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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* 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

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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 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 et al., 2017a) 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 effects of ocean acidification on *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 coralline crusts and the combination of the factors led to a further decrease in growth.

However, Xu et al. (2017) reported that ocean acidification and P enrichment did not enhance the growth of brown seaweed *Sargasssum muticum* further applied together, although either alone had a positive effect. The studies above indicate that the combined effects of ocean acidification and eutrophication might be species-specific.

Until now, most studies regarding the effect of ocean acidification on seaweeds have been based on nutrient replete conditions. In the field, the nutrient levels could be limiting and this is particularly true in the areas of intensive seaweed cultivation. For instance, the nitrogen and phosphorus concentrations in *Porphyra* cultivation areas could be half of those in non-cultivation areas (He et al., 2008; Wu et al., 2015). Little is known that how seaweeds grown under nutrient limited conditions respond to ocean acidification. In addition, the young and adult thalli may have differential responses to ocean acidification and nutrient limitation (Gao et al., 2017a). Therefore, here we investigated the effects of ocean acidification and nutrient limitation on the ecologically and commercially important seaweed *U. linza* at different life stages to understand how *Ulva* species respond to the combination of global climate change and local stressors.

2 Materials and methods

2.1 Sample preparation and culture conditions

To investigate whether life stage affects algal response to ocean acidification and nutrient, both spores and adults of *U. linza* were used in this study. Fertile and 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

111 °C) and taken to the laboratory within 1 h. They were then rinsed with filtered (0.2 μm)
112 natural seawater to remove any sediment and small grazers. Spores, released from the
113 fertile thalli after being exposed to high light (600 μmol) for 12 h, were allowed to
114 settle and attach to glass slides in darkness for 12 h.

115 The settled spores were cultured in four nutrient regimes (higher nitrate and
116 higher phosphate (HNHP), lower nitrate and higher phosphate (LNHP), higher nitrate
117 and lower phosphate (HNLP), lower nitrate and lower phosphate (LNLP)) and two
118 pCO_2 (400 (LC) and 1000 (HC) μatm) to explore the interactive effects of ocean
119 acidification and nutrient limitation. The treatment of LCHNHP was set as the control.
120 Twenty volumes of natural seawater (30.7 μmol L^{-1} N and 1.0 μmol L^{-1} P) were
121 diluted with 80 volumes of artificial seawater without N or P to make a LNLP
122 medium (6.1 μmol L^{-1} N and 0.2 μmol L^{-1} P). The medium for P limitation (HNLP,
123 106.1μmol L^{-1} N and 0.2 μmol L^{-1} P) was made of LNLP medium plus 100 μmol N.
124 The medium for N limitation (LNHP, 6.1 μmol L^{-1} N and 10.2 μmol L^{-1} P) was made
125 of LNLP medium plus 10 μmol P. The N&P replete medium (HNHP, 106.1 μmol L^{-1}
126 N and 10.2 μmol L^{-1} P) was made of LNLP medium plus 100 μmol N and 10 μmol P.
127 The 400 μatm pCO_2 level was maintained by bubbling ambient air, and the 1000 μatm
128 pCO_2 level was achieved using a CO_2 plant chamber (HP1000 GD, Wuhan Ruihua
129 Instrument & Equipment Ltd, China) with the variation of CO_2 less than 5% of the set
130 values. The incubation light intensity was 300 μmol photons m^{-2} s^{-1}, with a 12: 12
131 (light: dark) light period, and the incubation temperature was 20°C. The culture
132 conditions for adult *Ulva* were the same as for the spores. 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.

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

2.7 Estimation of soluble protein

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

2.8 Statistical analysis

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

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specific growth rate, net photosynthesis rate, rETR, Chl *a*, Chl *b*, soluble protein and nitrate reductase activity. 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^- but did not affect TA (Tukey HSD, $P < 0.05$). P limitation (LP) increased pCO_2 and CO_2, and reduced pH and CO_3^{2-} (Tukey HSD, $P < 0.05$).

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, $P < 0.05$). It is worth noting that LP dramatically inhibited the development of sporelings as the length under HP was 6,880−16,0290 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 or P limitation reduced growth and the combination of these nutrient limitations resulted in a further decrease (Tukey HSD, P < 0.05). In addition, young thalli had higher growth rates under each condition compared to adult plants (Paired t-test, P < 0.05).

The effects of pCO_2 and nutrients on the net photosynthetic rate of adult thalli were also investigated (Fig. 3). Both pCO_2 and nutrient had a significant effect on net photosynthetic rate (Table 5) and HC reduced NPR under each nutrient condition (Tukey HSD, P < 0.05). In terms of the effect of nutrients, LN or LP alone decreased NPR and the combination of LN and LP led to a further decrease under LC (Tukey HSD, P < 0.05). 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.

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). 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), P limitation had a larger negative effect and

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the combination of LN and LP resulted in the lowest rETR values (Tukey HSD, $P < 0.05$).

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 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, $P > 0.05$) but increased it when N or P 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, $P < 0.05$).

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). *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, $P < 0.05$) and the combination of N and P limitation did not lead to a further decrease in soluble protein content.

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

4.2 Photosynthetic response to OA and nutrients

HC was shown in the present study to decrease the Chl *a* and Chl *b* contents of *U. linza*. High CO_2 commonly down-regulates algal CO_2 concentrating mechanisms (CCMs), suggesting less energy is required to drive CCMs (Gao et al., 2012; Raven et al., 2012; Cornwall et al., 2017b; Raven et al., 2017). This may lead to decreased synthesis of pigment for energy capture. This phenomenon of `pigment economy' has also been found in our previous studies regarding *Ulva* species (Xu and Gao, 2012; 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 down-regulation of CCMs in thalli grown under HC might have reduced the intracellular CO_2 availability and have contributed to the lower net photosynthetic rate. 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.

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. 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. 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). 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 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

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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												

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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		

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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		

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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		

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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		

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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		

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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}.

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-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}. Different letters (low-case for LC and capital for HC) above the 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 rate 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}. Different letters (low-case for LC and capital for HC) above 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}. Different letters above 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}. Different letters (low-case for LC and capital for HC) above 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}. Different letters (low-case for LC and capital for HC) above 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}. Different letters (low-case for LC and capital for HC) above 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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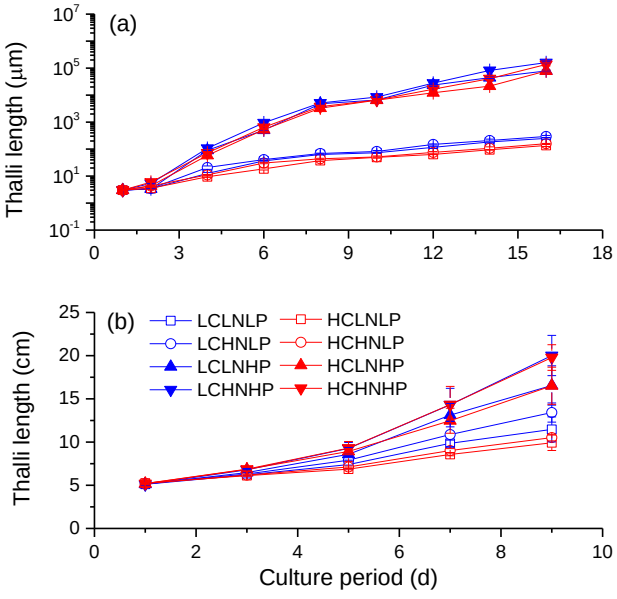

Fig. 1

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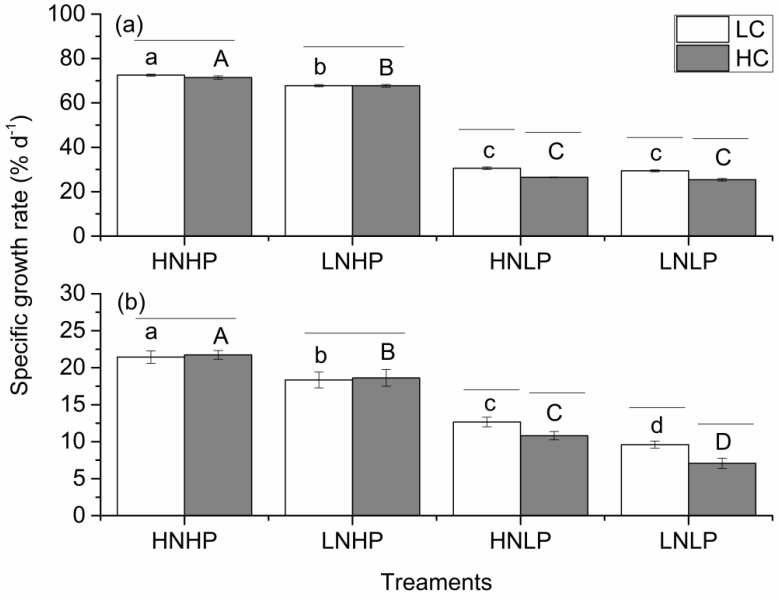

Fig. 2

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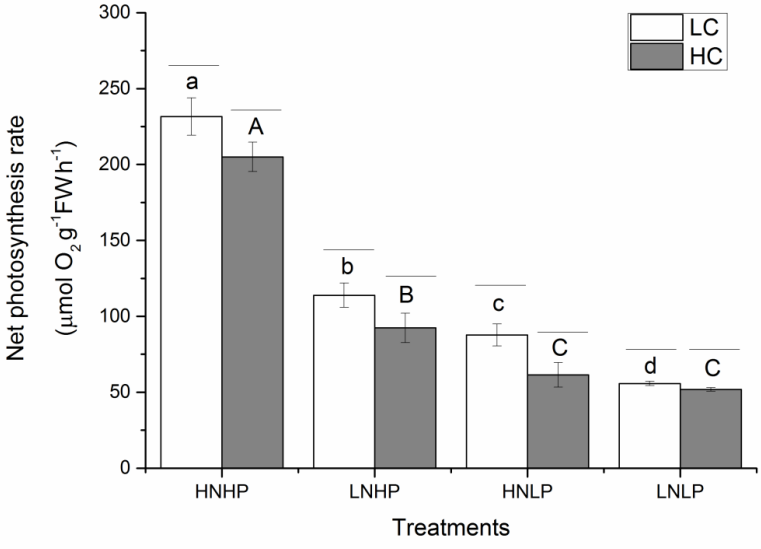

Fig. 3

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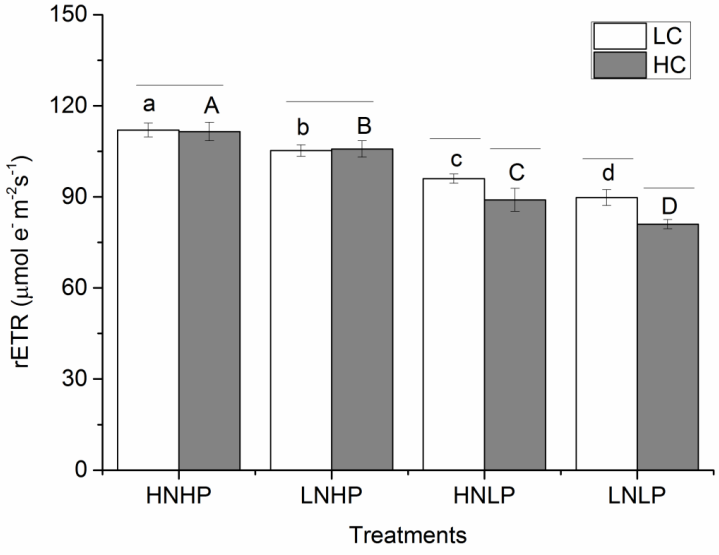

Fig. 4

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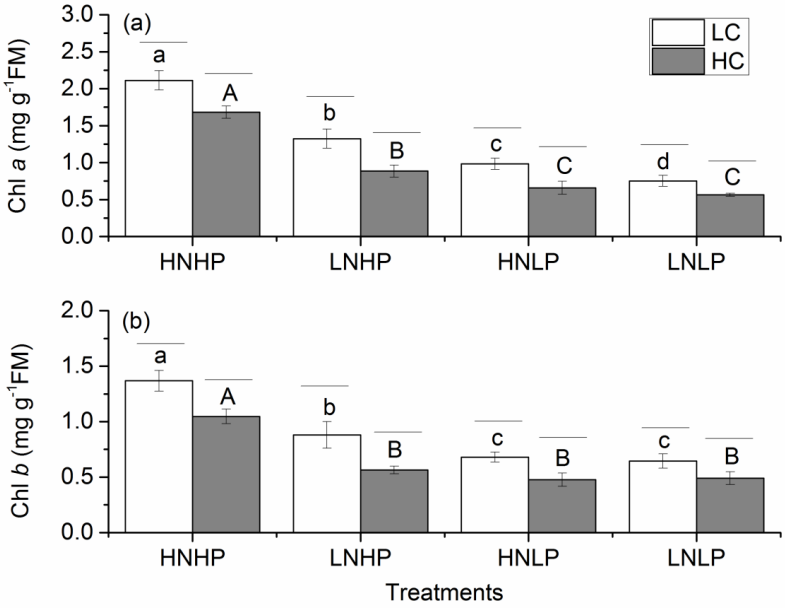

Fig.5

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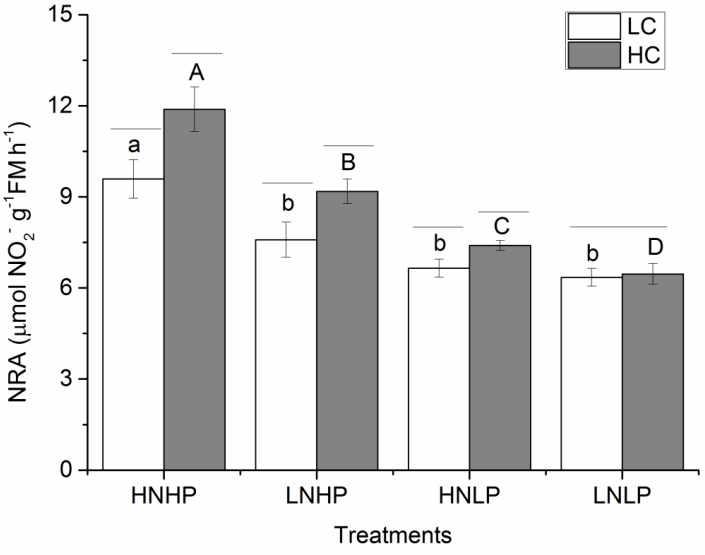

Fig. 6

Biogeosciences Discuss., https://doi.org/10.5194/bg-2018-1
Manuscript under review for journal Biogeosciences
Discussion started: 13 February 2018

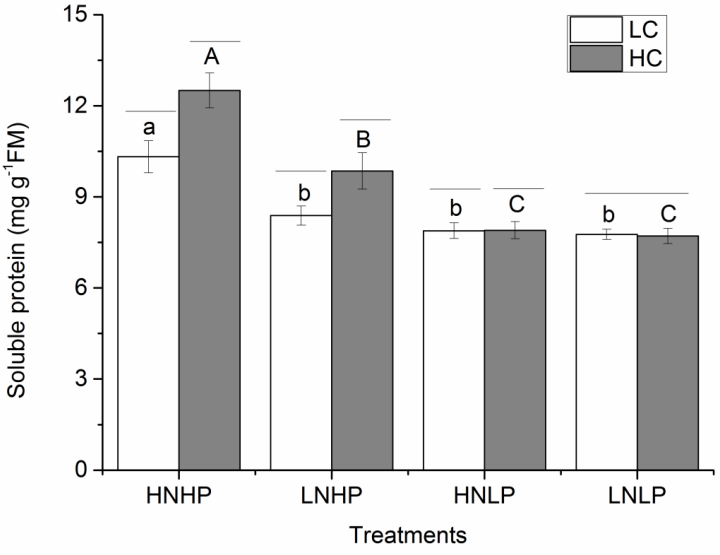

Fig. 7