Response to reviewers' comments

Anonymous Referee #2

Comments: The authors investigated the combined effects of ocean acidification and nutrient limitation on physiological performances, including growth, photosynthetic oxygen evolution, PSII fluorescence parameters, nitrogen assimilation, in a green tide alga, and found that ocean acidification did not affect growth and photosynthesis under the nutrient replete condition but reduced them when nutrient was limited. Nitrogen assimilation was stimulated by ocean acidification when nutrient was replete. The experiments were reasonably performed and the results were clearly presented. This study is of interest, indicating the interactive effects of global and local stressors on a green tide alga. But there are still some points to be revised before it could be published in Biogeosciences.

Response: We appreciate these comments very much and revised the manuscript based on the reviewer's comments.

Special revisions

1. Why were different cultivation periods used for sporeling and adult thalli? Are these periods enough for algal acclimation to ocean acidification?

Response: The cultures had been finished before the thalli became reproductive as the aim of this study focused on the growth and photosynthesis. Different cultivation periods were used because the periods were different for sporeling and adult to become reproductive. These cultivation periods are enough for *Ulva linza*'s acclimation to ocean acidification (Eggert, 2012; Gao et al., 2016). This information has been added to lines 147-151.

Eggert A. Seaweed responses to temperature. In: Wiencke C, Bischof K, editors. Seaweed biology. Berlin: Springer; 2012. pp.47-66.

Gao G, Liu Y, Li X, et al. An ocean acidification acclimatised green tide alga is robust to changes of seawater carbon chemistry but vulnerable to light stress. PloS one, 2016, 11(12): e0169040.

2. Please clarify the culture density used in this study and to what extent pH fluctuated during the culture period. How to maintain a stable pH in the cultures?

Response: The culture density was less than 0.1 g L^{-1} and the pH fluctuation was less than 0.03 units. Low culture density and aeration with ambient and CO_2-enriched air contributed to the stable pH in the cultures. This information has been added to lines 142-145.

3. Why was the light density of 300 photons m-2 s-1 used for the cultures since lower levels were used for the previous studies as mentioned in the text. Is the one used in this study close to ambient sunlight?

Response: The samples were collected in March 2017 and the light density of 300 photons m^{-2} s^{-1} used for the cultures was close to the ambient light level at the sample collecting site. This information has been added to lines 134-136.

Minor revisions

Line 113 change µmol to µmol photons m-2 s-1

Response: Corrected.

Line 123 add a space after 106.1

Response: Corrected.

Line 156 change weight to mass

Response: Corrected.

Line 329 delete activity and be consistent for using NRA or NR activity throughout the text.

Response: Corrected.

Figure 3 change FW to FM in Y axes legend

Response: Corrected.

Figure 7 I doubt there is a significant difference between HC and LC for the treatment of HNLP

Response: No, there is no significant difference between HC and LC for the treatment of HNLP. We apologize for this mistake and it has been corrected.

Anonymous Referee #3

This paper reports results from an interesting study aiming to test the effects of ocean acidification and nutrients limitation on Ulva. The study is pretty straightforward: adult and juvenile algae were exposed to different conditions of CO2 and nutrients and their physiological response was investigated. While this study is rather "classical", the originality comes from the use of nutrient limitation, while most studies have used so far nutrients addition. The results are rather interesting and demonstrate that the interaction between pCO2 and nutrient limitations are not straightforward. I find the discussion a bit complex and hard to read given the quantity of physiological parameters discussed. It might be worth considering adding a figure that would summarize all the results. Maybe a schematic representing the physiological impact of nutrients and carbon could be added.

I have listed below some specific comments.

Response: We appreciate these comments very much and a schematic figure (Fig. 8) has been added to summarize all the results.

Abstract: indicate the duration of the experiment

Response: it has been clarified to "We cultured *Ulva linza* for 9-16 days" at line 6.

L55: Wrong reference for Cornwall et al. 2017, they looked at coralline algae not phytoplankton.

Response: We are grateful for this comment and this reference has been removed.

L63-64: reformulate this sentence

Response: It has been revised to "By analyzing the literatures, it is found that life stage can affect the effects of ocean acidification on growth of *Ulva* species" at lines 63-65.

L119: "LCHNHP" is a bit hard to read/understand but I guess it's not really used later on.

Response: It has been revised to "The treatment of lower pCO_2, higher nitrate and higher phosphate (LCHNHP) was set as the control." at lines 121-122.

L130: How does this light level compare to in situ?

Response: The samples were collected in March 2017 and the light density of 300 photons m^{-2} s^{-1} used for the cultures was close to the ambient light level in situ. This information has been added to lines 134-136.

L132: What was the size of the tanks? Did you use any pumps, etc , to create water motion? This is critical as it could affect the capacity of the organisms to uptake nutrients.

Response: The thalli were grown in 1-L balloon flasks containing 900 mL of media. The cultures were bubbled with ambient or CO_2-enriched air at a rate of 300 mL min^{-1} to make the thalli roll up and down. Please see lines 140-141.

L133: Any reason to have chosen these durations? 9 days is rather short.

Response: The cultures had been finished before the thalli became reproductive as the aim of this study focused on the growth and photosynthesis. Different cultivation periods were used because the periods were different for sporeling and adult to become reproductive. These cultivation periods are enough for *Ulva linza*'s acclimation to ocean acidification (Eggert, 2012; Gao et al., 2016). This information has been added to lines 147-151.

L156: What were those fragments? Just a piece of algae? I always have problem with this method, as I highly doubt it represents the response of the entire organism. When where the incubations done, at the end of the experiment? How many replicates were used?

Response: The text has been specified to "Algal individuals were cut into 1-cm-long segments with a scissor. Approximately 0.02 g segments were randomly selected and transferred to the oxygen electrode cuvette with 8 ml of media from the culture flask." at lines 175-178 and " The following parameters were measured at the end of the culture periods for each flask under each treatment." at lines 151-152.

L176: This was also done at the end of the 9 d?

Response: Yes.

Results: I would favour indicating the actual p-values rather than < 0.05 or >0.05

Response: We have used the actual P-values for most cases, with $P < 0.001$ for those where actual P-values were less than 0.001. Meanwhile, we hope we can keep $P < 0.05$ or > 0.05 for some cases where there are too many comparisons in one sentence.

L-314-315: Any reason why the algae would do that? If they have more carbon available why would they reduce their photosynthesis? It doesn't make much sense from an organismal point of view.

Response: We appreciate these comments. The explanation has been specified to "Meanwhile, the saved energy due to down-regulation of CCMs in thalli grown under HC combined with higher light density used in this study may depress PSII activity and thus reduce net photosynthetic rate (Gao et al., 2012)." at lines 341-344.

L 331-332: Could this be due to pH rather than carbon?

Response: Yes, there is possibility that the change of NRA was due to pH. The following information has been added to the text "Meanwhile, the change of NRA under different pCO_2 levels might be also caused by varying pH as pH could affect NRA in seaweeds (Lopes et al., 1997)" at lines 369-370.

Lopes P F, Oliveira M C, Colepicolo P. Diurnal fluctuation of nitrate reductase activity in the marine red alga *Gracilaria tenuistipitata* (Rhodophyta). Journal of Phycology, 1997, 33: 225-231.

L344-345: CCM activity has often been linked to the light level. Could it explain some of these results?

Response: Yes, there are connections among CCM activity, CO_2 and light. The related discussion has been added to the text and it reads " The potential reason is that the saved energy from down-regulated CCMs under higher CO_2 levels could be used for growth at lower light levels but could inhibit PSII activity and thus growth at higher light levels" at lines 388-391.

As explained before, I think that an additional figure to summarize all of those results (and mostly the link between each other) would be highly valuable.

Response: It has been done. Please see section 4.4 for details.

L392-393: Could the seaweed culture also be affected by those limitations?

Response: The text has been revised to "This may hinder the occurrence of green tides and *Ulva* cultivation in future ocean." at lines 436-437.

Anonymous Referee #4

This manuscript details the results of a classical pCO2 x Nutrients experiment with seaweeds. In that respect its novelty relays in the distinction between N and P limitation, while most of the phenomena concerning pCO2 x N has been described before in Ulva sp. (eg. Gordillo et al. 2001 Planta and Gordillo et al. 2003 Planta).

Response: We agree with these comments. Gordillo et al (2001, 2003) did excellent work on the interaction of CO_2 and N. Another novel point of our study is that we used diluted natural seawater as nutrient limiting condition rather than natural seawater to mimic the situation in seaweed cultivation areas.

Main comments

A major concern is about net photosynthesis. As it is measured (O2 evolution), changes can derive either from photosynthesis or from respiration. Since respiration of seaweeds is commonly affected by pCO2 (Iñiguez et al. 2015 Polar Biol.; Iñiguez et al. 2016 Mar Biol) even in Ulva (e.g. Gordillo et al. 2003 Planta) and also by nutrients, authors must show respiration rates along with the net or gross photosynthesis. Otherwise, not much can be said about the effect of pCO2 and nutrients on photosynthetic O2 evolution.

Response: We totally agree with these comments. We measured dark respiration rate, but did not represent it as neither pCO_2 nor nutrient affected it, indicating that changes of O_2 evolution derived from photosynthesis rather than respiration. The data of dark respiration have been added to the text and also been discussed. Please see lines 259-260 and 354-360.

Line 304. The 'pigment economy' phenomenon occurring in algae at high pCO2 was

first described in Gordillo et al. (1999 J appl. Phycol) and described for Ulva using exactly the same name by Gordillo et al. 2003 (Planta), so credit must be given to those authors.

Response: We agree with these comments and the text has been corrected to "This phenomenon of `pigment economy' has also been found in the previous studies regarding *Ulva* species (Gordillo et al., 2003; Gao et al., 2016)." at lines 330-332.

Minor comments Methods Incubation setup needs more detail. What type of recipient was used for adult thalli? At what density? Was the bubbling enough to make them move or were they settling on the bottom? Incubation light need more detail. What source of light was used (fluorescent tubes of daylight type?). Also how was the irradiance measured? (type of sensor, air or underwater?, lambda range?PAR?)

Response: We appreciate these comments. The thalli were grown in 1-L balloon flasks containing 900 mL of media with the density less than 0.1 g L^{-1}. The cultures were bubbled with ambient or CO_2-enriched air at a rate of 300 mL min^{-1} to make the thalli roll up and down. Daylight fluorescent tubes (21W, Philips) were used and light density was measured by a Quantum Scalar Laboratory (QSL) radiometer (QSL-2100, Biospherical Instruments, Inc., USA) that detects photosynthetically active radiation (400-700 nm). Please see section 2.1.

53. 'also' instead of 'only'

Response: Corrected.

148-150. Sentence is nonsensical, please rephrase.

Response: It has been corrected to "The measuring light was 0.01 μmol photons m^{-2} s^{-1} and actinic light was set as the same as the growth light (300 μmol photons m^{-2} s^{-1})" at lines 167-169.

164. Units needed (nm)

Response: Corrected.

Tables 4 to 7 can be combined and look like table 2, so the information is not scattered.

Response: Tables 4 to 7 has been combined into a table, termed table 4.

Fig.2. The horizontal bar means significant differences between LC and HC, but that is hard to believe for some of the treatment at least like LNHP in (a), and HNHP and LNHP in (b). Please check your post-hoc comparisons. It is also highly convenient you mention the number of replicates (n) in the figure legends.

Response: The real indication of horizontal bars is that longer bars represent insignificant differences and shorter bars represent significant differences. We have realized that it is a little confusing. We have removed the longer horizontal bars to make it clear.

Comments from Dinghui Zou

The authors investigated the combined effects of ocean acidification and nutrient limitation on physiological performances, including growth, photosynthetic oxygen evolution, PSII fluorescence parameters, nitrogen assimilation, in a green tide alga, and found that ocean acidification did not affect growth and photosynthesis under the nutrient replete condition but reduced them when nutrient was limited. Nitrogen assimilation was stimulated by ocean acidification when nutrient was replete.

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

Guang Gao[a,c], John Beardall[d], Menglin Bao[a], Can Wang[a], Wangwang Ren[b], Juntian Xu[a,b*]

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[b]Co-Innovation Center of Jiangsu Marine Bio-industry Technology, Lianyungang 222005, China

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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

acidification, photosynthesis

1 Introduction

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

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

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

does not affect or even reduces growth of *Ulva* species at late life stages (Gao et al., 2017a). A possible explanation for the differential effects of ocean acidification is that higher CO_2 could induce more reproduction events for adult (Gao et al., 2017a).

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

In addition to independent effects, the combined effects of ocean acidification and nutrient on seaweeds have also been studied. Baydend et al. (2010) documented that 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}

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

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

2.2 Measurement of growth

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

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

2.3 Chlorophyll fluorescence assessment

The relative electron transport rate (rETR) was measured using a pulse amplitude modulation (PAM) fluorometer (PAM-2100, Walz, Germany). The measuring light ~~and actinic light~~ was 0.01 μmol photons m^{-2} s^{-1} and actinic light was set as the same as the growth light (300 μmol photons m^{-2} s^{-1})~~, respectively~~. The saturating pulse was set to 5, 000 μmol photons m^{-2} 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 maximal fluorescence levels from algae in the light. Ft is the fluorescence at an excitation level. PFD is the actinic light density.

2.4 Determination of photosynthesis

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

transferred to the oxygen electrode cuvette with 8 ml of media from the culture flask~~Approximately 0.02 g of fresh weight algae with 8 ml of media from the culture flask was transferred to the oxygen electrode cuvette~~, being stirred. The conditions for temperature and light were set the same as those for growth. The net photosynthetic rate was determined by the increase in the oxygen content in the media over five minutes. The unit for net photosynthetic rate (NPR) was μmol O_2 g^{-1} FM h^{-1}.

2.5 Measurement of photosynthetic pigments

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

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

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

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

2.6 Assessment of nitrate reductase activity

Nitrate reductase activity (NRA) of thalli was estimated based on a modified 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 with a maximum during the light period and a minimum in darkness (Velasco and Whitaker 1989; Deng et al. 1991). Approximately 0.3 g (FM) of thalli from each culture condition was incubated for 1 h at 20°C in darkness in the reaction

199 solution (10 mL), which contained 0.1 M phosphate buffer, 0.1% propanol (v/v), 50
200 mM KNO_3, 0.01 mM glucose, and 0.5 mM EDTA, with a pH of 8.0. The mixture was
201 flushed with pure N_2 gas (99.999%) for 2 minutes to obtain an anaerobic state before
202 the incubation. The concentration of nitrite produced was determined colorimetrically
203 at 540 nm (Xu et al., 2017). The NRA ~~activity~~ was expressed as μmol NO_2^- g^{-1} FM
204 h^{-1}.

205 **2.7 Estimation of soluble protein**

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

211 **2.8 Statistical analysis**

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

specific growth rate, net photosynthesis rate, rETR, Chl *a*, Chl *b*, soluble protein and ~~nitrate reductase activity~~NRA. Tukey's honest significant difference (Tukey HSD) was conducted for MNOVA and ANOVA *post hoc* investigation. Paired t-tests were used to compare the differences in specific growth rate between young and adult thalli under each treatment. A confidence interval of 95% was set for all tests.

3 Results

The carbonate system under each treatment was recorded (Table 1). Both pCO_2 and nutrient treatments had a significant effect on carbonate parameters (Table 2). Elevated pCO_2 reduced pH and CO_3^-, increased DIC, CO_2 and HCO_3^- ($P < 0.001$ for all parameters above) but did not affect TA (~~Tukey HSD,~~ P ~~<~~ = 0.740~~05~~). P limitation (LP) increased pCO_2 ($P = 0.002$) and CO_2 ($P = 0.002$), and reduced pH ($P = 0.001$) and CO_3^{2-} (~~Tukey HSD,~~ P ~~<~~ = 0.04~~65~~).

The length for both young and adult *U. linza* varied with culture time and the patterns under different pCO_2 and/ or nutrient conditions were inconsistent (Fig. 1 & Table 3). For example, the length gap between HP and LP increased with culture time (Bonferroni, $P < 0.$~~05~~01). It is worth noting that LP dramatically inhibited the development of sporelings as the length under HP was ~~6,880~~78,274−16~~,~~0~~,~~290 μm while it was only 137−250 μm under LP at the end of 16 days of culture.

Based on the initial and final length (young thalli) or mass (adult thalli), specific growth rate was calculated (Fig. 2). Nutrient and pCO_2 interacted to affect the growth of both young and adult *U. linza* (Table 4). Specifically, *post hoc* Tukey HSD comparison ($P = 0.05$) showed that HC reduced growth at LP but did not affect it at

HP, suggesting an interactive effect between P and C. Nutrient supply had an effect on growth but the patterns between young and adult thalli were different (Table 4). For young thalli, *post hoc* Tukey HSD comparison (P = 0.05) showed that N limitation did not reduce growth, P limitation dramatically reduced the growth and the combination of N and P limitation did not lead to a further decrease regardless of pCO_2 conditions. For adult thalli, either N ($P < 0.001$) or P ($P < 0.001$) limitation reduced growth and the combination of these nutrient limitations resulted in a further decrease (~~Tukey HSD,~~ P ~~<~~ =0.~~05~~011). In addition, young thalli had higher growth rates under each condition compared to adult plants (~~Paired t-test,~~ P < 0.~~05~~001).

The effects of pCO_2 and nutrients on the net photosynthetic rate of adult thalli were also investigated (Fig. 3a). Both pCO_2 and nutrient had a significant effect on net photosynthetic rate (Table ~~5~~4) and HC reduced NPR under each nutrient condition (~~Tukey HSD,~~ P < 0.05). In terms of the effect of nutrients, LN (P = 0.006) or LP ($P <$ 0.001) alone decreased NPR and the combination of LN and LP led to a further decrease under LC (~~Tukey HSD,~~ P < 0.~~05~~001). Under HC, *post hoc* Tukey HSD comparison (P = 0.05) showed that both LN and LP reduced NPR but the combination of LN and LP did not decrease NPR further. In terms of dark respiration rate (Fig. 3b), neither pCO_2 nor nutrient had a significant effect on it (Table 4).

To understand the photosynthetic performance of *U. linza* under various pCO_2 and nutrient conditions, relative electron transport rate (rETR) in PSII at 300 μmol photons m^{-2} s^{-1} was measured (Fig. 4). pCO_2 had an interactive effect with nutrient and each factor had a main effect (Table ~~5~~4). Specifically speaking, HC reduced rETR

under LP but did not change it under HP. Regardless of pCO_2 levels, N limitation reduced rETR (~~Tukey HSD, P <~~ = 0.~~05~~028), P limitation had a larger negative effect ($P < 0.001$) and the combination of LN and LP resulted in the lowest rETR values (~~Tukey HSD, P < 0.05~~001).

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

To investigate the effects of pCO_2 and nutrient on nitrogen acquisition, ~~nitrate reductase activity (~~RNA~~)~~ in adult *U. linza* grown under various conditions was measured (Fig. 6). Both pCO_2 and nutrient affected NRA and they had an interactive effect (Table 4~~7~~). Under LC, *post hoc* Tukey HSD comparison (P = 0.05) showed that either N or P limitation reduced NRA but the combination of them did not result in a further decrease. Under HC, N limitation and P limitation reduced NRA by 22.8% and 37.7% respectively and the combination of them increased NRA by 45.6%. In addition, HC did not affect NRA under LNLP (~~Tukey HSD, P >~~ = 0.700~~05~~) but increased it when N (P = 0.018) or P (P < 0.018) was replete and nitrate reductase had

the highest activity (11.9 ± 0.7 μmol NO_2^- g^{-1} FM h^{-1}) under HCHNHP condition (~~Tukey HSD,~~ $P < 0.$~~05~~001).

The content of soluble protein was assayed to investigate nitrogen assimilation of *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 ~~7~~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 (~~Tukey HSD,~~ $P < 0.$~~05~~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

309 cells is much slower than in blade cells (Han et al., 2003; Lüning et al., 2008), which
310 slows down the total growth of the thallus as it ages.

311 Lower P levels strongly inhibited the growth of both young and adult plants in this
312 study and the inhibitory effect was particularly significant for young plants. Gao et al.
313 (2017b) has reported that *U. rigida* becomes mature when the thalli reach a length of
314 around 1.5 cm and we also found a similar phenomenon in *U. linza*. Young plants
315 grown under P limitation were far away the mature stage even after 16 days of culture
316 while the plants grown under P repletion reached a mature stage by day 11 and the
317 length could be up to 16 cm by day 16. This finding supports the significant role of P
318 in development of *U. linza*. Phosphorus (P) is an essential element for seaweeds, in
319 the form of nucleic acids, phospholipids, ATP and ADP, but little is known regarding
320 the effect of P on development of seaweed. Our findings indicate that P limitation
321 may terminate the development of young *Ulva* and cause it to remain in the immature
322 stage. The separate addition of N did not change the growth rate of young plants but
323 increased the growth rate of adult plants, suggesting that adult plants could be more
324 resilient to P limitation compared to young plants.

325 **4.2 Photosynthetic response to OA and nutrients**

326 HC was shown in the present study to decrease the Chl *a* and Chl *b* contents of *U.*
327 *linza*. High CO_2 commonly down-regulates algal CO_2 concentrating mechanisms
328 (CCMs), suggesting less energy is required to drive CCMs (Gao et al., 2012; Raven et
329 al., 2012; Cornwall et al., 2017~~b~~; Raven et al., 2017). This may lead to decreased
330 synthesis of pigment for energy capture. This phenomenon of `pigment economy' has

also been found in ~~our~~ the previous studies regarding *Ulva* species (~~Xu and Gao, 2012~~Gordillo et al., 2003; Gao et al., 2016). Deficiency in N or/and P supply also reduced pigment content in this study. Nitrogen is a major component of Chl *a*. Although P is a non-constituent element in Chl *a*, higher P supply may stimulate the activity of Chl *a* synthesis-related enzymes (Xu et al., 2017). Accordingly, in this study nutrient (N & P) enrichment enhanced the synthesis of Chl *a*. This is consistent with other findings in *Ulva* species (Gordillo et al., 2001; Figueroa et al., 2009) and other macroalgae (Xu et al., 2017).

HC decreased net photosynthetic rate in *U. linza* in the present study. This could be due to the decrease of photosynthetic pigment in thalli grown under HC. Meanwhile, the saved energy due to down-regulation of CCMs in thalli grown under HC combined with higher light density used in this study may depress PSII activity and thus ~~might have reduced the intracellular CO_2 availability and have contributed to the lower~~ reduce net photosynthetic rate (Gao et al., 2012). An ocean acidification-induced decrease of net photosynthetic rate was also documented in *U. prolifera* (Xu and Gao, 2012). In terms of the effects of nutrient, N limitation reduced net photosynthetic rate in *U. linza* and P limitation resulted in a further decrease. The negative effects of N and P limitation on algal photosynthetic rate have been extensively reported (Longstaff et al., 2002; Kang and Chung, 2017; Xu et al., 2017), indicating the important role of N and P in algal photosynthesis. In addition to the separate effects of pCO_2 or nutrient, these factors also interplay on photosynthetic performances of *U. linza*. For instance, HC reduced rETR under LP but did not affect

it under HP, suggesting that P enrichment could offset the negative effect of ocean acidification. In contrast to net photosynthetic rate, the dark respiration rate of thalli was insensitive to the change of pCO_2 or nutrient. The higher pCO_2 did not affect the dark respiration rate in *U. prolifera* either (Xu et al., 2012) but reduced it in *U. rigida* (Gordillo et al., 2003). N enrichment did not affect the dark respiration rate under lower pCO_2 but reduced it under higher pCO_2 for *U. rigida* (Gordillo et al., 2003). The present finding combined with the previous studies indicates that response of dark respiration in *Ulva* may be species-dependent.

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4.3 N assimilation under OA and nutrient limitation

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

4.4 Interactive effects of OA and nutrient limitation on Growth

HC did not affect the growth of thalli when P was replete in the medium. Since HC

reduced photosynthesis rate but increased NRA and protein synthesis, the lack of effect of HC may be an integrated outcome of C and N assimilation (Fig. 8). This finding is different from our previous studies in which HC increased the growth rate of *U. linza* (Gao et al., 2016) and *U. prolifera* (Gao et al., 2017d). The possible reason causing this divergence might be due to the different light intensities that were used in the various studies. For the previous studies, a lower light intensity of 100 μmol photons m^{-2} s^{-1} was used for algal culture while a higher light intensity of 300 μmol photons m^{-2} s^{-1} was used in the present work. Ocean acidification could interact with light intensity to affect algal growth. It has commonly been reported that ocean acidification can increase algal photosynthesis/growth at lower light intensity and inhibit photosynthesis/growth at higher light intensity (Gao et al., 2012; Xu and Gao, 2012; Gao et al., 2016), with inversion points of PAR around 160, 125 and 178 μmol photons m^{-2} s^{-1} for *Phaeodactylum tricornutum*, *Thalassiosira pseudonana* and *Skeletonema costatum* respectively (Gao et al., 2012). The potential reason is that the saved energy from down-regulated CCMs under higher CO_2 levels could be used for growth at lower light levels but could inhibit PSII activity and thus growth at higher light levels (Gao et al., 2012; Gao et al., 2016). It seems that *U. linza* has a higher inversion point compared to diatoms.

Furthermore, HC reduced growth of *U. linza* when P was limited. In addition to the increased CO_2 supply, ocean acidification also reduces the pH of seawater, which has been deemed as a stressor disturbing the acid-base balance both at the cell surface and within cells and affecting algal photosynthetic performance (Flynn et al., 2012; Gao et

al., 2017d). Increased CO_2 and decreased pH also reduced rETR and net photosynthetic rate of *U. linza* in the present study. Xu et al. (2017) proposed that algae could synthesize HC transport-related proteins to combat that disturbance. Under P limitation conditions, such protein synthesis could be limited, which may lead to the decreased rETR and net photosynthetic rate and thus to decreased growth observed in the present study. Until now, most studies of ocean acidification on seaweed have been conducted under nutrient replete conditions. The present study thus demonstrates the contrasting effect of ocean acidification under nutrient deplete conditions.

4.5 Differential effects of N and P limitation

In the present study, compared to N limitation, P limitation seems to have a larger negative effect on physiological performances in *U. linza*. In other words, the addition of P resulted in a larger stimulating effect compared to N addition. Which one (N or P) is the nutrient most likely to limit marine primary productivity has been a controversial issue until now (Elser et al., 2007; Teichberg et al., 2010; Müller and Mitrovic, 2015). It has been proposed that the occurrence of N or P limitation depends on the difference in N:P ratio between in algal tissue and in seawater; when the ratio of N:P in algal tissue is higher than in seawater N limitation is indicated and the opposite is considered as P limitation (Harrison and Hurd, 2001). The ratio of N:P in tissue of *U. linza* grown in the field has not been documented and the mean value of N:P throughout a year is 15.4 for *U. prolifera* and 22.3 for *U. fenestrate* (Wheeler and Björnsäter, 1992). The ratio of N:P in natural seawater where the samples were

collected in the present study is 30.7:1, which is higher than the N:P ratio in the reported *Ulva* species. This suggests the existence of P limitation for *U. linza*, which could explain the larger stimulating effect with P addition.

In recent decades, P limitation has been suggested to commonly occur in coastal waters due to more effective P removal from industrial and domestic wastewater during de-eutrophication processes (Grizzetti et al., 2012). For instance, the ratio of dissolved inorganic N:P could be as high as 375:1 in nearshore waters of the North Sea, resulting in severe P limitation for algal growth (Burson et al., 2016).

5 Conclusions

With the continuous emission of CO_2, the trend of ocean acidification will continue through this century (Gattuso et al., 2015). Meanwhile, nutrient limitation would occur in coastal waters as a consequence of efforts on de-europhication. Measures to reduce eutrophication have often led to a more effective decline of phosphorus (P) than nitrogen (N) concentrations (Burson et al., 2016). In addition, intensive seaweed culture in coastal areas can also lead to noticeable decreases in N and P (He et al., 2008; Wu et al., 2015). Our study demonstrates that ocean acidification and nutrient limitation would synergistically inhibit development and growth of *Ulva* species. This may hinder the occurrence of green tides and *Ulva* cultivation in future ocean. In addition, it has been reported that fast-growing species require high nutrient inputs to sustain growth, while slow-growing species are better adapted to nutrient limiting conditions (Gordillo, 2012). The decrease in nutrient level may result in a shift in seaweed community composition in the future ocean

environment. Studies on other seaweeds are needed to have a comprehensive understanding in terms of the combined effects of global and local stressors on 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		

Table 4. Two-way analysis of variance for the effects of pCO_2 and nutrient on relative growth rate 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.

	Growth of young *U. linza*			Growth of adult *U. linza*		
Source	df	F	Sig.	df	F	Sig.
pCO_2	1	115.297	<0.001	1	20.039	<0.001
Nutrient	3	12678.566	<0.001	3	307.073	<0.001
pCO_2*nutrient	3	22.905	<0.001	3	1.723	0.011
Error	16			16		

Table 5. Two-way analysis of variance for the effects of pCO_2 and nutrient on net photosynthetic rate and rETR 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.

	Net photosynthetic rate			rETR		
Source	df	F	Sig.	df	F	Sig.
pCO_2	1	35.096	<0.001	1	14.592	0.002
Nutrient	3	493.992	<0.001	3	135.690	<0.001
pCO_2*nutrient	3	2.619	0.087	3	5.023	0.012
Error	16			16		

Table 6. Two-way analysis of variance for the effects of pCO_2 and nutrient on content of Chl *a* and Chl *b* in *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.

	Chl *a*			Chl *b*		
Source	df	F	Sig.	df	F	Sig.
pCO_2	1	85.900	<0.001	1	71.600	<0.001
Nutrient	3	217.334	<0.001	3	104.483	<0.001
pCO_2*nutrient	3	2.440	0.102	3	2.005	0.154
Error	16			16		

Table 7. Two-way analysis of variance for the effects of pCO_2 and nutrient on nitrate reductase activity and soluble protein 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.

	Nitrate reductase activity			Soluble protein		
Source	df	F	Sig.	df	F	Sig.
pCO_2	1	38.271	<0.001	1	30.212	<0.001
Nutrient	3	100.487	<0.001	3	106.523	<0.001
pCO_2*nutrient	3	6.246	0.005	3	11.295	<0.001
Error	16			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 ~~16~~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.

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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.

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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.

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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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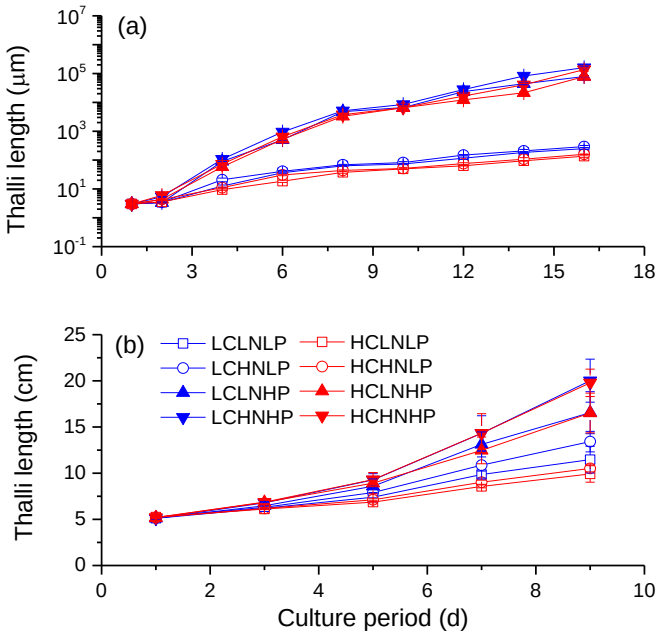

(a)
Thalli length (μm)
10^7
10^5
10^3
10^1
10^{-1}
0
3
6
9
12
15
18
(b)
LCLNLP
LCHNLP
LCLNHP
LCHNHP
HCLNLP
HCHNLP
HCLNHP
HCHNHP
Thalli length (cm)
25
20
15
10
5
0
0
2
4
6
8
10
Culture period (d)

Fig. 1

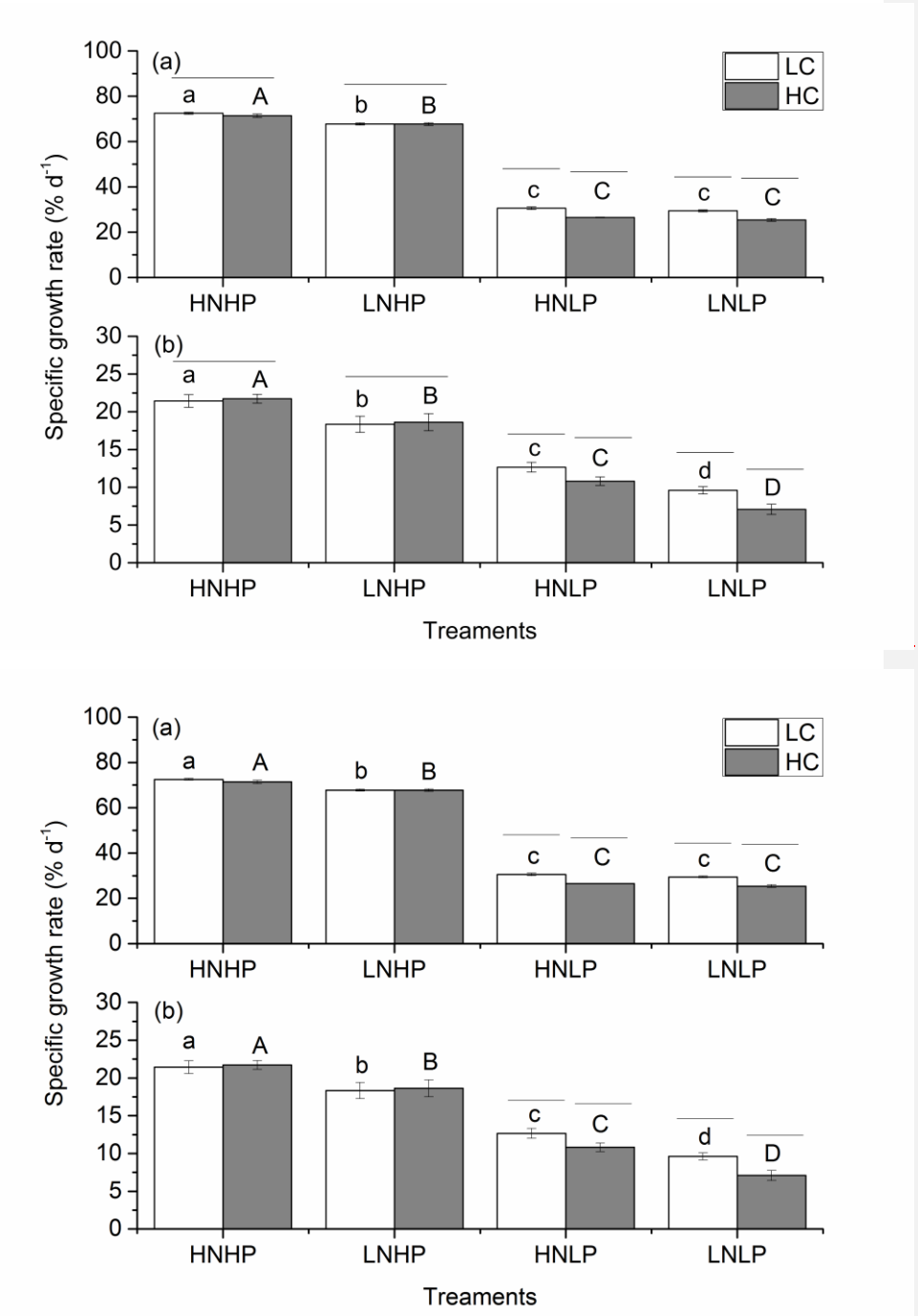
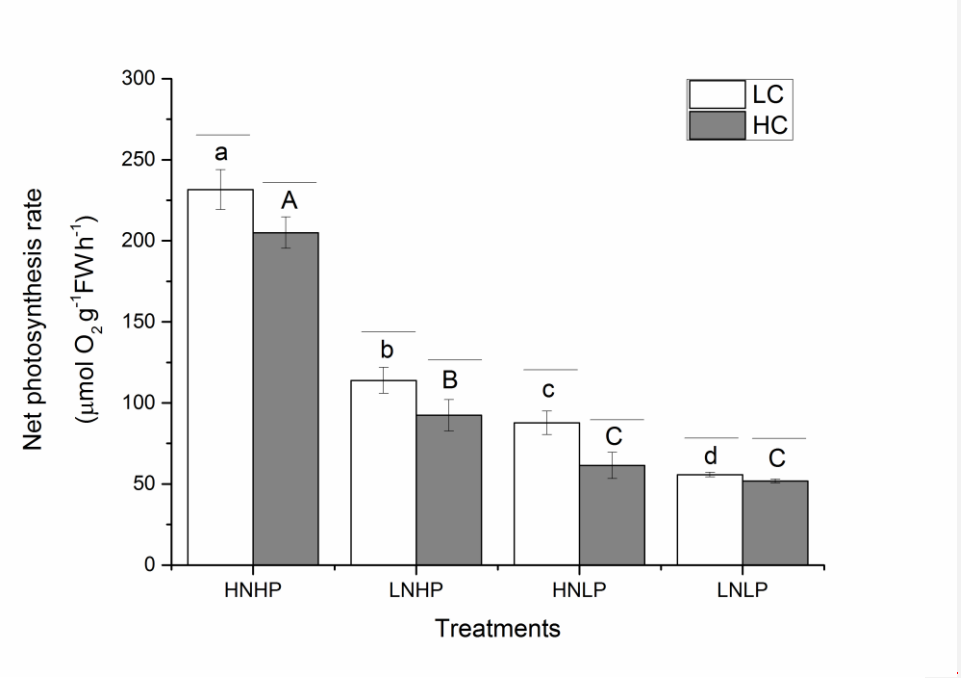

(a)
(b)
LC
HC
Specific growth rate (% d-1)
Treaments
HNHP
LNHP
HNLP
LNLP
a
A
b
B
c
C
d
D

Fig. 2


Net photosynthesis rate
(μmol O_2 g^{-1}FW h^{-1})
300
250
200
150
100
50
0
LC
HC
a
A
b
B
c
C
d
C
HNHP
LNHP
HNLP
LNLP
Treatments

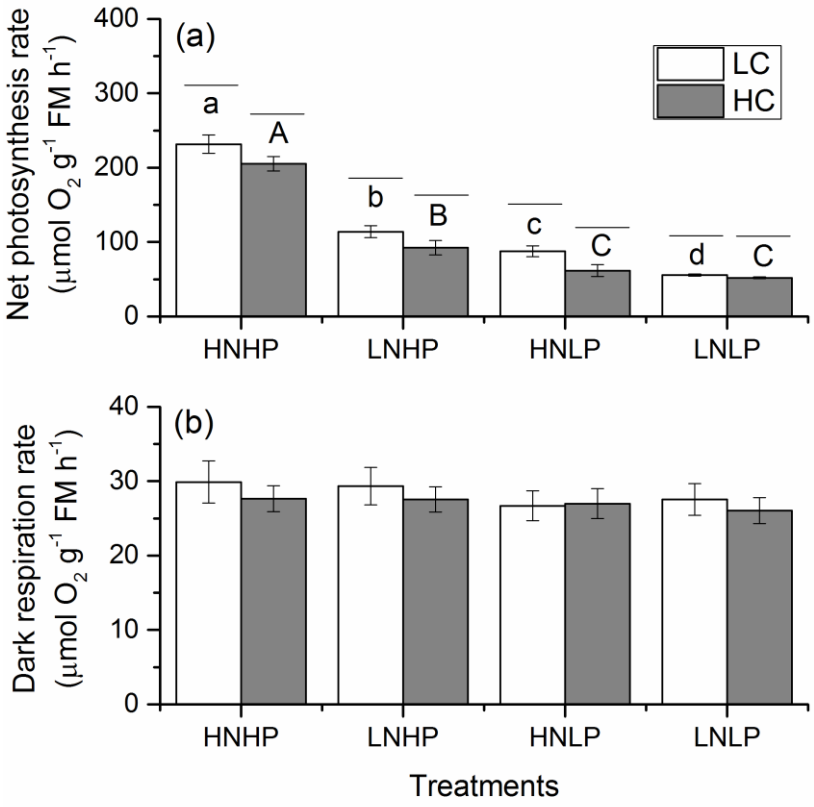

(a)
Net photosynthesis rate
(μmol O2 g-1 FM h-1)
400
300
200
100
0
a
A
b
B
c
C
d
C
LC
HC
HNHP
LNHP
HNLP
LNLP
(b)
Dark respiration rate
(μmol O2 g-1 FM h-1)
40
30
20
10
0
HNHP
LNHP
HNLP
LNLP
Treatments

Fig. 3

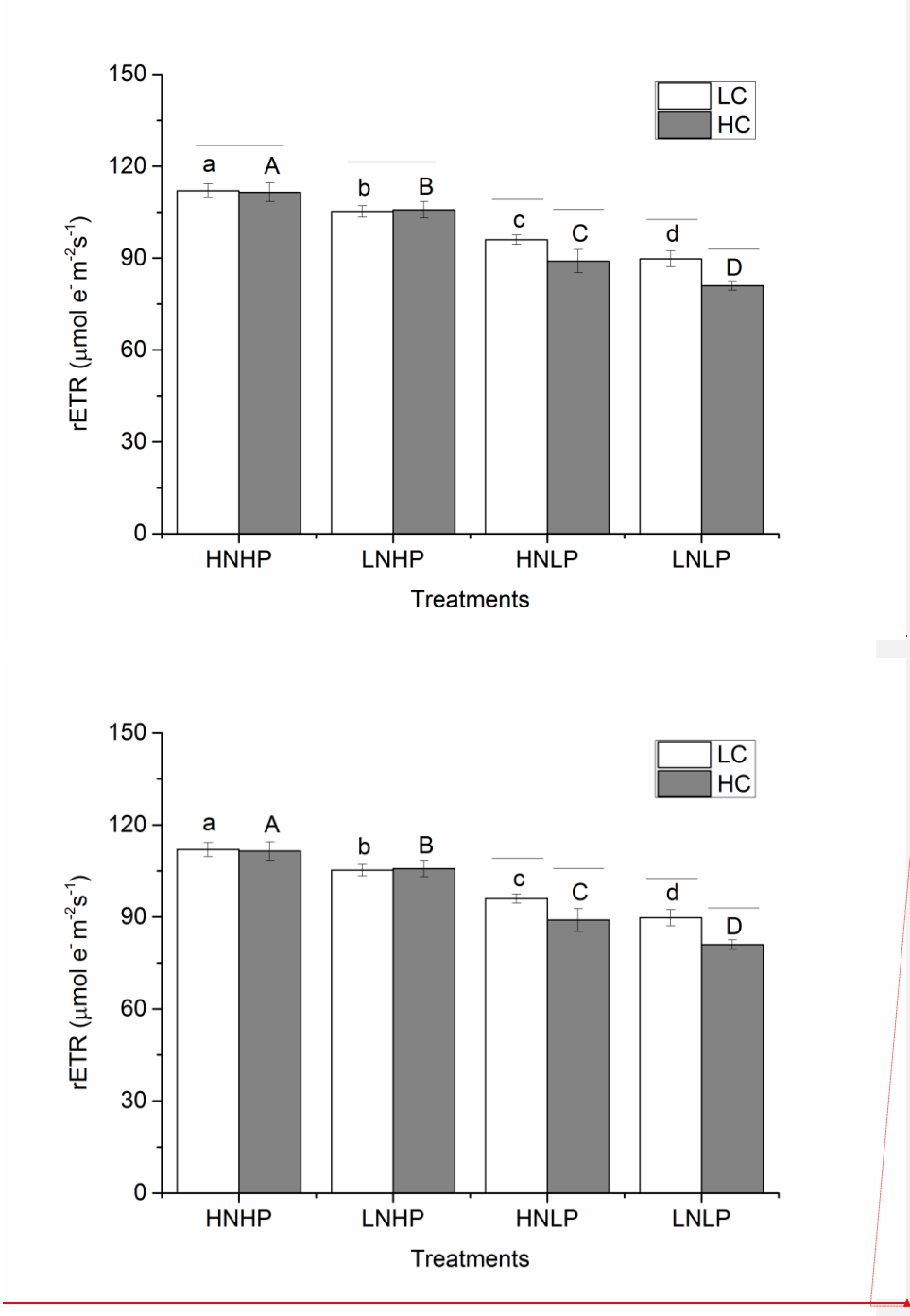


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Fig. 4

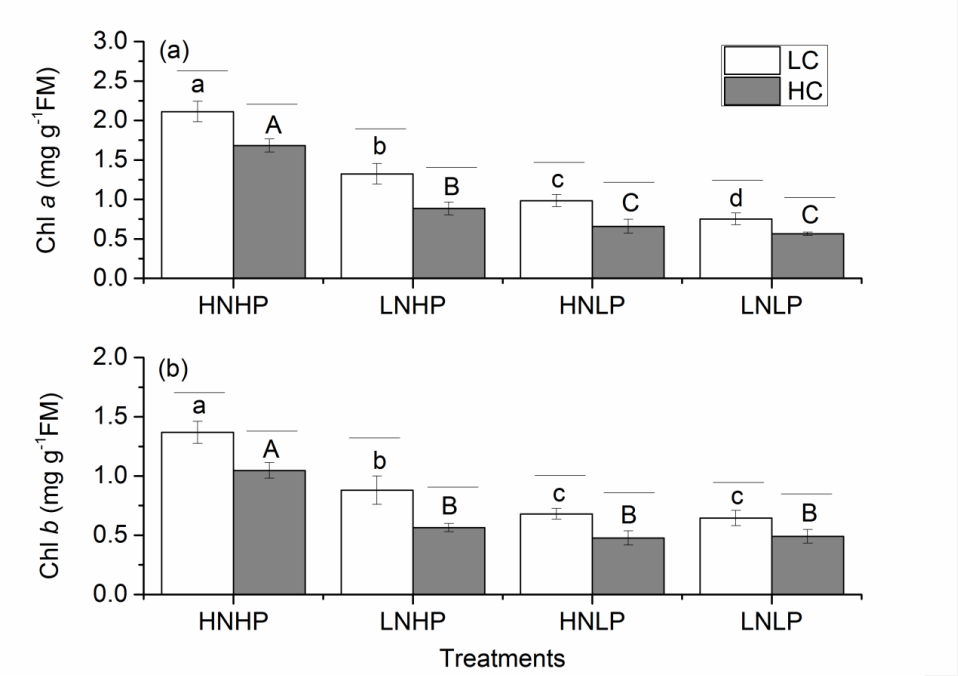

(a)
LC
HC
Chl a (mg g-1FM)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
a
A
b
B
c
C
d
C
HNHP
LNHP
HNLP
LNLP
(b)
Chl b (mg g-1FM)
2.0
1.5
1.0
0.5
0.0
a
A
b
B
c
B
c
B
HNHP
LNHP
HNLP
LNLP
Treatments

Fig.5

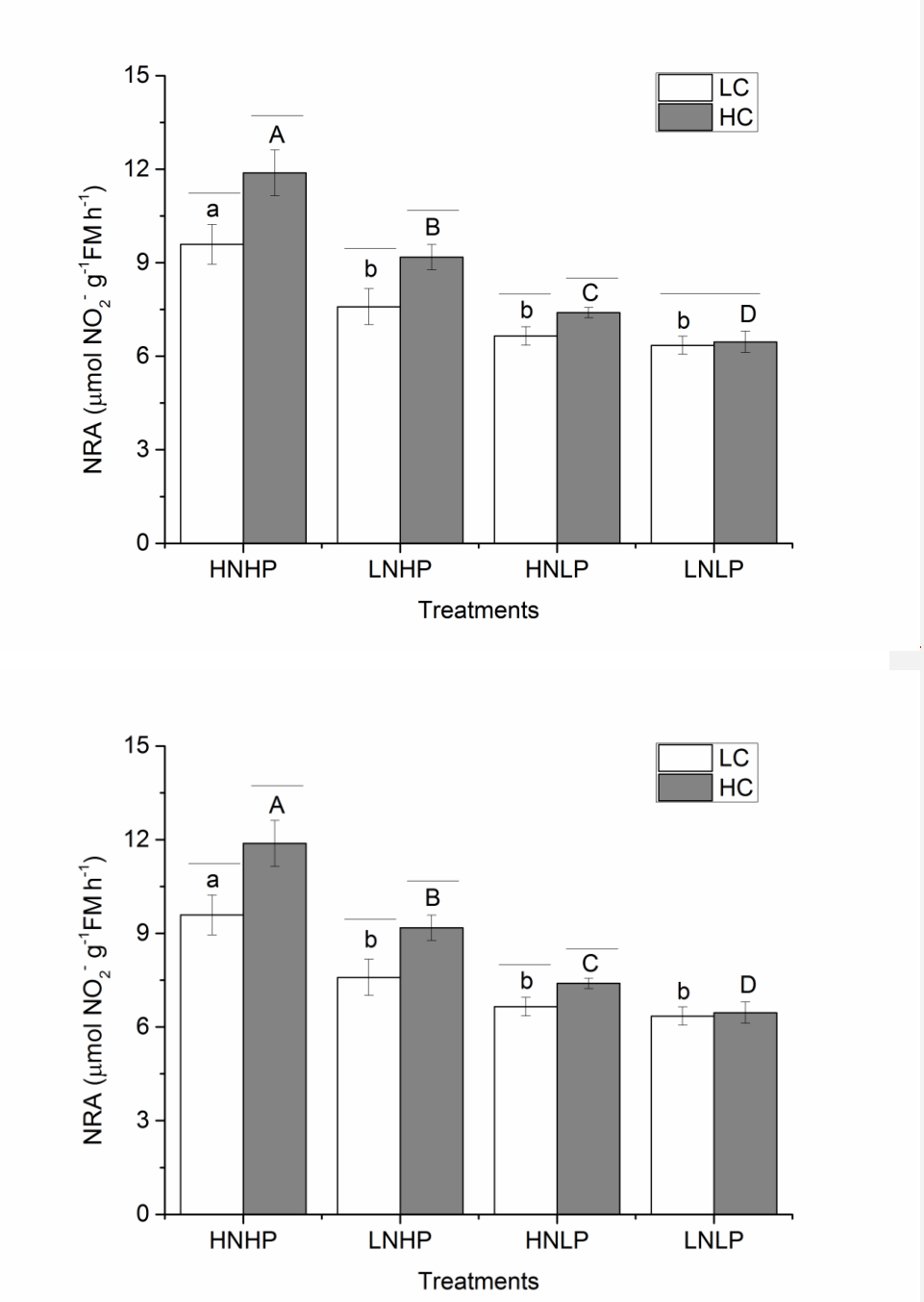
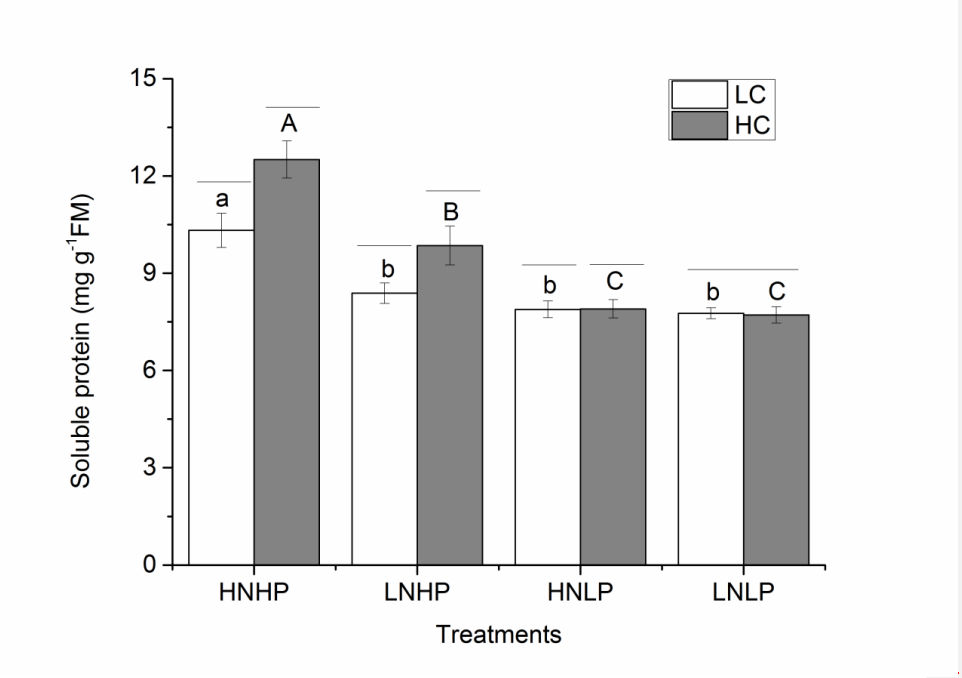

15
12
9
6
3
0
NRA (μmol NO2- g-1FM h-1)
LC
HC
A
B
C
D
a
b
b
b
HNHP
LNHP
HNLP
LNLP
Treatments

Fig. 6


15
12
9
6
3
0
Soluble protein (mg g-1FM)
LC
HC
A
a
B
b
b
C
b
C
HNHP
LNHP
HNLP
LNLP
Treatments

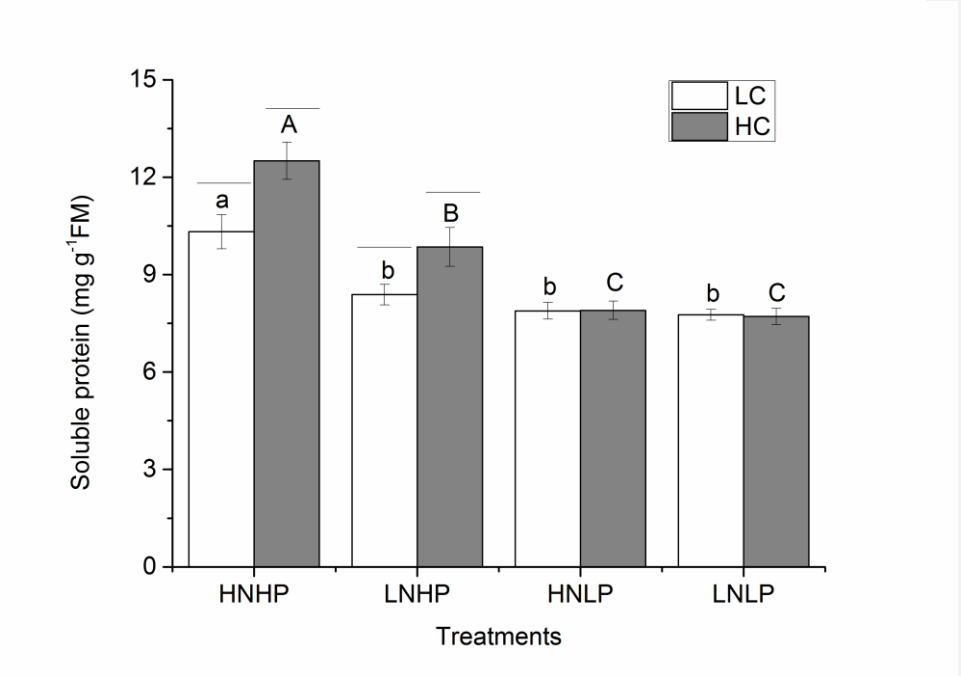

15
12
9
6
3
0
Soluble protein (mg g-1FM)
LC
HC
A
a
B
b
b
C
b
C
HNHP
LNHP
HNLP
LNLP
Treatments

Fig. 7

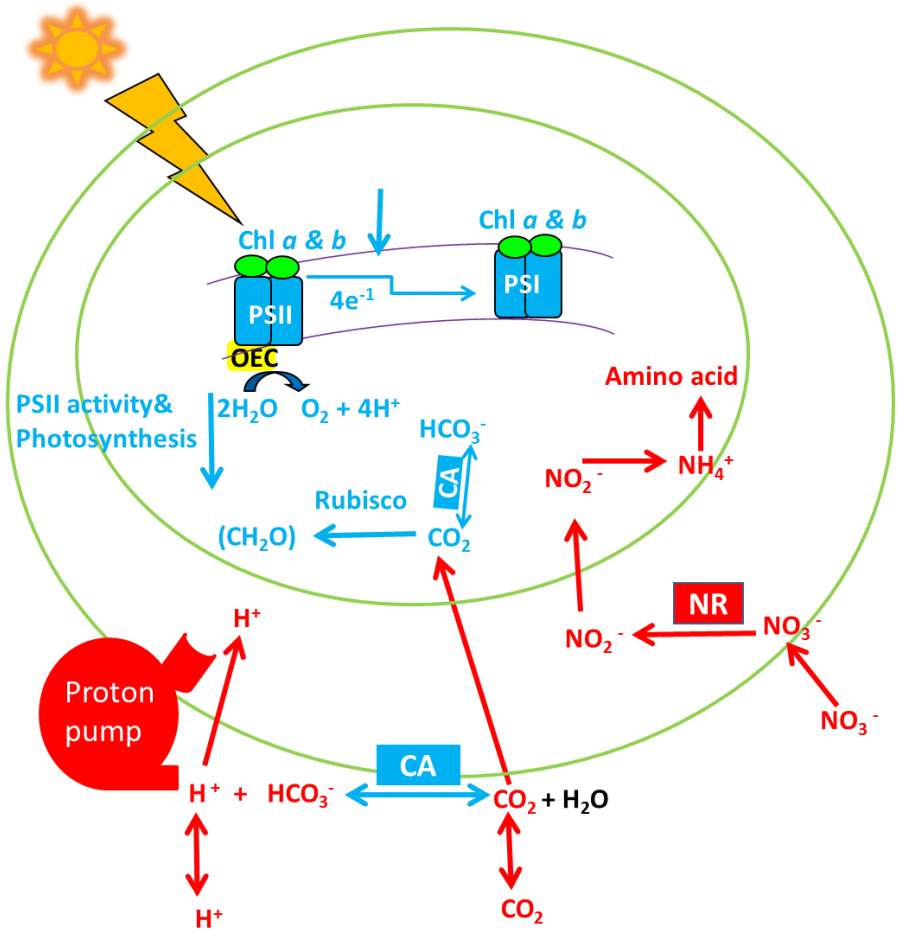


Fig. 8

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