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

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

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

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

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

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

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

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

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

(HNLP), lower nitrate and lower phosphate (LNLP)) and two pCO$_2$ (400 (LC) and

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

limitation. The treatment of lower pCO$_2$, higher nitrate and higher phosphate

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

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

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

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

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

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

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

and 10 μmol P. The 400 μatm pCO$_2$ level was maintained by bubbling ambient air,

and the 1000 μatm pCO$_2$ level was achieved using a CO$_2$ plant chamber (HP1000 GD,

Wuhan Ruihua Instrument & Equipment Ltd, China) with the variation of CO$_2$ less

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 $20^{o}C$. 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 (%) = $(\ln M_{t2} - \ln M_{t1}) / t \times 100$, 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}) = $(F_m' - F_t) / F_m' \times 0.5 \times PFD$, 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^oC

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^oC in darkness in the reaction

198 solution (10 mL), which contained 0.1 M phosphate buffer, 0.1% propanol (v/v), 50

10

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 $\mu mol\ NO_2^-\ g^{-1}\ FM\ h^{-1}$.

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

levels affected the content of soluble protein and showed interactive effects (Table 4).

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

4 Discussions

4.1 Differential response of young and adult *Ulva*

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

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

15

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.

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.

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.

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	df	pH		DIC		HCO_3^-		CO_3^{2-}		CO_2		TA	
		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.

Source	Growth of young *U. linza*			Growth of adult *U. linza*		
	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.
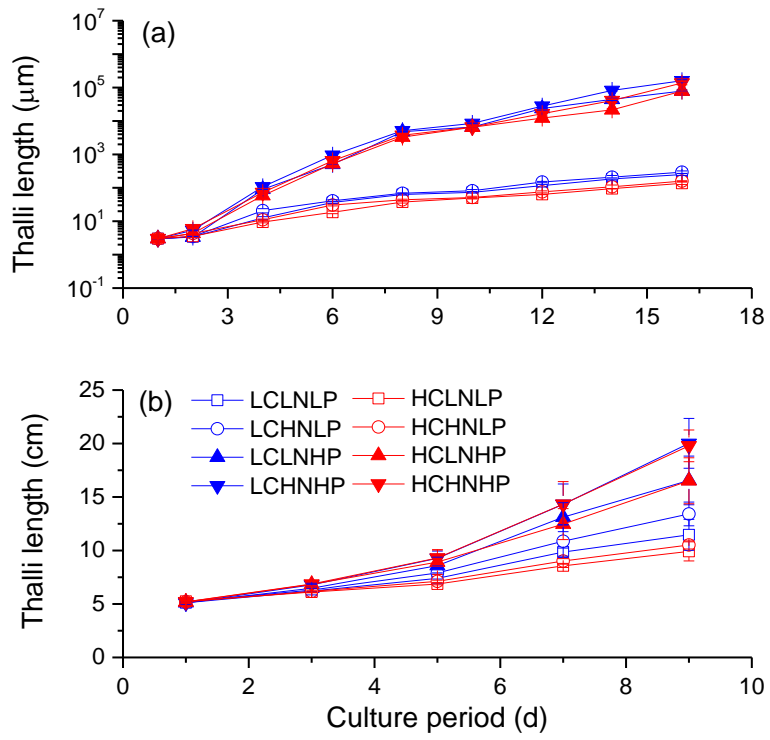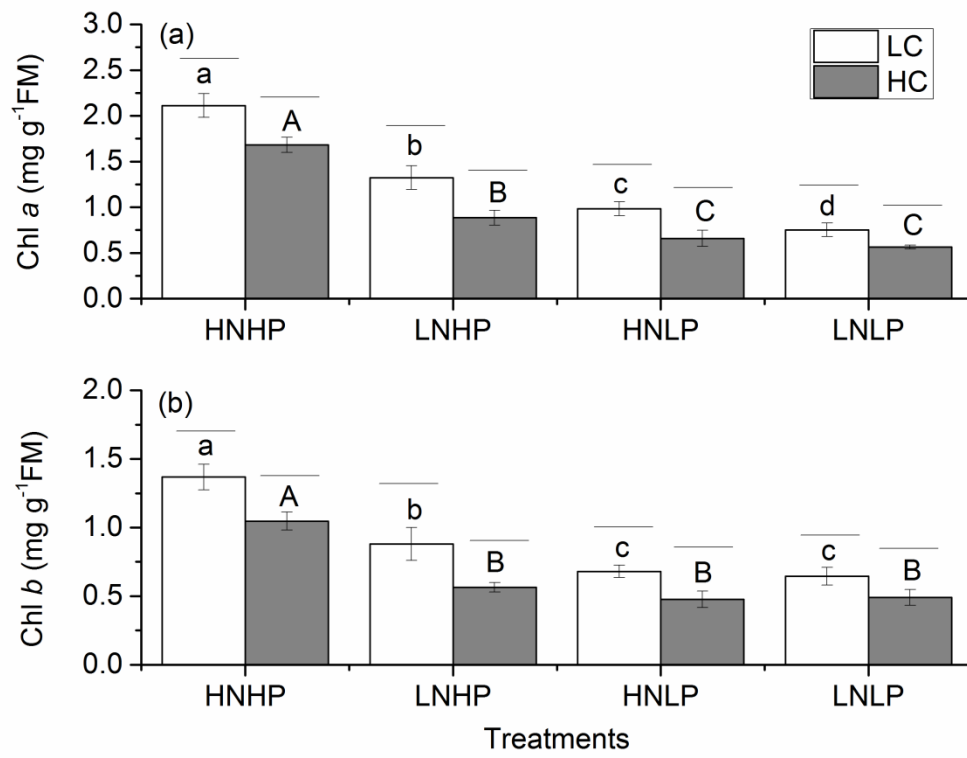
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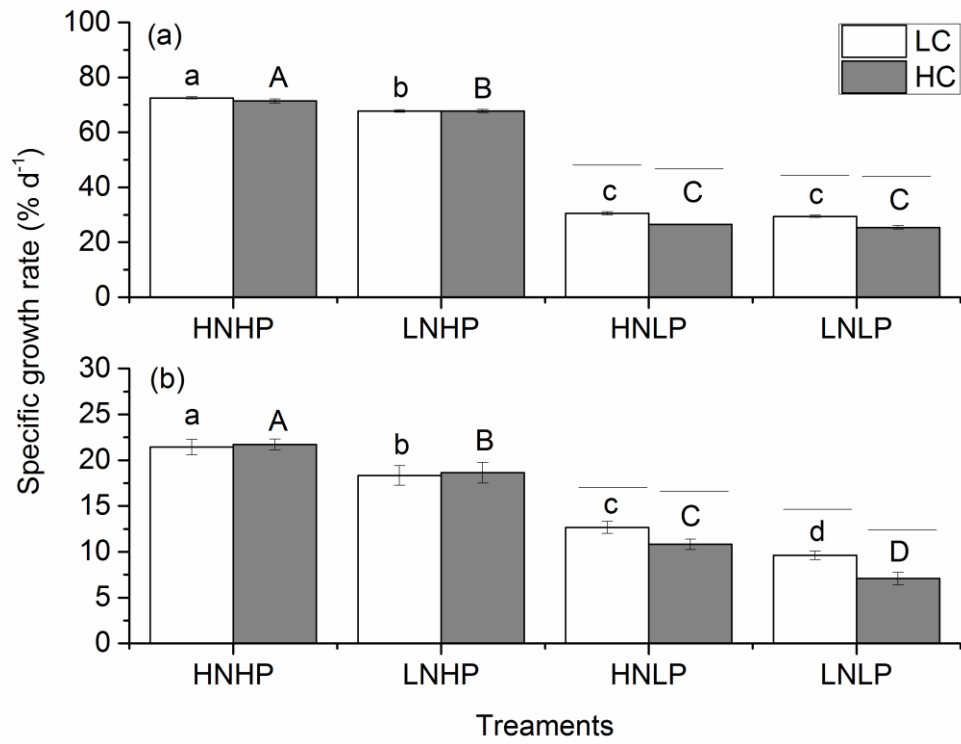
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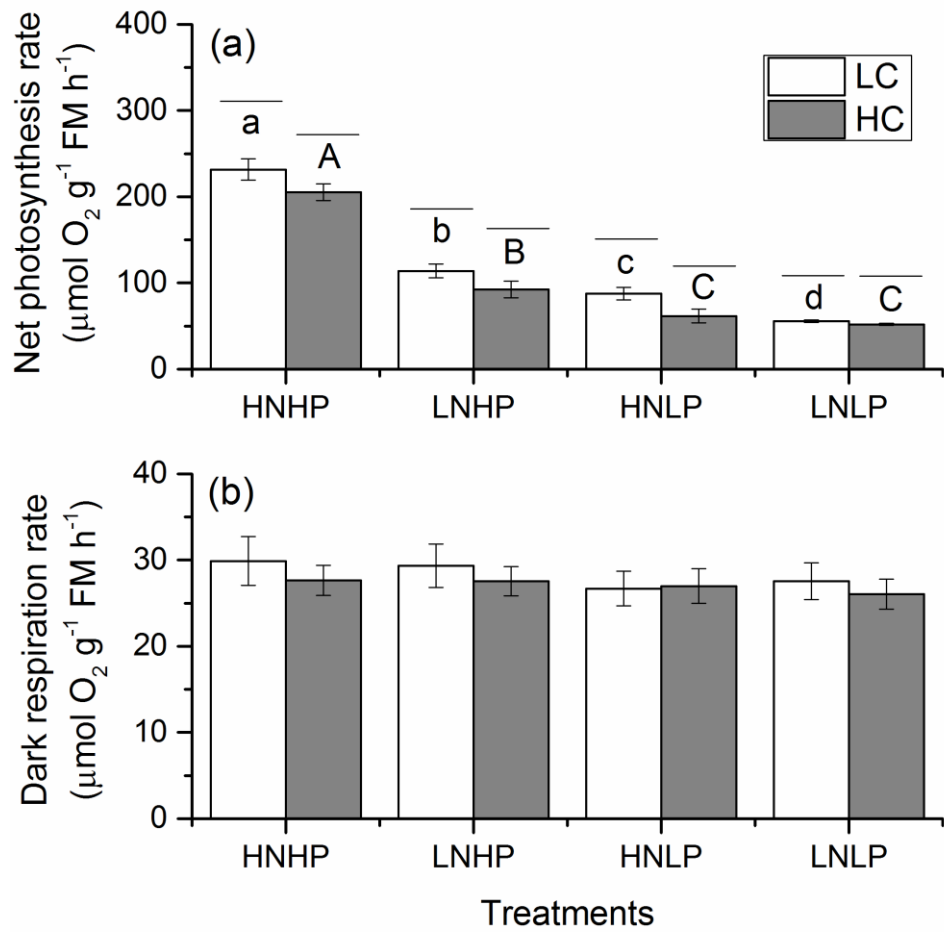
Fig. 3

Fig. 4

Fig.5

Fig. 6

Fig. 7

Fig. 8