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 acclimatisation to ocean acidification (Eggert, 2012; Gao et al., 2016). This information has been added to lines 147-151.


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 CO₂-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 pCO₂ levels might be also caused by varying pH as pH could affect NRA in seaweeds (Lopes et al., 1997)” at lines 369-370.


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, CO₂ 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 CO₂ 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 CO₂ 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 pCO₂ nor nutrient affected it, indicating that changes of O₂ 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 CO₂-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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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.
Ocean acidification and nutrient limitation synergistically reduce growth and photosynthetic performances of a green tide alga *Ulva linza*

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\textsuperscript{d}School of Biological Sciences, Monash University, Clayton, Victoria 3800, Australia

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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 pCO₂ (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 pCO₂ 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 pCO₂ 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
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\(_2\)S) 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 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; 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
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 *Sargassum 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
ο, 34.5 °N), Jiangsu province, China. The fronds were put into a cooling box (4–6 °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$^2$) 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 \(\text{CO}_2\)-enriched air at a rate of 300 mL min\(^{-1}\) to make the thalli roll up and down.

The culture conditions for adult \textit{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 \(\text{CO}_2\)-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 \textit{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: \( \text{SGR (\%)} = \frac{\ln M_{t2} - \ln M_{t1}}{t} \times 100 \), 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 \( 0.01 \mu\text{mol photons m}^{-2} \text{s}^{-1} \) and actinic light was set as the same as the growth light (300 \( \mu\text{mol photons m}^{-2} \text{s}^{-1} \)), respectively. The saturating pulse was set to 5, 000 \( \mu\text{mol photons m}^{-2} \text{s}^{-1} \) (0.8 s). \( \text{rETR (\mu mol e}^{- \text{m}^{-2} \text{s}^{-1}} \) = \( \frac{(F_m' - F_i)}{F_m' \times 0.5 \times PFD} \), 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 $\mu$mol 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 nm ($A_{652}$) 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:

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

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

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
solution (10 mL), which contained 0.1 M phosphate buffer, 0.1% propanol (v/v), 50 mM KNO₃, 0.01 mM glucose, and 0.5 mM EDTA, with a pH of 8.0. The mixture was flushed with pure N₂ 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 NRA activity was expressed as µmol NO₂⁻ g⁻¹ FM h⁻¹.

2.7 Estimation of soluble protein

Approximately 0.2 g of FM thallus 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 pCO₂ 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 pCO₂ and nutrient on
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 pCO₂ and nutrient treatments had a significant effect on carbonate parameters (Table 2). Elevated pCO₂ reduced pH and CO₃⁻, increased DIC, CO₂ and HCO₃⁻ (P < 0.001 for all parameters above) but did not affect TA (Tukey HSD, P < 0.7405). P limitation (LP) increased pCO₂ (P = 0.002) and CO₂ (P = 0.002), and reduced pH (P = 0.001) and CO₃²⁻ (Tukey HSD, P < 0.0465).

The length for both young and adult U. linza varied with culture time and the patterns under different pCO₂ 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 < 0.0501). 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 pCO₂ 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, $P < 0.0501$). In addition, young thalli had higher growth rates under each condition compared to adult plants (Paired t-test, $P < 0.0501$).

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) 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, $P < 0.05001$). 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). 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, $P < 0.05028$), 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, $P < 0.05001$).

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). 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, $P > 0.7005$) 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 NO₂⁻ g⁻¹ FM h⁻¹) under HCHNHP condition
(Tukey HSD, P < 0.05001).

The content of soluble protein was assayed to investigate nitrogen assimilation of
U. linza under various pCO₂ and nutrient conditions (Fig. 7). Both pCO₂ 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 < 0.05001) and the
combination of N and P limitation did not lead to a further decrease in soluble protein
content (P = 0.953).

4 Discussions

4.1 Differential response of young and adult Ulva

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

Lower P levels strongly inhibited the growth of both young and adult plants in this study and the inhibitory effect was particularly significant for young plants. Gao et al. (2017b) has reported that *U. rigida* becomes mature when the thalli reach a length of 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 CO₂ commonly down-regulates algal CO₂ 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 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 CO₂ availability and have contributed to the lower 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 pCO₂ 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.

### 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 
adacidification 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. fenestrata* (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 CO₂, 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-eutrophication. 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.

**Acknowledgements**

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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).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>$p$CO₂ (µatm)</th>
<th>pH</th>
<th>DIC</th>
<th>CO₂</th>
<th>HCO₃⁻</th>
<th>CO₃²⁻</th>
<th>TA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>(µmol kg⁻¹)</td>
<td>(µmol kg⁻¹)</td>
<td>(µmol kg⁻¹)</td>
<td>(µmol kg⁻¹)</td>
<td>(µmol kg⁻¹)</td>
</tr>
<tr>
<td>LCHNHP</td>
<td>373.8±6.3</td>
<td>8.21±0.01</td>
<td>1991.9±49.4</td>
<td>12.3±0.2</td>
<td>1805.3±43.3</td>
<td>174.2±6.1</td>
<td>2243.7±55.8</td>
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<tr>
<td>LCLNHP</td>
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<td>8.20±0.01</td>
<td>2015.1±50.4</td>
<td>12.6±0.1</td>
<td>1827.4±42.0</td>
<td>175.1±8.5</td>
<td>2267.0±60.5</td>
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<tr>
<td>LCHNLNP</td>
<td>400.4±9.0</td>
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<td>2029.9±50.1</td>
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<td>LCLNLNP</td>
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<td>13.1±0.3</td>
<td>1818.7±35.1</td>
<td>166.3±4.3</td>
<td>2226.7±42.9</td>
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<td>HCHNHP</td>
<td>929.1±24.8</td>
<td>7.86±0.01</td>
<td>2154.6±52.5</td>
<td>30.7±0.8</td>
<td>2034.9±49.5</td>
<td>89.1±2.6</td>
<td>2263.3±54.2</td>
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<td>HCLNHP</td>
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<td>7.85±0.01</td>
<td>2155.0±42.0</td>
<td>31.6±0.2</td>
<td>2036.8±38.5</td>
<td>86.5±3.6</td>
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<td>HCHNLNP</td>
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<td>HCLNLNP</td>
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<td>7.82±0.03</td>
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<td>2005.3±28.0</td>
<td>78.9±5.2</td>
<td>2199.3±37.4</td>
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Table 2. Two-way multivariate analysis of variance for the effects of pCO$_2$ and nutrient 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.

<table>
<thead>
<tr>
<th>Source</th>
<th>pH</th>
<th>DIC</th>
<th>HCO$_3^-$</th>
<th>CO$_3^{2-}$</th>
<th>CO$_2$</th>
<th>TA</th>
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<tbody>
<tr>
<td></td>
<td>df</td>
<td>F</td>
<td>Sig.</td>
<td>F</td>
<td>Sig.</td>
<td>F</td>
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<tr>
<td>pCO$_2$</td>
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<td>5237.765</td>
<td>&lt;0.001</td>
<td>57.132</td>
<td>&lt;0.001</td>
<td>158.536</td>
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<td>Nutrient</td>
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<td>0.001</td>
<td>0.747</td>
<td>0.540</td>
<td>0.741</td>
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<td>pCO$_2$*Nutrient</td>
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<td>1.294</td>
<td>0.311</td>
<td>0.256</td>
<td>0.856</td>
<td>0.332</td>
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<tr>
<td>Error</td>
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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.

<table>
<thead>
<tr>
<th>Source</th>
<th>df</th>
<th>F</th>
<th>Sig.</th>
<th>df</th>
<th>F</th>
<th>Sig.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Growth of young <em>U. linza</em></td>
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<td></td>
<td></td>
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<tr>
<td>Time</td>
<td>8</td>
<td>1153.328</td>
<td>&lt;0.001</td>
<td>4</td>
<td>571.769</td>
<td>&lt;0.001</td>
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<tr>
<td>Time*pCO$_2$</td>
<td>8</td>
<td>23.582</td>
<td>&lt;0.001</td>
<td>4</td>
<td>3.158</td>
<td>0.020</td>
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<tr>
<td>Time*Nutrient</td>
<td>24</td>
<td>457.170</td>
<td>&lt;0.001</td>
<td>12</td>
<td>28.505</td>
<td>&lt;0.001</td>
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<tr>
<td>Time*pCO$_2$*nutrient</td>
<td>24</td>
<td>10.585</td>
<td>&lt;0.001</td>
<td>12</td>
<td>0.689</td>
<td>0.756</td>
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<tr>
<td>Error</td>
<td>128</td>
<td></td>
<td></td>
<td>64</td>
<td></td>
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</table>
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.

<table>
<thead>
<tr>
<th>Source</th>
<th>pCO$_2$</th>
<th>Nutrient</th>
<th>pCO$_2$*nutrient</th>
<th>Error</th>
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<tr>
<td></td>
<td>df</td>
<td>F</td>
<td>Sig.</td>
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<td>Growth of sporeling</td>
<td>1</td>
<td>115.297</td>
<td>&lt;0.001</td>
<td>3</td>
</tr>
<tr>
<td>Growth of adult</td>
<td>1</td>
<td>20.039</td>
<td>&lt;0.001</td>
<td>3</td>
</tr>
<tr>
<td>Net photosynthetic rate</td>
<td>1</td>
<td>35.096</td>
<td>&lt;0.001</td>
<td>3</td>
</tr>
<tr>
<td>Dark respiration rate</td>
<td>1</td>
<td>2.306</td>
<td>0.148</td>
<td>3</td>
</tr>
<tr>
<td>rETR</td>
<td>1</td>
<td>14.592</td>
<td>0.002</td>
<td>3</td>
</tr>
<tr>
<td>Chl $a$</td>
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<td>85.900</td>
<td>&lt;0.001</td>
<td>3</td>
</tr>
<tr>
<td>Chl $b$</td>
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<td>71.600</td>
<td>&lt;0.001</td>
<td>3</td>
</tr>
<tr>
<td>Nitrate reductase activity</td>
<td>1</td>
<td>38.271</td>
<td>&lt;0.001</td>
<td>3</td>
</tr>
<tr>
<td>Soluble protein</td>
<td>1</td>
<td>30.212</td>
<td>&lt;0.001</td>
<td>3</td>
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</table>
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.

<table>
<thead>
<tr>
<th>Source</th>
<th>Growth of young <em>U. linza</em></th>
<th>Growth of adult <em>U. linza</em></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>df</td>
<td>F</td>
</tr>
<tr>
<td>pCO$_2$</td>
<td>1</td>
<td>115.297</td>
</tr>
<tr>
<td>Nutrient</td>
<td>3</td>
<td>12678.566</td>
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<tr>
<td>pCO$_2$*nutrient</td>
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</tr>
<tr>
<td>Error</td>
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<td></td>
</tr>
<tr>
<td>Source</td>
<td>Net photosynthetic rate</td>
<td>rETR</td>
</tr>
<tr>
<td>-----------------</td>
<td>-------------------------</td>
<td>-------</td>
</tr>
<tr>
<td></td>
<td>df</td>
<td>E</td>
</tr>
<tr>
<td>pCO₂</td>
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<tr>
<td>Nutrient</td>
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<td>493.992</td>
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<tr>
<td>pCO₂*nutrient</td>
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<td>2.619</td>
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<tr>
<td>Error</td>
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</table>

Table 5. Two-way analysis of variance for the effects of pCO₂ and nutrient on net photosynthetic rate and rETR of *U. linza*. pCO₂*nutrient means the interactive effect of pCO₂ and nutrient, df means degree of freedom, F means the value of F statistic, and Sig. means p-value.
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.

<table>
<thead>
<tr>
<th>Source</th>
<th>Chl $a$</th>
<th>Chl $b$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>df</td>
<td>F</td>
</tr>
<tr>
<td>pCO$_2$</td>
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<td>85.900</td>
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<tr>
<td>Nutrient</td>
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<td>pCO$_2$*nutrient</td>
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<td>2.440</td>
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<td>Error</td>
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</tbody>
</table>


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.

<table>
<thead>
<tr>
<th>Source</th>
<th>Nitrate reductase activity</th>
<th></th>
<th></th>
<th>Soluble protein</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>df</td>
<td>F</td>
<td>Sig.</td>
<td>df</td>
<td>F</td>
<td>Sig.</td>
</tr>
<tr>
<td>pCO$_2$</td>
<td>1</td>
<td>38.271</td>
<td>&lt;0.001</td>
<td>1</td>
<td>30.242</td>
<td>&lt;0.001</td>
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<tr>
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<td>pCO$_2$*nutrient</td>
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<tr>
<td>Error</td>
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<td>16</td>
<td></td>
<td>16</td>
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</tr>
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</table>
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 462-day). LC, 400 μatm; HC, 1000 μatm; LN, 6.1 μmol L⁻¹; LP, 0.2 μmol L⁻¹; HN, 106.1 μmol L⁻¹; HP, 10.2 μmol L⁻¹. The error bars indicate the standard deviations (n = 3). Different letters (low-case for LC and capital for HC) above the error bars represent significant differences (P < 0.05) among nutrient treatments while horizontal bars represent significant differences (P < 0.05) between LC and HC within a nutrient treatment.

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

Fig. 4. Relative electron transport rate (rETR) of adult *U. linza* grown under various
conditions. LC, 400 μatm; HC, 1000 μatm; LN, 6.1 μmol L⁻¹; LP, 0.2 μmol L⁻¹; HN, 106.1 μmol L⁻¹; HP, 10.2 μmol L⁻¹. The error bars indicate the standard deviations (n = 3). Different letters above the error bars (low-case for LC and capital for HC) represent significant differences (P < 0.05) among nutrient treatments while horizontal bars represent significant differences (P < 0.05) between LC and HC within a nutrient treatment.

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

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

**Fig. 7.** Content of soluble protein in adult *U. linza* grown under various conditions. LC, 400 μatm; HC, 1000 μatm; LN, 6.1 μmol L⁻¹; LP, 0.2 μmol L⁻¹; HN, 106.1 μmol
L$^{-1}$, HP, 10.2 μmol L$^{-1}$. The error bars indicate the standard deviations (n = 3).

Different letters (low-case for LC and capital for HC) above the error bars represent significant differences ($P < 0.05$) among nutrient treatments while horizontal bars represent significant differences ($P < 0.05$) between LC and HC within a nutrient treatment.

**Fig. 8.** Physiological responses of *U. linza* to the combination of ocean acidification and nutrient availability. The blue and the red symbols represent down- and up-regulated metabolic pathways respectively. PSI: Photosystem I; PSII: Photosystem II; OEC: oxygen-evolving complex; NPQ: non-photochemical quenching; CA: carbonic anhydrase; NR: nitrate reductase.
Fig. 1
Fig. 3
Fig. 4
Fig. 5

(a) Chl a (mg g⁻¹ FM)

(b) Chl b (mg g⁻¹ FM)
Fig. 6
Fig. 7