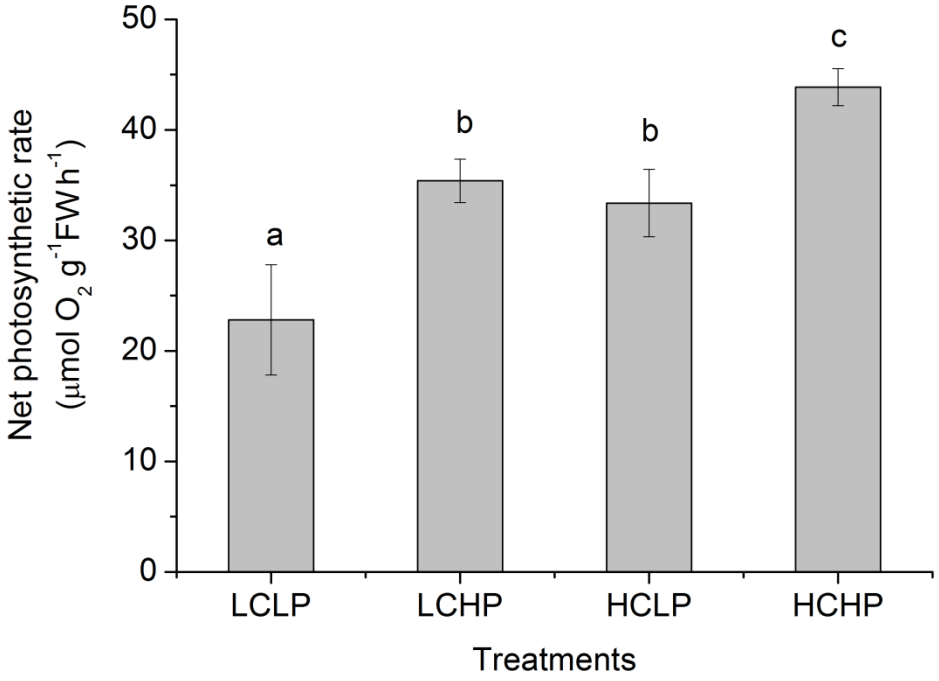
Net photosynthetic rate
(μmol O_2 g^{-1}FW h^{-1})
50
40
30
20
10
0
a
b
b
c
LCLP
LCHP
HCLP
HCHP
Treatments

Fig. 2

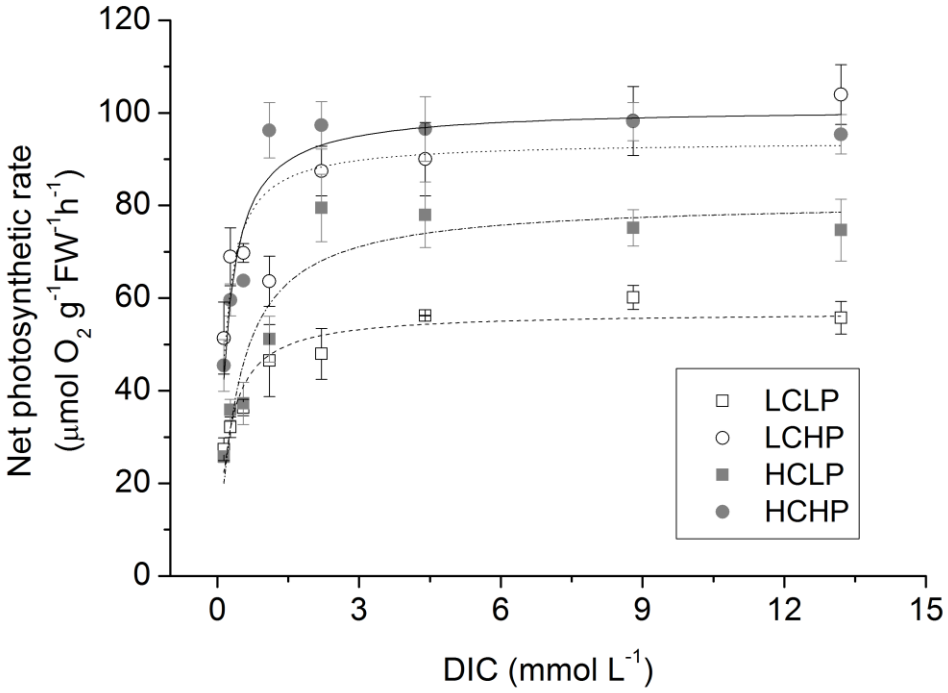
Net photosynthetic rate
(μmol O2 g-1FW-1h-1)
120
100
80
60
40
20
0
0
3
6
9
12
15
DIC (mmol L-1)
LCLP
LCHP
HCLP
HCHP

Fig. 3

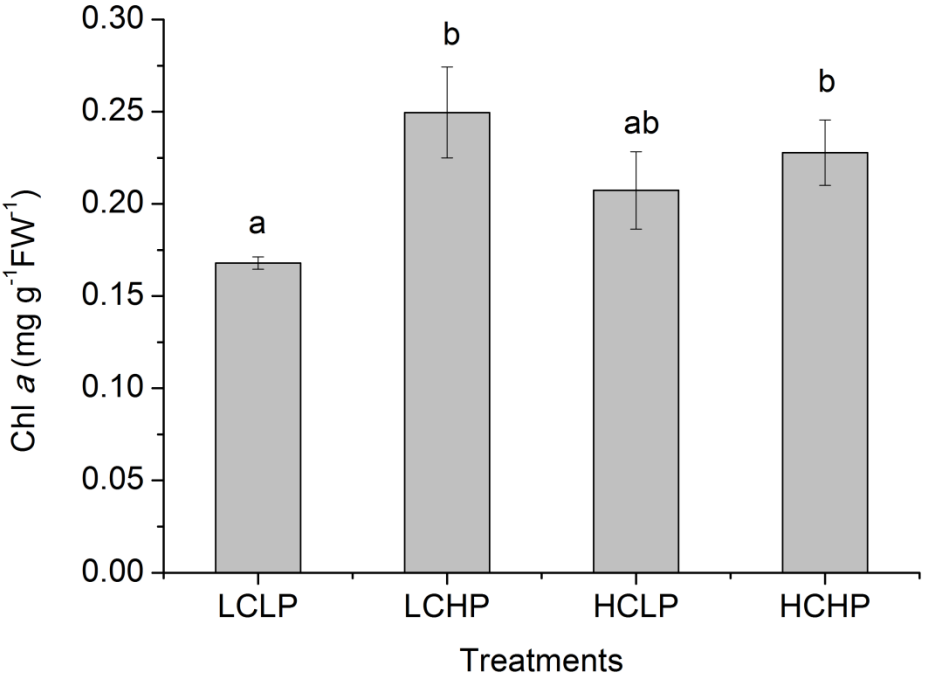
0.30
0.25
0.20
0.15
0.10
0.05
0.00
Chl a (mg g-1FW-1)
a
b
ab
b
LCLP
LCHP
HCLP
HCHP
Treatments

Fig. 4

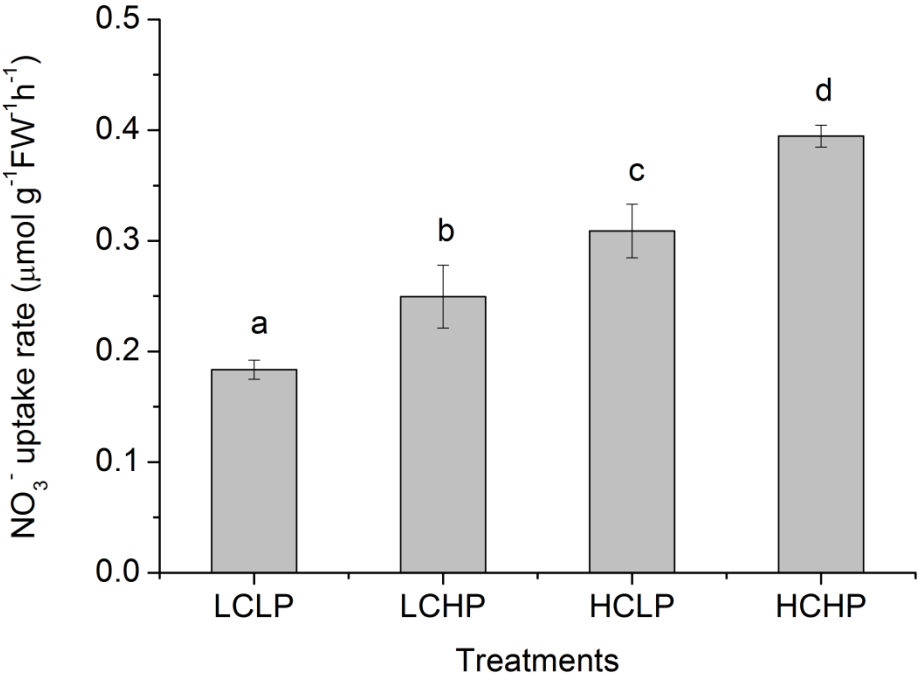
0.5
0.4
0.3
0.2
0.1
0.0
NO3- uptake rate (μmol g-1FW-1h-1)
a
b
c
d
LCLP
LCHP
HCLP
HCHP
Treatments

Fig. 5

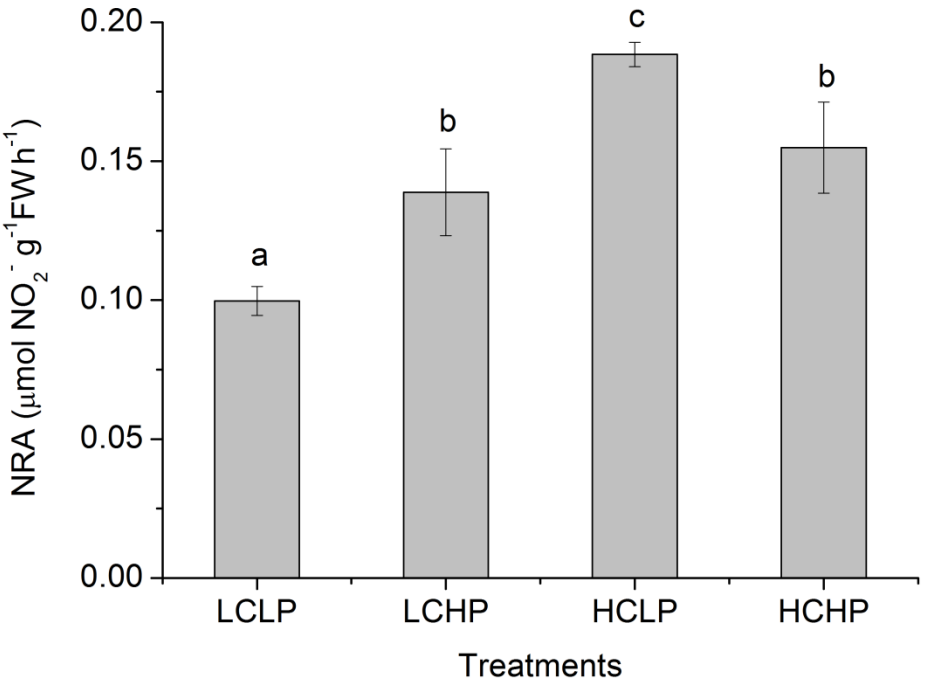
0.20
0.15
0.10
0.05
0.00
NRA (μmol NO2- g-1FW h-1)
a
b
c
b
LCLP
LCHP
HCLP
HCHP
Treatments

Fig. 6

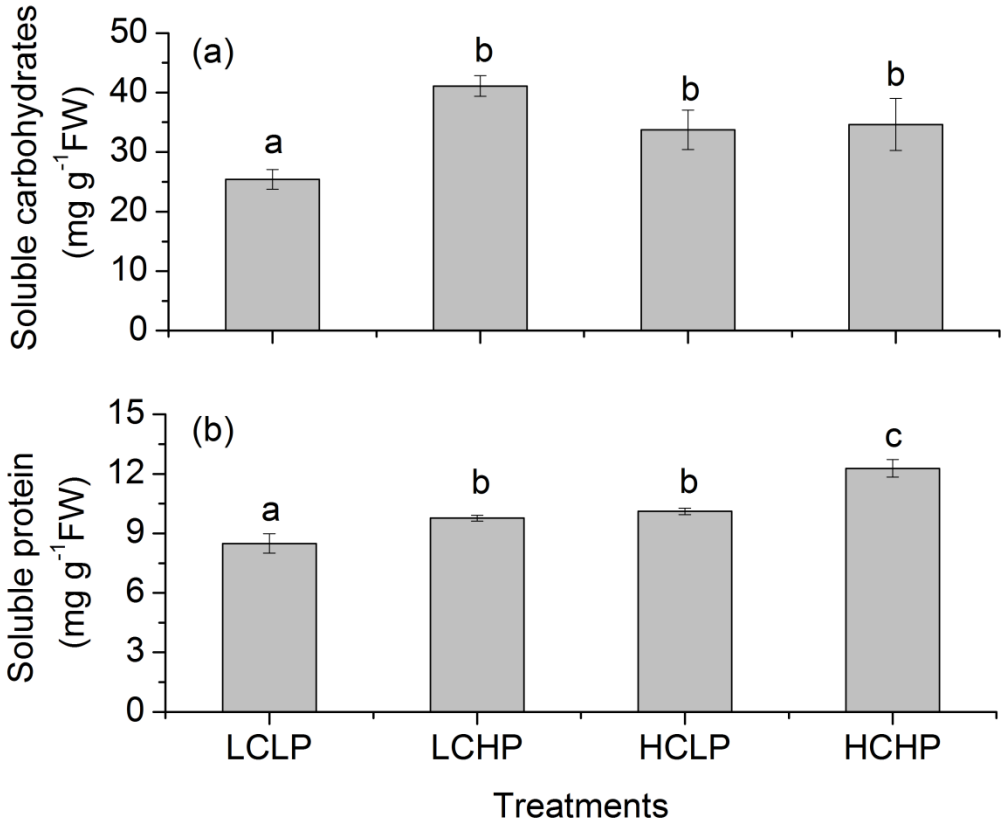
(a)
Soluble carbohydrates (mg g-1FW)
50
40
30
20
10
0
a
b
b
b
(b)
Soluble protein (mg g-1FW)
15
12
9
6
3
0
a
b
b
c
LCLP
LCHP
HCLP
HCHP
Treatments

Fig. 7

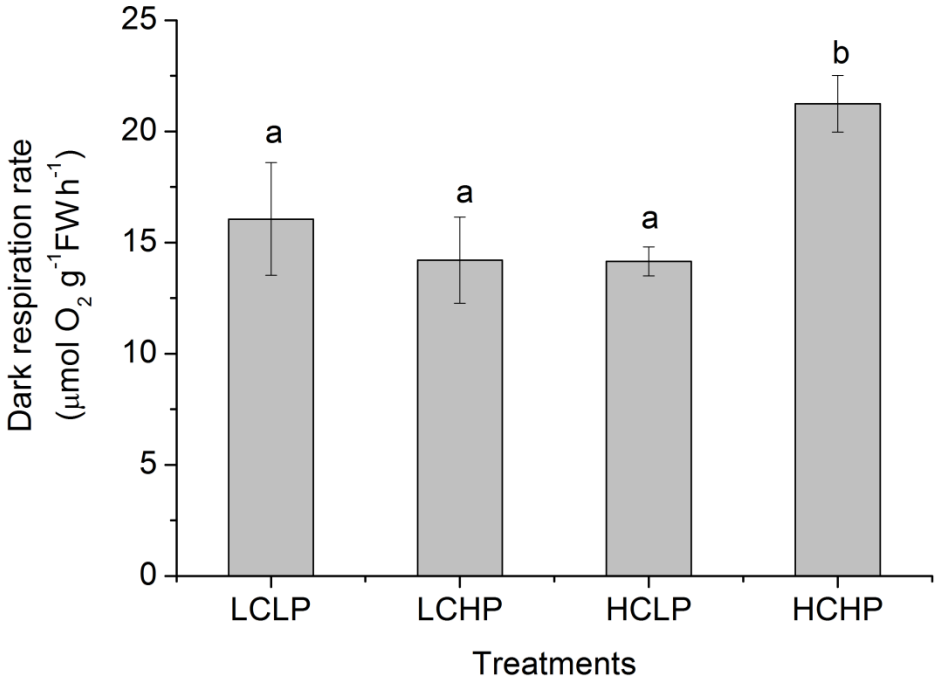
Dark respiration rate
(μmol O_2 $g^{-1}FWh^{-1}$)
25
20
15
10
5
0
a
a
a
b
LCLP
LCHP
HCLP
HCHP
Treatments

Fig. 8