Ocean acidification and nutrient limitation synergistically reduce growth and photosynthetic performances of a green tide alga *Ulva linza*

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Abstract. Large-scale green tides have been invading the coastal zones of the western Yellow Sea annually since 2008. Meanwhile, oceans are becoming more acid due to continuous absorption of anthropogenic carbon dioxide and intensive seaweed cultivation in Chinese coastal areas is leading to severe regional nutrient limitation. However, little is known the combined effects of global and local stressors on the eco-physiology of bloom-forming algae. We cultured *Ulva linza* for 9-16 days under two levels of pCO_2 (400 and 1000 μatm) and four treatments of nutrient (nutrient repletion, N limitation, P limitation, and N & P limitation) to investigate the physiological responses of this green tide alga to the combination of ocean acidification and nutrient limitation. For both sporelings and adult plants, elevated pCO_2 did not affect the growth rate when cultured under nutrient replete conditions but reduced it under P limitation; N or P limitation by themselves reduced growth rate. P limitation resulted in a larger inhibition in growth for sporelings compared to adult plants. Sporelings under P limitation did not reach the mature stage after 16 days of culture while those under P repletion became mature by day 11. Elevated pCO_2 reduced net photosynthetic rate for all nutrient treatments but increased nitrate reductase activity and soluble protein content under P replete conditions. N or P limitation reduced nitrate reductase activity and soluble protein content. These findings indicate that ocean acidification and nutrient limitation would synergistically reduce the growth of *Ulva* species and may thus hinder the occurrence of green tides in a future ocean environment.

Keywords: green tide, growth, nitrate reductase, nutrient limitation, ocean

23 acidification, photosynthesis

24 1 Introduction

25 Seaweeds are a group of organisms that play a vital role in the function of coastal
26 ecosystems. They provide diverse habitats and breeding areas for a large number of
27 organisms including crustaceans, other invertebrates and fishes. In addition, in spite of
28 only occupying a small part of the world's oceans, seaweeds account for
29 approximately 10% of the total oceanic primary productivity due to their high
30 densities (Wiencke and Bischof, 2012). Consequently, they are of importance in
31 global carbon cycle and modulating climate change. In addition to high ecological
32 significance, seaweeds are also economically important. They have been widely used
33 in the industry for food, chemical products, pharmaceuticals, cosmetics, etc. (Wang et
34 al., 2017). The increasing demand has resulted in the fast development of seaweed
35 cultivation and successful cultivation has been carried out worldwide, particularly in
36 Asian countries. Among the diverse range of seaweeds, *Ulva*, a cosmopolitan genus in
37 green seaweeds, is common from tropical to polar areas, from fresh water to fully
38 saline environments due to its robustness in acclimating to a variety of salinity and
39 water temperature conditions (Gao et al., 2017b). Thanks to their strong capacity for
40 nutrient uptake and quick growth, *Ulva* is the only genus that causes green tides due
41 to massive growth (Smetacek and Zingone, 2013; Gao et al., 2017d). Green tides have
42 received increasing concerns globally due to their ecological and economic impacts.
43 Firstly, they can hinder shore-based activities by preventing small boats, swimmers
44 and tourists from accessing the sea due to their sheer physical mass. Furthermore,

45 nutrients could be re-released to the seawaters and toxic hydrogen sulphide (H_2S)
46 could be produced when thalli decompose, leading to highly eutrophic, anoxic
47 conditions and the spread of coastal dead zones (Smetacek and Zingone, 2013).

48 Atmospheric carbon dioxide (CO_2) has continuously increased from 278 to 407
49 parts per million since the industrial revolution (NOAA 2017). The global ocean has
50 absorbed around 30% of anthropogenic CO_2 emissions since 1750, leading to the
51 decrease of seawater pH termed ocean acidification (Gattuso et al., 2015). Ocean
52 acidification is not only changing the fundamental chemistry and physics in the
53 oceans but also imposing significant impacts on marine organisms (Mostofa, 2016).
54 In terms of marine algae, extensive studies have been conducted on phytoplankton
55 species (Mccarthy et al., 2012; Li et al., 2015; Gao et al., 2018; Yuan et al., 2018) or
56 communities (Gao et al., 2012; Eberlein et al., 2017; Gao et al., 2017c). Depending on
57 experimental conditions or species, the effects of ocean acidification on growth and
58 photosynthesis of phytoplankton could be positive (Mccarthy et al., 2012), neutral
59 (Boelen et al., 2011) or negative (Gao et al., 2012). Compared to phytoplankton,
60 studies regarding seaweeds are relatively few. Recently, however, there have been
61 increasing concerns about the responses of seaweeds, particularly *Ulva* species, to
62 ocean acidification (Xu and Gao, 2012; Rautenberger et al., 2015; Gao et al., 2016;
63 Gao et al., 2017a). By analyzing the literatures, it is found that life stage can affect the
64 effects of ocean acidification on growth of *Ulva* species. Ocean acidification generally
65 increases growth of *Ulva* species at early life stages (Xu and Gao, 2012; Gao et al.,
66 2016) but does not affect or even reduces growth of *Ulva* species at late life stages

67 (Gao et al., 2017a). A possible explanation for the differential effects of ocean
68 acidification is that higher CO_2 could induce more reproduction events for adult (Gao
69 et al., 2017a).

70 Nutrients are crucial for growth and development of seaweeds. Nitrogen and
71 phosphorus, two key nutrient components for seaweeds, are commonly thought to be
72 limiting in natural seawater (Elser et al., 2007; Müller and Mitrovic, 2015).
73 Accordingly, enrichment of nitrogen and phosphorus generally stimulate growth of
74 seaweeds (Msuya and Neori, 2008; Luo et al., 2012; Xu et al., 2017). There are
75 studies indicating that N availability controls the biomass of seaweeds in temperate
76 coastal areas (Nixon and Pilson, 1983; Oviatt et al., 1995; Howarth et al., 2000) and P
77 limitation is the dominating factor for macroalgal growth in tropical latitudes
78 (Lapointe, 1997; Lapointe et al., 2010). However, phosphorus appears to play a more
79 important role in limiting the growth of *Ulva* sp. compared to nitrogen in a temperate
80 coastal area (Villares et al., 1999). In addition, Teichberg et al. (2010) investigated the
81 effects of nitrogen and phosphorus enrichment on growth of *U.* spp. at nine sites
82 across temperate and tropical areas. It was found that *Ulva*'s growth was controlled by
83 dissolved inorganic nitrogen (DIN) when ambient DIN levels were low and by
84 phosphorus when DIN levels were higher, irrespective of geographic or latitudinal
85 differences (Teichberg et al., 2010).

86 In addition to independent effects, the combined effects of ocean acidification and
87 nutrient on seaweeds have also been studied. Baydend et al. (2010) documented that
88 both ocean acidification and elevated nutrient concentration reduced the growth of

89 coralline crusts and the combination of the factors led to a further decrease in growth.
90 However, Xu et al. (2017) reported that ocean acidification and P enrichment did not
91 enhance the growth of brown seaweed *Sargasssum muticum* further applied together,
92 although either alone had a positive effect. The studies above indicate that the
93 combined effects of ocean acidification and eutrophication might be species-specific.
94 Until now, most studies regarding the effect of ocean acidification on seaweeds
95 have been based on nutrient replete conditions. In the field, the nutrient levels could
96 be limiting and this is particularly true in the areas of intensive seaweed cultivation.
97 For instance, the nitrogen and phosphorus concentrations in *Porphyra* cultivation
98 areas could be half of those in non-cultivation areas (He et al., 2008; Wu et al., 2015).
99 Little is known that how seaweeds grown under nutrient limited conditions respond to
100 ocean acidification. In addition, the young and adult thalli may have differential
101 responses to ocean acidification and nutrient limitation (Gao et al., 2017a). Therefore,
102 here we investigated the effects of ocean acidification and nutrient limitation on the
103 ecologically and commercially important seaweed *U. linza* at different life stages to
104 understand how *Ulva* species respond to the combination of global climate change
105 and local stressors.

106 2 Materials and methods

107 2.1 Sample preparation and culture conditions

108 To investigate whether life stage affects algal response to ocean acidification and
109 nutrient, both spores and adults of *U. linza* were used in this study. Fertile and
110 vegetative thalli (~5 cm) were collected from the coastal water of Lianyungang (119.3

111 ^{o}E, 34.5 ^{o}N), Jiangsu province, China. The fronds were put into in a cooling box (4–6

112 ^{o}C) and taken to the laboratory within 1 h. They were then rinsed with filtered (0.2 μm)

113 natural seawater to remove any sediment and small grazers. Spores, released from the

114 fertile thalli after being exposed to high light (600 μmol photons m^{-2} s^{-1}) for 12 h,

115 were allowed to settle and attach to glass slides in darkness for 12 h.

116 The settled spores were cultured in 1-L balloon flasks containing 900 mL of

117 media under four nutrient regimes (higher nitrate and higher phosphate (HNHP),

118 lower nitrate and higher phosphate (LNHP), higher nitrate and lower phosphate

119 (HNLP), lower nitrate and lower phosphate (LNLP)) and two pCO_2 (400 (LC) and

120 1000 (HC) μatm) to explore the interactive effects of ocean acidification and nutrient

121 limitation. The treatment of lower pCO_2, higher nitrate and higher phosphate

122 (LCHNHP) was set as the control. Twenty volumes of natural seawater (30.7 μmol L^{-1}

123 N and 1.0 μmol L^{-1} P) were diluted with 80 volumes of artificial seawater without N

124 or P to make a LNLP medium (6.1 μmol L^{-1} N and 0.2 μmol L^{-1} P). The medium for P

125 limitation (HNLP, 106.1 μmol L^{-1} N and 0.2 μmol L^{-1} P) was made of LNLP medium

126 plus 100 μmol N. The medium for N limitation (LNHP, 6.1 μmol L^{-1} N and 10.2 μmol

127 L^{-1} P) was made of LNLP medium plus 10 μmol P. The N&P replete medium (HNHP,

128 106.1 μmol L^{-1} N and 10.2 μmol L^{-1} P) was made of LNLP medium plus 100 μmol N

129 and 10 μmol P. The 400 μatm pCO_2 level was maintained by bubbling ambient air,

130 and the 1000 μatm pCO_2 level was achieved using a CO_2 plant chamber (HP1000 GD,

131 Wuhan Ruihua Instrument & Equipment Ltd, China) with the variation of CO_2 less

132 than 5% of the set values. The incubation light intensity was 300 μmol photons m^{-2} s^{-1}

133 (daylight fluorescent tubes, 21W, Philips), with a 12: 12 (light: dark) light period, and
134 the incubation temperature was 20°C. The samples were collected in March 2017 and
135 the light density of 300 photons m^{-2} s^{-1} used for the cultures was close to the ambient
136 light level at the sample collecting site. Light density was measured by a Quantum
137 Scalar Laboratory (QSL) radiometer (QSL-2100, Biospherical Instruments, Inc., USA)
138 that detects photosynthetically active radiation (400-700 nm). Once the sporelings had
139 attained a length of 1 cm they were detached from the glass slides and dispersed
140 directly into the treatment flasks. The cultures were bubbled with ambient or
141 CO_2-enriched air at a rate of 300 mL min^{-1} to make the thalli roll up and down.

142 The culture conditions for adult *Ulva* were the same as for the spores. The culture
143 density was less than 0.1 g L^{-1} and the pH fluctuation was less than 0.03 units. Low
144 culture density and aeration with ambient and CO_2-enriched air contributed to the
145 stable pH in the cultures. The cultures were carried out in triplicates and lasted 16
146 days for spores and 9 days for adult thalli. The media were renewed every two days.
147 The cultures had been finished before the thalli became reproductive as the aim of this
148 study focused on the growth and photosynthesis. Different cultivation periods were
149 used because the periods were different for sporeling and adult to become
150 reproductive. These cultivation periods are enough for *U. linza*'s acclimation to ocean
151 acidification (Eggert, 2012; Gao et al., 2016). The following parameters were
152 measured at the end of the culture periods for each flask under each treatment.

153 **2.2 Measurement of growth**

154 The variations in sporeling length and adult fresh mass (FM) were recorded every

155 two days. The length of sporelings was measured by a microscope (Leica DM500,
156 Germany) with a micro ruler. The fresh mass of adults was determined by weighing
157 using a balance (BS 124S, Sartorius, Germany) after removing surface water by
158 gently blotting the thalli with tissue paper. The specific growth rate (SGR) was
159 estimated as follows: SGR (%) = ($\ln M_{t2} - \ln M_{t1}$) / t × 100, where M_{t1} is the initial
160 length for sporelings or initial fresh mass for adults; M_{t2} is the length or fresh mass
161 after t days (16 days for sporeling and 9 days for adult) culture. Due to the tiny mass
162 of sporelings, length rather than mass was used to determine SGR for sporelings and
163 only adult thalli were used for measurements of the following aspects of physiological
164 performance.

165 **2.3 Chlorophyll fluorescence assessment**

166 The relative electron transport rate (rETR) was measured using a pulse amplitude
167 modulation (PAM) fluorometer (PAM-2100, Walz, Germany). The measuring light
168 was 0.01 μmol photons m^{-2} s^{-1} and actinic light was set as the same as the growth light
169 (300 μmol photons m^{-2} s^{-1}). The saturating pulse was set to 5, 000 μmol photons m^{-2}
170 s^{-1} (0.8 s). rETR (μmol e^- m^{-2} s^{-1}) = (F_m' − F_t) / F_m' × 0.5 × PFD, where F_m' is the
171 maximal fluorescence levels from algae in the light. Ft is the fluorescence at an
172 excitation level. PFD is the actinic light density.

173 **2.4 Determination of photosynthesis**

174 The net photosynthetic rate of thalli was measured by a Clark-type oxygen
175 electrode (YSI model 5300A). Algal individuals were cut into 1-cm-long segments
176 with a scissor. Approximately 0.02 g segments were randomly selected and

177 transferred to the oxygen electrode cuvette with 8 ml of media from the culture flask,
178 being stirred. The conditions for temperature and light were set the same as those for
179 growth. The net photosynthetic rate was determined by the increase in the oxygen
180 content in the media over five minutes. The unit for net photosynthetic rate (NPR)
181 was μmol O_2 g^{-1} FM h^{-1}.

182 **2.5 Measurement of photosynthetic pigments**

183 Approximately 20 mg of fresh mass thalli was extracted in 5 mL methanol at 4°C
184 for 24 hours in darkness. Then the absorbance values of samples at 665 (A_{665}) and 652
185 (A_{652}) nm were read with a UV/Visible spectrophotometer (Ultrospect 3300 pro,
186 Amersham Bioscience, Sweden). The content of Chl *a* and Chl *b* was determined as
187 follows:

188 $$\text{Chl } a \text{ (mg gFM}^{-1}) = (16.29 \times A_{665} - 8.54 \times A_{652}) \times V / (M \times 1000)$$

189 $$\text{Chl } b \text{ (mg gFM}^{-1}) = (30.66 \times A_{652} - 13.58 \times A_{665}) \times V / (M \times 1000),$$

190 where V is the volume of methanol used and M is the mass of thalli used.

191 **2.6 Assessment of nitrate reductase activity**

192 Nitrate reductase activity (NRA) of thalli was estimated based on a modified
193 method of Corzo and Niell (1991). The measurement was conducted during the local
194 noon period (13:00) since the activity of nitrate reductase usually displays circadian
195 periodicity with a maximum during the light period and a minimum in darkness
196 (Velasco and Whitaker 1989; Deng et al. 1991). Approximately 0.3 g (FM) of thalli
197 from each culture condition was incubated for 1 h at 20°C in darkness in the reaction
198 solution (10 mL), which contained 0.1 M phosphate buffer, 0.1% propanol (v/v), 50

199 mM KNO_3, 0.01 mM glucose, and 0.5 mM EDTA, with a pH of 8.0. The mixture was
200 flushed with pure N_2 gas (99.999%) for 2 minutes to obtain an anaerobic state before
201 the incubation. The concentration of nitrite produced was determined colorimetrically
202 at 540 nm (Xu et al., 2017). The NRA was expressed as μmol NO_2^- g^{-1} FM h^{-1}.

203 **2.7 Estimation of soluble protein**

204 Approximately 0.2 g of FM thalli under each treatment at the end of the culture
205 period were ground in a mortar with extraction solution (0.1 mol L^{-1} phosphate buffer,
206 pH 6.8) and then centrifuged for 10 minutes at 5, 000 *g*. Content of soluble protein
207 was estimated from the supernatant using the Bradford (1976) assay, with bovine
208 serum albumin as a standard.

209 **2.8 Statistical analysis**

210 The results in this study were expressed as means of replicates ± standard
211 deviation and the data were analyzed using the software SPSS v.22. The data under
212 every treatment conformed to a normal distribution (Shapiro-Wilk, $P > 0.05$) and the
213 variances could be considered equal (Levene's test, $P > 0.05$). Two-way multivariate
214 analysis of variance (MANOVA) was conducted to assess the effects of pCO_2 and
215 nutrient on seawater carbonate parameters. Repeated measures analysis of variance
216 (RM-ANOVA) was conducted to analyze the effects of culture time on length of
217 young and adult thalli, with Bonferroni for *post hoc* investigation. Two-way analysis
218 of variance (ANOVA) was conducted to assess the effects of pCO_2 and nutrient on
219 specific growth rate, net photosynthesis rate, rETR, Chl *a*, Chl *b*, soluble protein and
220 NRA. Tukey's honest significant difference (Tukey HSD) was conducted for MNOVA

221 and ANOVA *post hoc* investigation. Paired t-tests were used to compare the
222 differences in specific growth rate between young and adult thalli under each
223 treatment. A confidence interval of 95% was set for all tests.

224 **3 Results**

225 The carbonate system under each treatment was recorded (Table 1). Both pCO_2
226 and nutrient treatments had a significant effect on carbonate parameters (Table 2).
227 Elevated pCO_2 reduced pH and CO_3^-, increased DIC, CO_2 and HCO_3^- ($P < 0.001$ for
228 all parameters above) but did not affect TA ($P = 0.740$). P limitation (LP) increased
229 pCO_2 ($P = 0.002$) and CO_2 ($P = 0.002$) , and reduced pH ($P = 0.001$) and CO_3^{2-} ($P =$
230 0.046).

231 The length for both young and adult *U. linza* varied with culture time and the
232 patterns under different pCO_2 and/ or nutrient conditions were inconsistent (Fig. 1 &
233 Table 3). For example, the length gap between HP and LP increased with culture time
234 (Bonferroni, $P < 0.01$). It is worth noting that LP dramatically inhibited the
235 development of sporelings as the length under HP was 78,274−160,290 μm while it
236 was only 137−250 μm under LP at the end of 16 days of culture.

237 Based on the initial and final length (young thalli) or mass (adult thalli), specific
238 growth rate was calculated (Fig. 2). Nutrient and pCO_2 interacted to affect the growth
239 of both young and adult *U. linza* (Table 4). Specifically, *post hoc* Tukey HSD
240 comparison ($P = 0.05$) showed that HC reduced growth at LP but did not affect it at
241 HP, suggesting an interactive effect between P and C. Nutrient supply had an effect on
242 growth but the patterns between young and adult thalli were different (Table 4). For

243 young thalli, *post hoc* Tukey HSD comparison (P = 0.05) showed that N limitation
244 did not reduce growth, P limitation dramatically reduced the growth and the
245 combination of N and P limitation did not lead to a further decrease regardless of
246 pCO_2 conditions. For adult thalli, either N (P < 0.001) or P (P < 0.001) limitation
247 reduced growth and the combination of these nutrient limitations resulted in a further
248 decrease (P = 0.011). In addition, young thalli had higher growth rates under each
249 condition compared to adult plants (P < 0.001).

250 The effects of pCO_2 and nutrients on the net photosynthetic rate of adult thalli
251 were also investigated (Fig. 3a). Both pCO_2 and nutrient had a significant effect on
252 net photosynthetic rate (Table 4) and HC reduced NPR under each nutrient condition
253 (P < 0.05). In terms of the effect of nutrients, LN (P = 0.006) or LP (P < 0.001) alone
254 decreased NPR and the combination of LN and LP led to a further decrease under LC
255 (P < 0.001). Under HC, *post hoc* Tukey HSD comparison (P = 0.05) showed that both
256 LN and LP reduced NPR but the combination of LN and LP did not decrease NPR
257 further. In terms of dark respiration rate (Fig. 3b), neither pCO_2 nor nutrient had a
258 significant effect on it (Table 4).

259 To understand the photosynthetic performance of *U. linza* under various pCO_2
260 and nutrient conditions, relative electron transport rate (rETR) in PSII at 300 μmol
261 photons m^{-2} s^{-1} was measured (Fig. 4). pCO_2 had an interactive effect with nutrient
262 and each factor had a main effect (Table 4). Specifically speaking, HC reduced rETR
263 under LP but did not change it under HP. Regardless of pCO_2 levels, N limitation
264 reduced rETR (P = 0.028), P limitation had a larger negative effect (P < 0.001) and

265 the combination of LN and LP resulted in the lowest rETR values ($P < 0.001$).

266 Changes in photosynthetic pigments are shown in Fig. 5. Both pCO_2 and nutrient 267 had an effect on the content of Chl *a* and Chl *b* (Table 4) but slight differences 268 between Chl *a* and Chl *b* were found after post hoc Tukey HSD ($P = 0.05$) tests had 269 been conducted. Under LC, either N or P limitation reduced Chl *a* content, with P 270 limitation having a larger effect. LNLP decreased Chl *a* content further. Under HC, 271 LN or LP reduced Chl *a* but the combination of limiting nutrients did not lead to a 272 further decrease. As far as Chl *b* is concerned, either LN or LP decreased Chl *b*, with 273 LP having a larger effect under LC. The combination of LN and LP did not lead to a 274 further decrease compared with LP.

275 To investigate the effects of pCO_2 and nutrient on nitrogen acquisition, RNA in 276 adult *U. linza* grown under various conditions was measured (Fig. 6). Both pCO_2 and 277 nutrient affected NRA and they had an interactive effect (Table 4). Under LC, *post* 278 *hoc* Tukey HSD comparison ($P = 0.05$) showed that either N or P limitation reduced 279 NRA but the combination of them did not result in a further decrease. Under HC, N 280 limitation and P limitation reduced NRA by 22.8% and 37.7% respectively and the 281 combination of them increased NRA by 45.6%. In addition, HC did not affect NRA 282 under LNLP ($P = 0.700$) but increased it when N ($P = 0.018$) or P ($P < 0.001$) was 283 replete and nitrate reductase had the highest activity (11.9 ± 0.7 μmol NO_2^- g^{-1} FM h^{-1}) 284 under HCHNHP condition ($P < 0.001$).

285 The content of soluble protein was assayed to investigate nitrogen assimilation of 286 *U. linza* under various pCO_2 and nutrient conditions (Fig. 7). Both pCO_2 and nutrient

287 levels affected the content of soluble protein and showed interactive effects (Table 4).
288 *Post hoc* Tukey HSD comparison ($P = 0.05$) showed that HC did not affect the
289 content of soluble protein under LP but increased it under HP. Under LC, separate N
290 or P limitation and their combination showed a similar negative effect on soluble
291 protein content. Under HC, P limitation had a larger inhibition effect on soluble
292 protein content compared to N limitation ($P < 0.001$) and the combination of N and P
293 limitation did not lead to a further decrease in soluble protein content ($P = 0.953$).

294 4 Discussions

295 4.1 Differential response of young and adult *Ulva*

296 Compared to adult plants, young *Ulva* grew much faster regardless of culture
297 conditions. This trend was also found in *U. rigida* (Gao et al., 2017a). The noticeable
298 difference in growth rate between young and adult *Ulva* could be attributed to cell
299 differentiation. In the early life history of *Ulva*, cell division proceeds fast as all cells
300 are of the same type, developing from one single cell. Thereafter, cells differentiate
301 into two types: rhizoidal cells in the basal part and blade cells in the marginal part
302 (Gao et al., 2017b). Differences in cell size and photosynthetic pigments between
303 these two cell types result in unequal growth in the thallus; the growth of rhizoidal
304 cells is much slower than in blade cells (Han et al., 2003; Lüning et al., 2008), which
305 slows down the total growth of the thallus as it ages.

306 Lower P levels strongly inhibited the growth of both young and adult plants in this
307 study and the inhibitory effect was particularly significant for young plants. Gao et al.
308 (2017b) has reported that *U. rigida* becomes mature when the thalli reach a length of

309 around 1.5 cm and we also found a similar phenomenon in *U. linza*. Young plants
310 grown under P limitation were far away the mature stage even after 16 days of culture
311 while the plants grown under P repletion reached a mature stage by day 11 and the
312 length could be up to 16 cm by day 16. This finding supports the significant role of P
313 in development of *U. linza*. Phosphorus (P) is an essential element for seaweeds, in
314 the form of nucleic acids, phospholipids, ATP and ADP, but little is known regarding
315 the effect of P on development of seaweed. Our findings indicate that P limitation
316 may terminate the development of young *Ulva* and cause it to remain in the immature
317 stage. The separate addition of N did not change the growth rate of young plants but
318 increased the growth rate of adult plants, suggesting that adult plants could be more
319 resilient to P limitation compared to young plants.

320 **4.2 Photosynthetic response to OA and nutrients**

321 HC was shown in the present study to decrease the Chl *a* and Chl *b* contents of *U.*
322 *linza*. High CO_2 commonly down-regulates algal CO_2 concentrating mechanisms
323 (CCMs), suggesting less energy is required to drive CCMs (Gao et al., 2012; Raven et
324 al., 2012; Cornwall et al., 2017; Raven et al., 2017). This may lead to decreased
325 synthesis of pigment for energy capture. This phenomenon of `pigment economy' has
326 also been found in the previous studies regarding *Ulva* species (Gordillo et al., 2003;
327 Gao et al., 2016). Deficiency in N or/and P supply also reduced pigment content in
328 this study. Nitrogen is a major component of Chl *a*. Although P is a non-constituent
329 element in Chl *a*, higher P supply may stimulate the activity of Chl *a* synthesis-related
330 enzymes (Xu et al., 2017). Accordingly, in this study nutrient (N & P) enrichment

331 enhanced the synthesis of Chl *a*. This is consistent with other findings in *Ulva* species
332 (Gordillo et al., 2001; Figueroa et al., 2009) and other macroalgae (Xu et al., 2017).
333 HC decreased net photosynthetic rate in *U. linza* in the present study. This could be
334 due to the decrease of photosynthetic pigment in thalli grown under HC. Meanwhile,
335 the saved energy due to down-regulation of CCMs in thalli grown under HC
336 combined with higher light density used in this study may depress PSII activity and
337 thus reduce net photosynthetic rate (Gao et al., 2012). An ocean acidification-induced
338 decrease of net photosynthetic rate was also documented in *U. prolifera* (Xu and Gao,
339 2012). In terms of the effects of nutrient, N limitation reduced net photosynthetic rate
340 in *U. linza* and P limitation resulted in a further decrease. The negative effects of N
341 and P limitation on algal photosynthetic rate have been extensively reported
342 (Longstaff et al., 2002; Kang and Chung, 2017; Xu et al., 2017), indicating the
343 important role of N and P in algal photosynthesis. In addition to the separate effects of
344 pCO_2 or nutrient, these factors also interplay on photosynthetic performances of *U.*
345 *linza*. For instance, HC reduced rETR under LP but did not affect it under HP,
346 suggesting that P enrichment could offset the negative effect of ocean acidification. In
347 contrast to net photosynthetic rate, the dark respiration rate of thalli was insensitive to
348 the change of pCO_2 or nutrient. The higher pCO_2 did not affect the dark respiration
349 rate in *U. prolifera* either (Xu et al., 2012) but reduced it in *U. rigida* (Gordillo et al.,
350 2003). N enrichment did not affect the dark respiration rate under lower pCO_2 but
351 reduced it under higher pCO_2 for *U. rigida* (Gordillo et al., 2003). The present finding
352 combined with the previous studies indicates that response of dark respiration in *Ulva*

353 may be species-dependent.

354 **4.3 N assimilation under OA and nutrient limitation**

355 Contrary to C assimilation, HC did not affect the content of soluble protein under
356 LP and even increased it when the P level was sufficient. The increased protein
357 synthesis under HC could be put down to the stimulation of NRA under HC. Gordillo
358 et al. (2001) proposed that the positive effect of HC on N assimilation may be due to a
359 direct action on synthesis of NR rather than the physiological consequences of C
360 metabolism as occurs in higher plants. Our results support this hypothesis because HC
361 increased NRA in thalli grown under HP in this study. Meanwhile, the change of
362 NRA under different pCO_2 levels might be also caused by varying pH as pH could
363 affect NRA in seaweeds (Lopes et al., 1997). P is considered to be playing a critical
364 role in enzyme synthesis and may interact with CO_2 to promote the synthesis and
365 activity of nitrate reductase.

366 **4.4 Interactive effects of OA and nutrient limitation on Growth**

367 HC did not affect the growth of thalli when P was replete in the medium. Since HC
368 reduced photosynthesis rate but increased NRA and protein synthesis, the lack of
369 effect of HC may be an integrated outcome of C and N assimilation (Fig. 8). This
370 finding is different from our previous studies in which HC increased the growth rate
371 of *U. linza* (Gao et al., 2016) and *U. prolifera* (Gao et al., 2017d). The possible reason
372 causing this divergence might be due to the different light intensities that were used in
373 the various studies. For the previous studies, a lower light intensity of 100 μmol
374 photons m^{-2} s^{-1} was used for algal culture while a higher light intensity of 300 μmol

375 photons m^{-2} s^{-1} was used in the present work. Ocean acidification could interact with
376 light intensity to affect algal growth. It has commonly been reported that ocean
377 acidification can increase algal photosynthesis/growth at lower light intensity and
378 inhibit photosynthesis/growth at higher light intensity (Gao et al., 2012; Xu and Gao,
379 2012; Gao et al., 2016), with inversion points of PAR around 160, 125 and 178 µmol
380 photons m^{-2} s^{-1} for *Phaeodactylum tricornutum*, *Thalassiosira pseudonana* and
381 *Skeletonema costatum* respectively (Gao et al., 2012). The potential reason is that the
382 saved energy from down-regulated CCMs under higher CO_2 levels could be used for
383 growth at lower light levels but could inhibit PSII activity and thus growth at higher
384 light levels (Gao et al., 2012; Gao et al., 2016). It seems that *U. linza* has a higher
385 inversion point compared to diatoms.
386 Furthermore, HC reduced growth of *U. linza* when P was limited. In addition to the
387 increased CO_2 supply, ocean acidification also reduces the pH of seawater, which has
388 been deemed as a stressor disturbing the acid-base balance both at the cell surface and
389 within cells and affecting algal photosynthetic performance (Flynn et al., 2012; Gao et
390 al., 2017d). Increased CO_2 and decreased pH also reduced rETR and net
391 photosynthetic rate of *U. linza* in the present study. Xu et al. (2017) proposed that
392 algae could synthesize HC transport-related proteins to combat that disturbance.
393 Under P limitation conditions, such protein synthesis could be limited, which may
394 lead to the decreased rETR and net photosynthetic rate and thus to decreased growth
395 observed in the present study. Until now, most studies of ocean acidification on
396 seaweed have been conducted under nutrient replete conditions. The present study

397 thus demonstrates the contrasting effect of ocean acidification under nutrient deplete
398 conditions.

399 **4.5 Differential effects of N and P limitation**

400 In the present study, compared to N limitation, P limitation seems to have a larger
401 negative effect on physiological performances in *U. linza*. In other words, the addition
402 of P resulted in a larger stimulating effect compared to N addition. Which one (N or P)
403 is the nutrient most likely to limit marine primary productivity has been a
404 controversial issue until now (Elser et al., 2007; Teichberg et al., 2010; Müller and
405 Mitrovic, 2015). It has been proposed that the occurrence of N or P limitation depends
406 on the difference in N:P ratio between in algal tissue and in seawater; when the ratio
407 of N:P in algal tissue is higher than in seawater N limitation is indicated and the
408 opposite is considered as P limitation (Harrison and Hurd, 2001). The ratio of N:P in
409 tissue of *U. linza* grown in the field has not been documented and the mean value of
410 N:P throughout a year is 15.4 for *U. prolifera* and 22.3 for *U. fenestrate* (Wheeler and
411 Björnsäter, 1992). The ratio of N:P in natural seawater where the samples were
412 collected in the present study is 30.7:1, which is higher than the N:P ratio in the
413 reported *Ulva* species. This suggests the existence of P limitation for *U. linza*, which
414 could explain the larger stimulating effect with P addition.

415 In recent decades, P limitation has been suggested to commonly occur in coastal
416 waters due to more effective P removal from industrial and domestic wastewater
417 during de-eutrophication processes (Grizzetti et al., 2012). For instance, the ratio of
418 dissolved inorganic N:P could be as high as 375:1 in nearshore waters of the North

419 Sea, resulting in severe P limitation for algal growth (Burson et al., 2016).

420 **5 Conclusions**

421 With the continuous emission of CO_2, the trend of ocean acidification will
422 continue through this century (Gattuso et al., 2015). Meanwhile, nutrient limitation
423 would occur in coastal waters as a consequence of efforts on de-europhication.
424 Measures to reduce eutrophication have often led to a more effective decline of
425 phosphorus (P) than nitrogen (N) concentrations (Burson et al., 2016). In addition,
426 intensive seaweed culture in coastal areas can also lead to noticeable decreases in N
427 and P (He et al., 2008; Wu et al., 2015). Our study demonstrates that ocean
428 acidification and nutrient limitation would synergistically inhibit development and
429 growth of *Ulva* species. This may hinder the occurrence of green tides and *Ulva*
430 cultivation in future ocean. In addition, it has been reported that fast-growing species
431 require high nutrient inputs to sustain growth, while slow-growing species are better
432 adapted to nutrient limiting conditions (Gordillo, 2012). The decrease in nutrient level
433 may result in a shift in seaweed community composition in the future ocean
434 environment. Studies on other seaweeds are needed to have a comprehensive
435 understanding in terms of the combined effects of global and local stressors on
436 seaweed communities.

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Table 1. Parameters of the seawater carbonate system in different cultures. LC, 400 μatm; HC, 1000 μatm; LN, 6.1 μmol L^{-1}; LP, 0.2 μmol L^{-1}; HN, 106.1 μmol L^{-1}; HP, 10.2 μmol L^{-1}. DIC = dissolved inorganic carbon, TA = total alkalinity. Data are the means ± SD (n = 3).

Treatment	pCO_2 (μatm)	pH	DIC (μmol kg^{-1})	CO_2 (μmol kg^{-1})	HCO_3^- (μmol kg^{-1})	CO_3^{2-} (μmol kg^{-1})	TA (μmol kg^{-1})
LCHNHP	373.8±6.3	8.21±0.01	1991.9±49.4	12.3±0.2	1805.3±43.3	174.2±6.1	2243.7±55.8
LCLNHP	381.3±2.9	8.20±0.01	2015.1±50.4	12.6±0.1	1827.4±42.0	175.1±8.5	2267.0±60.5
LCHNLP	400.4±9.0	8.19±0.01	2029.9±50.1	13.2±0.3	1846.4±43.1	170.2±7.7	2262.7±58.2
LCLNLP	397.4±8.4	8.18±0.01	1998.2±39.0	13.1±0.3	1818.7±35.1	166.3±4.3	2226.7±42.9
HCHNHP	929.1±24.8	7.86±0.01	2154.6±52.5	30.7±0.8	2034.9±49.5	89.1±2.6	2263.3±54.2
HCLNHP	958.8±5.2	7.85±0.01	2155.0±42.0	31.6±0.2	2036.8±38.5	86.5±3.6	2259.0±46.5
HCHNLP	976.1±10.9	7.84±0.01	2159.3±38.7	32.2±0.4	2041.7±36.1	85.4±2.5	2250.7±41.3
HCLNLP	1020.2±51.8	7.82±0.03	2117.9±31.0	33.7±1.7	2005.3±28.0	78.9±5.2	2199.3±37.4

Table 2. Two-way multivariate analysis of variance for the effects of of pCO_2 and nutrient on on pH, dissolved inorganic carbon (DIC), HCO_3^-, CO_3^{2-}, CO_2, total alkalinity (TA) in the seawater. pCO_2*nutrient means the interactive effect of pCO_2 and nutrient, df means degree of freedom, F means the value of F statistic, and Sig. means *p*-value.

Source		pH		DIC		HCO_3^-		CO_3^{2-}		CO_2		TA	
	df	F	Sig.	F	Sig.	F	Sig.	F	Sig.	F	Sig.	F	Sig.
pCO_2	1	5237.765	<0.001	57.132	<0.001	158.536	<0.001	1504.349	<0.001	4486.773	<0.001	0.114	0.740
Nutrient	3	9.765	0.001	0.747	0.540	0.741	0.543	3.336	0.046	7.999	0.002	1.225	0.333
pCO_2*Nutrient	3	1.294	0.311	0.256	0.856	0.332	0.802	0.162	0.921	2.683	0.082	0.228	0.876
Error	16												

Table 3. Repeated analysis of variance for the effects of culture time on length changes of young and adult *U. linza* grown under various pCO_2 and nutrient conditions. Time*pCO_2 means the interactive effect of Time and pCO_2, Time* nutrient means the interactive effect of Time and nutrient, and Time*pCO_2*nutrient means the interactive effect of Time, pCO_2 and nutrient. df means degree of freedom, F means the value of F statistic, and Sig. means *p*-value.

	Growth of young *U. linza*			Growth of adult *U. linza*		
Source	df	F	Sig.	df	F	Sig.
Time	8	1153.328	<0.001	4	571.769	<0.001
Time*pCO_2	8	23.582	<0.001	4	3.158	0.020
Time*Nutrient	24	457.170	<0.001	12	28.505	<0.001
Time*pCO_2*nutrient	24	10.585	<0.001	12	0.689	0.756
Error	128			64		

Table 4. Two-way analysis of variance for the effects of pCO_2 and nutrient on physiological parameters of *U. linza*. pCO_2*nutrient means the interactive effect of pCO_2 and nutrient, df means degree of freedom, F means the value of F statistic, and Sig. means *p*-value.

Source	pCO_2			Nutrient			pCO_2*nutrient			Error		
	df	F	Sig.	df	F	Sig.	df	F	Sig.	df	F	Sig.
Growth of sporeling	1	115.297	<0.001	3	12678.566	<0.001	3	22.905	<0.001	16		
Growth of adult	1	20.039	<0.001	3	307.073	<0.001	3	1.723	0.011	16		
Net photosynthetic rate	1	35.096	<0.001	3	493.992	<0.001	3	2.619	0.087	16		
Dark respiration rate	1	2.306	0.148	3	1.445	0.267	3	0.410	0.748	16		
rETR	1	14.592	0.002	3	135.690	<0.001	3	5.023	0.012	16		
Chl *a*	1	85.900	<0.001	3	217.334	<0.001	3	2.440	0.102	16		
Chl *b*	1	71.600	<0.001	3	104.483	<0.001	3	2.005	0.154	16		
Nitrate reductase activity	1	38.271	<0.001	3	100.487	<0.001	3	6.246	0.005	16		
Soluble protein	1	30.212	<0.001	3	106.523	<0.001	3	11.295	<0.001	16		

Figure legends

Fig. 1. Length changes of young (a) and adult (b) *U. linza* grown under various conditions during the culture periods. LC, 400 μatm; HC, 1000 μatm; LN, 6.1 μmol L^{-1}; LP, 0.2 μmol L^{-1}; HN, 106.1 μmol L^{-1}; HP, 10.2 μmol L^{-1}. The error bars indicate the standard deviations (n = 3).

Fig. 2. Specific growth rate (% d^{-1}) of young (a) and adult (b) *U. linza* grown under various conditions. The specific growth rate for young and adult thalli were calculated based on the initial and final length (for young thalli over a 16-day culture) or mass (for adult thalli over a 9-day). LC, 400 μatm; HC, 1000 μatm; LN, 6.1 μmol L^{-1}; LP, 0.2 μmol L^{-1}; HN, 106.1 μmol L^{-1}; HP, 10.2 μmol L^{-1}. The error bars indicate the standard deviations (n = 3). Different letters (low-case for LC and capital for HC) above the error bars represent significant differences ($P < 0.05$) among nutrient treatments while horizontal bars represent significant differences ($P < 0.05$) between LC and HC within a nutrient treatment.

Fig. 3. Net photosynthetic (a) and dark respiration rates (b) of adult *U. linza* grown under various conditions. LC, 400 μatm; HC, 1000 μatm; LN, 6.1 μmol L^{-1}; LP, 0.2 μmol L^{-1}; HN, 106.1 μmol L^{-1}; HP, 10.2 μmol L^{-1}. The error bars indicate the standard deviations (n = 3). Different letters (low-case for LC and capital for HC) above error bars represent significant differences ($P < 0.05$) among nutrient treatments while horizontal bars represent significant differences ($P < 0.05$) between LC and HC within a nutrient treatment.

Fig. 4. Relative electron transport rate (rETR) of adult *U. linza* grown under various

conditions. LC, 400 μatm; HC, 1000 μatm; LN, 6.1 μmol L^{-1}; LP, 0.2 μmol L^{-1}; HN, 106.1 μmol L^{-1}; HP, 10.2 μmol L^{-1}. The error bars indicate the standard deviations (n = 3). Different letters above the error bars (low-case for LC and capital for HC) represent significant differences ($P < 0.05$) among nutrient treatments while horizontal bars represent significant differences ($P < 0.05$) between LC and HC within a nutrient treatment.

Fig. 5. Content of Chl *a* (a) and Chl *b* (b) in adult *U. linza* grown under various conditions. LC, 400 μatm; HC, 1000 μatm; LN, 6.1 μmol L^{-1}; LP, 0.2 μmol L^{-1}; HN, 106.1 μmol L^{-1}; HP, 10.2 μmol L^{-1}. The error bars indicate the standard deviations (n = 3). Different letters (low-case for LC and capital for HC) above the error bars represent significant differences ($P < 0.05$) among nutrient treatments while horizontal bars represent significant differences ($P < 0.05$) between LC and HC within a nutrient treatment.

Fig. 6. Nitrate reductase activity (NRA) in adult *U. linza* grown under various conditions. LC, 400 μatm; HC, 1000 μatm; LN, 6.1 μmol L^{-1}; LP, 0.2 μmol L^{-1}; HN, 106.1 μmol L^{-1}; HP, 10.2 μmol L^{-1}. The error bars indicate the standard deviations (n = 3). Different letters (low-case for LC and capital for HC) above the error bars represent significant differences ($P < 0.05$) among nutrient treatments while horizontal bars represent significant differences ($P < 0.05$) between LC and HC within a nutrient treatment.

Fig. 7. Content of soluble protein in adult *U. linza* grown under various conditions. LC, 400 μatm; HC, 1000 μatm; LN, 6.1 μmol L^{-1}; LP, 0.2 μmol L^{-1}; HN, 106.1 μmol

L^{-1}; HP, 10.2 μmol L^{-1}. The error bars indicate the standard deviations (n = 3). Different letters (low-case for LC and capital for HC) above the error bars represent significant differences ($P < 0.05$) among nutrient treatments while horizontal bars represent significant differences ($P < 0.05$) between LC and HC within a nutrient treatment.

Fig. 8. Physiological responses of *U. linza* to the combination of ocean acidification and nutrient availability. The blue and the red symbols represent down- and up-regulated metabolic pathways respectively. PSI: Photosystem I; PSII: Photosystem II; OEC: oxygen- evolving complex; NPQ: non-photochemical quenching; CA: carbonic anhydrase; NR: nitrate reductase.

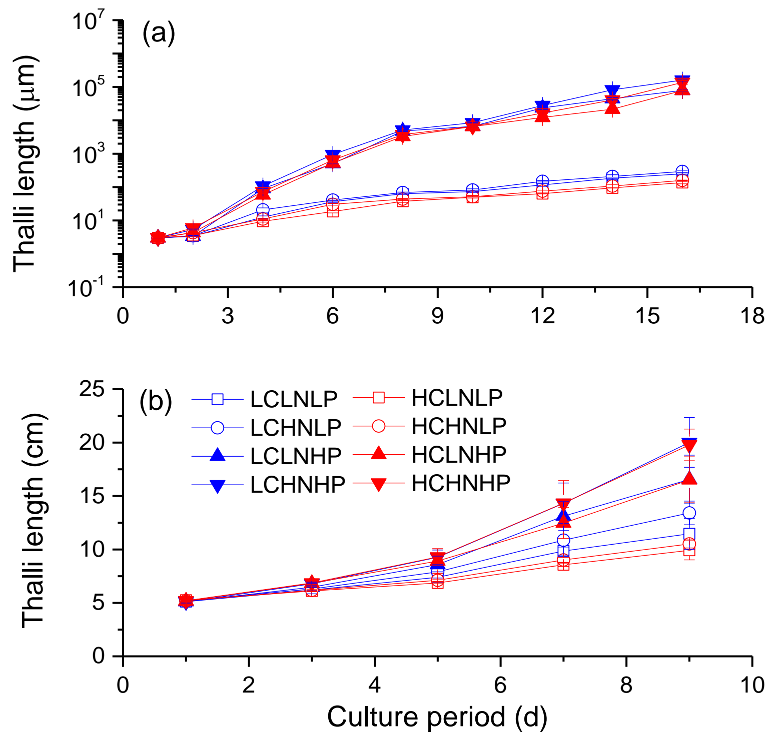

Fig. 1

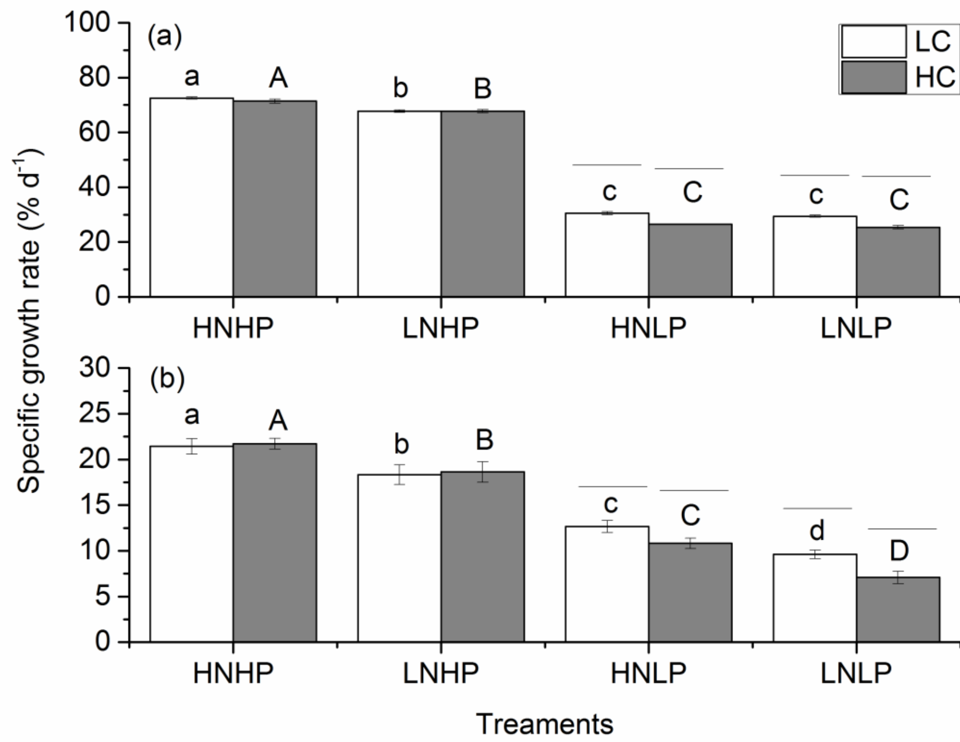

Fig. 2

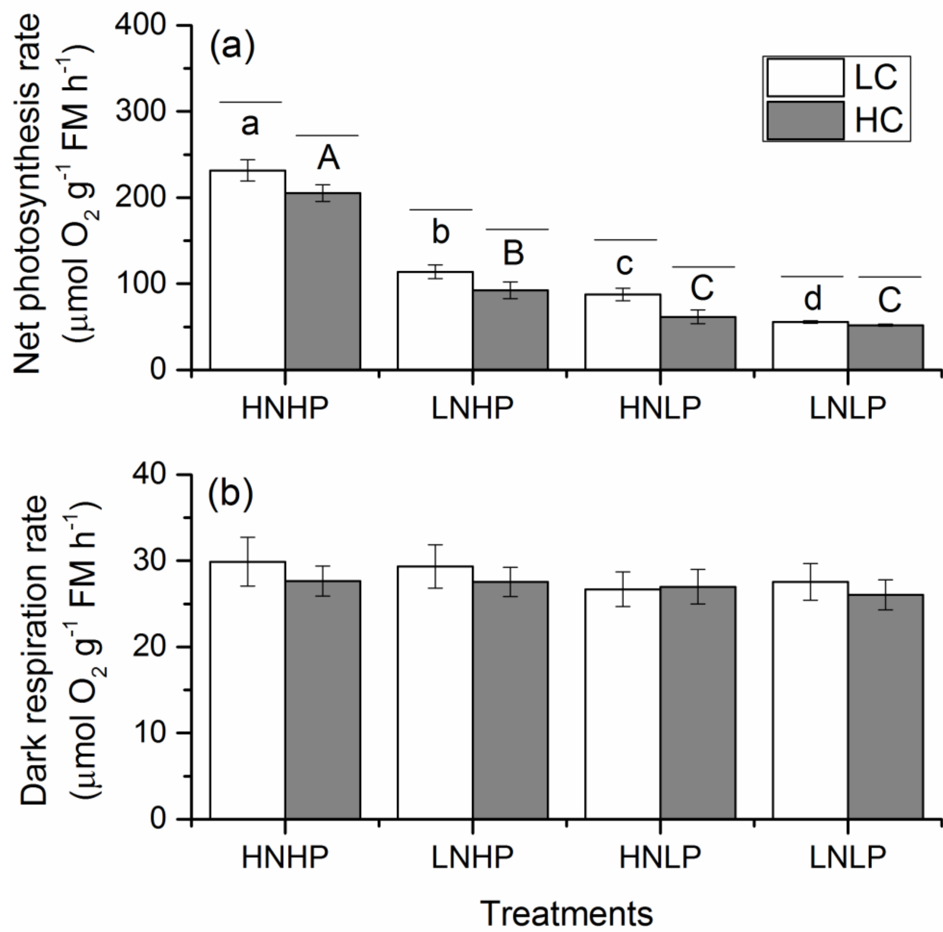

Fig. 3

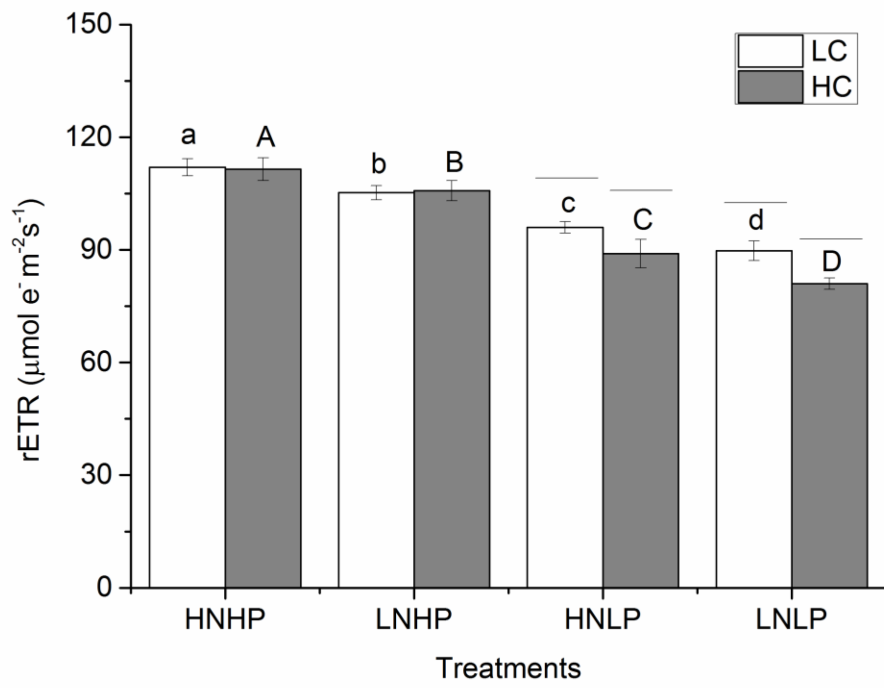

Fig. 4

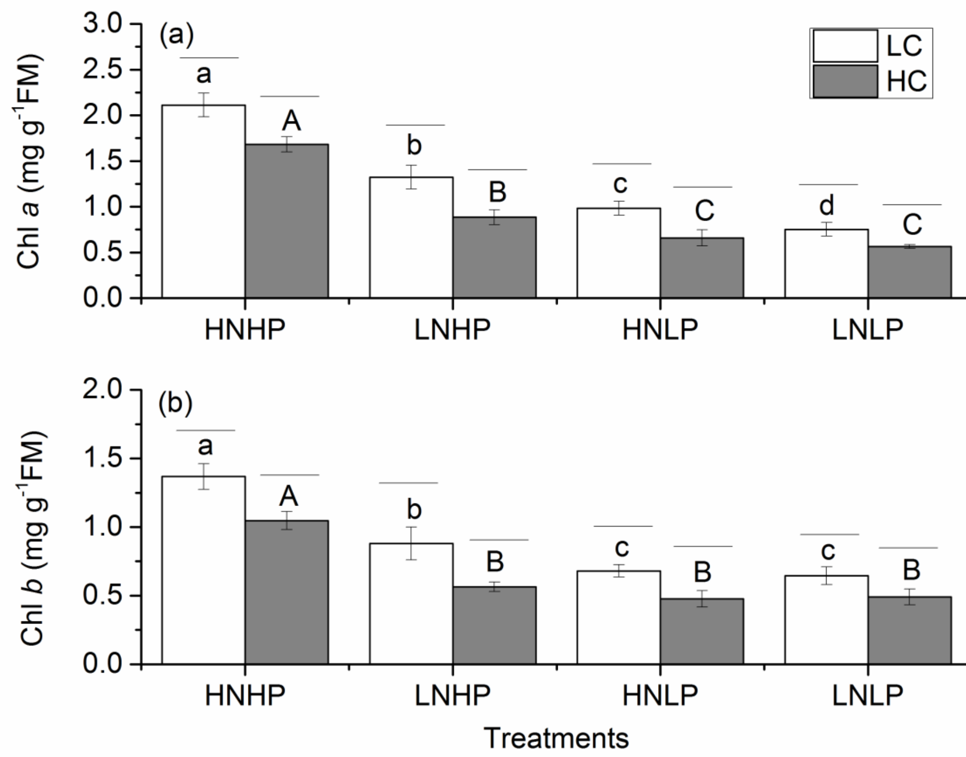

Fig.5

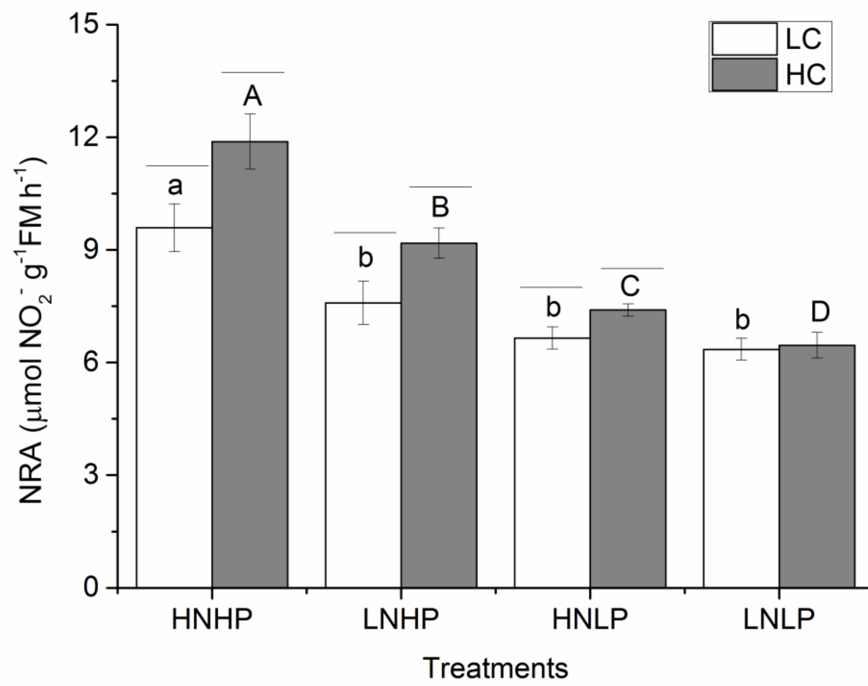

Fig. 6

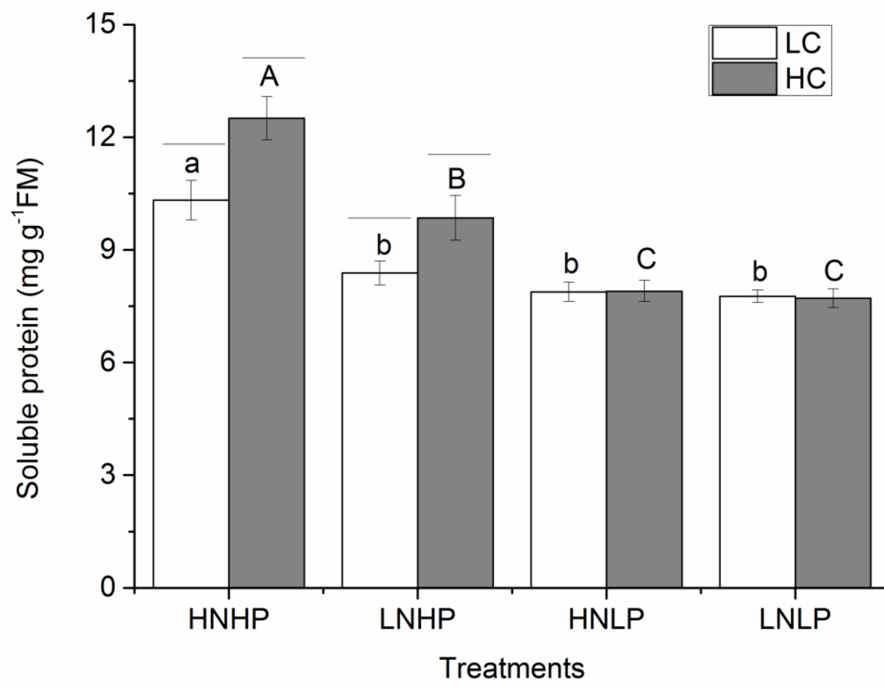

Fig. 7

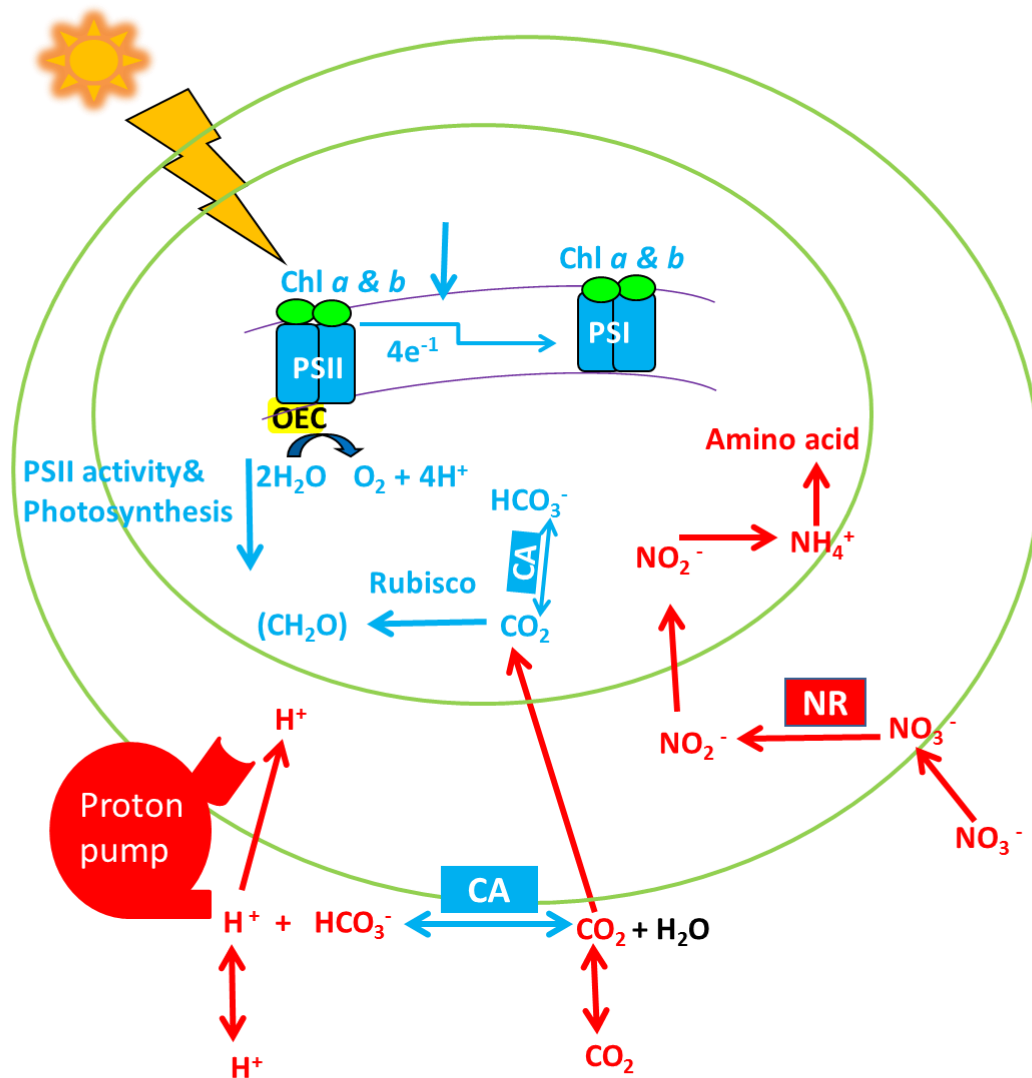

Fig. 8