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Future CO_2-induced ocean acidification enhances resilience of a green tide alga to low-salinity stress

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To understand how *Ulva* species might respond to salinity stress during future ocean acidification we cultured a green tide alga *Ulva linza* at various salinities (control salinity, 30 PSU; medium salinity, 20 PSU; low salinity, 10 PSU) and CO_2 concentrations (400 and 1000 ppmv) for over 30 days. The results showed that, under the low salinity conditions, the thalli could not complete its whole life cycle. The specific growth rate (SGR) of juvenile thalli decreased significantly with reduced salinity but increased with a rise in CO_2. Compared to the control, medium salinity also decreased the SGR of adult thalli at low CO_2 but did not affect it at high CO_2. Similar patterns were also found in relative electron transport rate (rETR), non-photochemical quenching, saturating irradiance, and Chl *b* content. Although medium salinity reduced net photosynthetic rate and maximum rETR at each CO_2 level, these negative effects were significantly alleviated at high CO_2 levels. In addition, nitrate reductase activity was reduced by medium salinity but enhanced by high CO_2. These findings indicate that future ocean acidification would enhance *U. linza*'s tolerance to low salinity stress and may thus facilitate the occurrence of green tides dominated by *U. linza*.

Keywords: green tides, life cycle, ocean acidification, photosynthesis, salinity, *Ulva linza*

Introduction

Green macroalgae started to grow excessively and became a nuisance along the shores of industrialized countries in the 1970s and these outgrowths were termed green tides (Fletcher, 1996). Large-scale green tides have occurred in the coastal zones of the western Yellow Sea annually since 2008 and China has experienced the largest macroalgal blooms in the world (Liu *et al.*, 2013). Green tides have received increasing attention globally due to their ecological and economic impacts (Zhang *et al.*, 2015; Gao *et al.*, 2018). Previous studies reported that the green-tide-forming species in the Yellow Sea were *Ulva prolifera* and co-occurring *Ulva linza* (Kim *et al.*, 2011; Luo *et al.*, 2012). While the free-floating green mats off the southwest coast of Korea were mainly composed of *U. linza* (Kang *et al.*, 2014). Based on numerous hypotheses about the original sources of microscopic propagules, which include gametes, zygotes, spores, microscopic germlings and vegetative fragments in the Yellow Sea, the most widely accepted viewpoint is that they exist in marine sediments and coastal waters and provide a "seed bank" for the rapid generation of green algal blooms under the favourable conditions (Liu *et al.*, 2012; Huo *et al.*, 2014).

The coastal waters of Jiangsu province are affected by numerous freshwater inputs stemming from high rain fall during the monsoon season, leading to large variations in coastal salinities (Kakinuma *et al.*, 2006; Chen *et al.*, 2008; Kang *et al.*, 2016). Decreased salinity can influence many species of macroalgae, such as *Sargassum fusiforme* (Xie *et al.*, 2016), *Pterocladiella capillacea* (Schmidt *et al.*, 2015), and *Fucus vesiculosus* (Takolander *et al.*, 2017). The effects of salinity on *Ulva* species have also been intensively studied (Xia *et al.*, 2004; Ichihara *et al.*, 2013; Gao *et al.*, 2016b; Rybak and Gąbka, 2018). The most suitable salinity

for growth of *Ulva* spp. ranges from 16 to 40 PSU under laboratory conditions although *Ulva* spp. does show different tolerances to low salinities (Wang *et al.*, 2007; Turner *et al.*, 2008; Ichihara *et al.*, 2013; Yamochi, 2013). For instance, after 7 days of freshwater exposure, the viability of *U. linza* decreased to ∼20% while *U. prolifera* showed nearly 100% viability (Ichihara *et al.*, 2013). Rybak (2018) reported that the best conditions for development of *U. linza* was at a salinity of ∼30 PSU and this species was found in less diverse habitats, with populations restricted to sea bays, on rocky shores and in estuaries. Meanwhile, our previous work showed that *U. prolifera* has a high tolerance to low salinity and a decrease in salinity (from 30 to 10 PSU) did not affect the growth of thalli (Gao *et al.*, 2016b). In contrast, *U. prolifera* has a diverse habitat and has been found in seven different types of aquatic ecosystems (Rybak, 2018). The high tolerance to low salinities shown by *U. prolifera* may be an important factor in its ability to dominate green tides occurring in the western Yellow Sea.

Due to the expansion of industrial activities since the Industrial Revolution, atmospheric CO_2 has been continuously increasing. The oceans have absorbed approximately one-third of the atmospheric CO_2, leading to ocean acidification (Gattuso *et al.*, 2015). Based on the RCP8.5 scenario, atmospheric CO_2 could further increase up to ∼1000 ppm by the end of this century resulting in further ocean acidification (Gattuso *et al.*, 2015). Ocean acidification represents a severe challenge to the physiology and ecology of marine organisms in coastal areas (Kroeker *et al.*, 2013; Cornwall and Hurd, 2016; Kram *et al.*, 2016). The low CO_2 levels in seawater, combined with a higher half saturation constant of RuBisCO, and slow CO_2 diffusion in seawater, makes CO_2 a common limiting factor for algal growth (Raven *et al.*, 2011; Mishra *et al.*, 2018). The influence of ocean acidification and the effects of the changed seawater carbonate system on macroalgae are species-specific (Raven and Beardall, 2014). Those species lacking carbon concentrating mechanisms (CCMs) or with weak CCMs usually benefit more from ocean acidification (Raven and Beardall, 2014; Gao *et al.*, 2016a). However, despite the presence of robust CCMs, the green-tide macroalgae (*Ulva* spp.) still showed a positive response to ocean acidification, such as increased growth rate and photosynthesis under elevated CO_2 concentrations (Xu and Gao, 2012; Gao *et al.*, 2016a; Kang and Chung, 2017).

For most species of green macroalgae, their life cycle consists of two stages, the growth of microscopic propagules (gametes, zygotes, spores, microscopic germlings, and vegetative fragments) followed by a macroscopic stage (Song *et al.*, 2015a, b). The response of micro-propagules to environmental factors plays an essential role in determining the development of macroalgal blooms. These two stages respond differentially to environmental factors (Gao *et al.*, 2017a). For instance, elevated CO_2 increased the growth of juvenile *U. rigida* but did not affect growth of adult thalli (Gao *et al.*, 2017a).

The effects of either salinity or ocean acidification on *Ulva* species have been well studied however there are no reports of the combined effect of salinity and acidification on *Ulva*. The salinity of coastal waters in Jiangsu province can be as low as 10 PSU due to the Changjiang plume (Wu *et al.*, 2014). In addition, leaf-like (bistratose laminar) *Ulva* species such as *U. linza*, has been considered to be vulnerable to low salinity stress (Ichihara *et al.*, 2013; Rybak, 2018). Tolerance to salinity stress is related to photosynthesis and nitrogen assimilation (Touchette, 2007; Liang *et al.*, 2018). We therefore hypothesized that ocean acidification may enhance the resilience of *U. linza* to low salinity by stimulating its carbon and nitrogen assimilation. To test this hypothesis, we cultured *U. linza* at two concentrations of CO_2 and three different salinities and monitored the variation of growth, relative electron transport rate (rETR), non-photochemical quenching (NPQ), net photosynthetic rate (NPR), respiration rate (R_d), photosynthetic pigment content, and nitrite reductase activity. Our study provides important insights into how future climate change may interact with local stressors to influence the occurrence of green tides.

Material and methods

Algal collection and culture

The thalli of *U. linza* (8–10 cm long) were collected from *Porphyra* rafts in coastal waters of Lianyungang, Jiangsu province, China (119.3°E 34.50°N), where green tides occur. Identification of *U. linza* was first by morphology and then confirmed by DNA barcoding (Gao, 2016) because *Ulva* species have a high plasticity in their morphology (Hayden and Waaland, 2004; Kirkendale *et al.*, 2013). Healthy thalli were transported to the laboratory in a cool container (4°C) within 2 h of collection and then cleaned with 0.2 μm filtered natural seawater to remove epiphytes, sediment, and small grazers. The thalli were cultured in a glass aquarium (50 × 45 × 30 cm) at 20 ± 0.5°C and a light intensity of 240 μmol photons m^{-2} s^{-1} (light: dark = 12 h:12 h) in 0.2 μm filtered natural seawater (enriched with 60 μM NO_3^- and 8 μM PO_4^{3-}). After spores were released from the reproductive thalli, they were allowed to settle on glass slides (7.1 × 2.2 × 0.2 cm) in darkness for 12 h at a concentration of 6000 spores ml^{-1}.

The settled spores were cultured in 1 l balloon flasks (one glass slide per flask) containing 900 ml of media at three salinities (control salinity, CS, 30 PSU; medium salinity, MS, 20 PSU; low salinity, LS, 10 PSU) and two CO_2 levels (LC, 400 ppmv; HC, 1000 ppmv), with other conditions consistent with those described above. The low and high CO_2 concentrations represent the current atmospheric level and the atmospheric levels which are predicted to occur in the 2100s, respectively (Gattuso *et al.*, 2015; Gao *et al.*, 2018) and the salinities fall in the range found in the coastal waters of Jiangsu province (Wu *et al.*, 2014). The LC level was maintained by pumping ambient air from the roof of the laboratory building and the HC level was achieved with a CO_2 plant chamber (Ruihua, HP1000G-D, Wuhan, China) that can supply ambient-2000 ppm CO_2 by mixing ambient air with pure CO_2. The cultures were bubbled with ambient or CO_2-enriched air at a rate of 300 ml min^{-1} to facilitate CO_2 equilibration between the atmosphere and the medium (Gao *et al.*, 2018). The medium and low salinity seawater (20 and 10 PSU) was obtained by diluting the filtered seawater with distilled water and 60 μM NO_3^- and 8 μM PO_4^{3-} were added after dilution to avoid nutrient limitation. Salinity was checked using a portable refractometer (Tianlei, RHS-10ATC, Shanghai, China). The medium was renewed every other day and the flasks were randomly reallocated after renewing. Each treatment was carried out in triplicate. Germlings were detached from glass slides when they approached a size of 10 mm and 60 individuals were kept in a flask. To maintain a stable carbonate system in the seawater, biomass was kept <0.4 g fresh weight l^{-1} until the thalli became reproductive. The cultures were stopped when thalli became reproductive. The culture periods ranged from 26 to 36 days because thalli grown under different conditions had different generation times.

Seawater carbonate system

The seawater pH was monitored with a pH metre (Eutech Instruments, pH 700, Singapore) and total alkalinity (TA) was determined by acid–base titration (Gao *et al.*, 2019). The other parameters of the carbonate system were calculated with CO2SYS software based on the known values of TA and pH, using the equilibrium constants of K1 and K2 for carbonic acid dissociation (Roy *et al.*, 1993).

Measurement of generation span and growth

Generation span is defined as the time taken from spores settling on glass slides to the reproductive thalli releasing spores. Thalli under low salinity conditions (10 PSU) had died before they approached the adult stage, so the generation span under low salinity condition could not be calculated.

The specific growth rate (SGR) of *U. linza* at juvenile and adult stages, was calculated as follows: $SGR = 100 \times (\ln N_t - \ln N_0)/t$, where N_0 is the initial length, N_t is the length after t days. The juvenile stage means the period when the thalli grow from spores to a length of ~10 mm, and the adult stage is when the thalli grow from a length of 10 mm to a reproductive adult. During the juvenile stage, the thallus length was measured with a microscope (Leica, DM500, Germany) and 300 spores were randomly selected for measurement for each glass slide. The length of adult thalli was measured with a ruler.

The biomass of juvenile thalli was too low to carry out biochemical tests and thus only adult thalli were used. Thalli incubated under the low salinity conditions (10 PSU) died before they approached the adult stage. Therefore, parameters at low salinity conditions were not available for adult thalli.

Photosystem II (PSII) characterization

The PSII fluorescence parameters were measured using a pulse-amplitude modulated fluorometer (WATER-PAM, Walz, Germany). The rETR was calculated as follows: $rETR\ (\mu mol\ e^-\ m^{-2}\ s^{-1}) = F_v'/F_m' \times 0.5 \times PAR$, where Fv'/Fm' represents the effective PSII quantum yield, PAR is the photosynthetically active radiation, and 0.5 is a factor allowing for the fraction of the absorbed light utilized by PSII. NPQ was obtained as follows: $NPQ = (F_m - F_m')/F_m'$, where F_m is the maximum fluorescence yield after dark adaptation for 15 min and F_m' is the maximum fluorescence yield under actinic light. The rapid light curves for rETR were measured at eight different light intensities and the parameters were calculated according to the modified function from Jassby and Platt (1976): $rETR = rETR_{max} \times \tanh(\alpha \times PAR/rETR_{max})$, $I_k = rETR_{max}/\alpha$, where α represents photosynthetic electron transport efficiency (the initial slope of the curve), the $rETR_{max}$ is the maximum rETR, and I_k is saturating irradiance for maximum rETR.

Measurement of rates of photosynthesis and respiration

The photosynthesis and R_d were determined by the O_2 concentration changes in light and dark chambers. Measurement of oxygen evolution was performed using a Clark-type oxygen electrode (YSI, Model 5300, USA). The thalli were cut into 1 cm long segments and then incubated in the relevant growth conditions for at least 1 h to minimize the effect of cutting damage (Gao *et al.*, 2017b). About 0.04 g fresh weight of thalli were transferred to a chamber filled with 8 ml of cultured seawater, and the seawater was mixed using a magnetic stirring bar during the experiment. The temperature within the chamber was maintained at 20 ± 0.5°C with a light intensity of 240 μmol photons $m^{-2}\ s^{-1}$ for the measurement of NPR. The increase in the oxygen content in seawater within 5 min was defined as the NPR and the decrease in oxygen content in seawater in darkness within 10 min was defined as the R_d. The NPR and the R_d were presented as mmol $O_2\ g^{-1}$ FW h^{-1}.

Determination of photosynthetic pigments

Approximately 0.02 g FW of *U. linza* was extracted with 10 ml of absolute methanol at 4°C for 24 h in darkness. The samples were not ground as 24 h is enough to extract the pigments completely (Gao *et al.*, 2016b). The quantification of Chl *a*, Chl *b*, and carotenoid was based on the absorbance values of samples at 470, 653, and 666 nm in a UV/visible spectrophotometer (Ultrospec 3300 pro, Amersham Bioscience, Sweden) (Wellburn, 1994).

Measurement of nitrate reductase activity

Nitrate reductase activity of *U. linza* was determined based on the method of Corzo and Niell (1991). Approximately 0.1 g fresh thalli was placed into a tube containing 10 ml medium buffer (pH 7.5, phosphate buffer) and then bubbled with N_2 gas (99.999%) for 2 min to remove oxygen before being placed into a water bath (20°C) in the dark for 30 min. After incubation, 2 ml of reaction media was removed and 2 ml reaction solution (1 g sulfanilamide dissolved in 3 mM l^{-1} HCl) was added. After reacting for 5 min, 2 ml naphthyle thylenediamine was added to the reaction medium and incubated for 30 min. Finally, the absorbance values at 540 nm were recorded in a UV/visible spectrophotometer (Ultrospec 3300 pro, Amersham Bioscience, Sweden). Nitrate reductase activity was calculated as μmol NO_2^- g FW^{-1} h^{-1}.

Statistics

All data are shown as mean ± *SD* (standard deviation). The software SPSS v.21 was used in data processing and statistical analysis. The data for 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 salinity and CO_2 on seawater carbonate parameters. Two-way analysis of variance (ANOVA) was conducted to analyse the effects of CO_2 and salinity on generation span, SGR, rETR, NPQ, NPR, dark R_d, $rETR_{max}$, α, I_k, pigment content, and nitrate reductase activity. Least significant difference was used for *post hoc* analysis. A p-value <0.05 was considered as statistically significant.

Results

Carbonate system

The carbonate systems under various culture conditions were monitored (Table 1). Salinity affected all carbonate parameters (Table 2). The concentration of CO_2 in seawater increased while other parameters decreased at lower salinities (Table 2). It is worth noting that the decrease in DIC was smaller than expected. This is mainly due to incomplete purification of the distilled water and the following aeration. The DIC in the distilled water before aeration was 323 ± 38 μmol kg^{-1} and it became 418 ± 51 and 485 ± 76 μmol kg^{-1} after 24-h aeration of 400 and 1000 ppmv CO_2. Elevated atmosphere CO_2 increased the CO_2 concentration in seawater, reduced pH and CO_3^{2-}, but did not affect DIC, HCO_3^-, or TA.

Table 1. Parameters of the seawater carbonate system under ambient and enriched CO_2 levels.

	pH_{NBS}	DIC (μmol kg^{-1})	HCO_3^- (μmol kg^{-1})	CO_3^{2-} (μmol kg^{-1})	CO_2 (μmol kg^{-1})	TA (μmol kg^{-1})
CS-LC	8.14 ± 0.03^a	2481 ± 21^a	2279 ± 9^a	184 ± 13^a	18.4 ± 1.2^d	2722 ± 40^a
CS-HC	7.86 ± 0.02^c	2570 ± 93^a	2430 ± 85^a	103 ± 8^b	37.3 ± 0.4^b	2682 ± 103^a
MS-LC	8.05 ± 0.03^b	2013± 84^b	1896 ± 84^b	96 ± 2^b	21.6 ± 2.4^d	2127 ± 77^b
MS-HC	7.87 ± 0.03^c	2047 ± 47^b	1949 ± 43^b	65 ± 6^c	33.5 ± 1.6^c	2109 ± 56^b
LS-LC	7.85 ± 0.04^c	1545 ± 91^c	1481 ± 87^c	31 ± 5^d	32.7 ± 1.3^c	1560 ± 97^c
LS-HC	7.67 ± 0.02^d	1586 ± 178^c	1514 ± 172^c	21 ± 3^d	50.5 ± 3.4^a	1570 ± 179^c

TA, pH_{NBS}, and the temperature were used to derive all other parameters using CO_2 system analysing software (CO2YSY). Data are the means ± *SD* of three measurements. Different letters represent significant difference ($p < 0.05$) among the treatments.

Table 2. Two-way MANOVA for the effects of CO_2 and salinity on pH, dissolved inorganic carbon (DIC), HCO_3^-, CO_3^{2-}, CO_2, TA in the seawater.

		pH		DIC		HCO_3^-		CO_3^{2-}		CO_2		TA	
Source	**df**	***F***	**Sig.**	***F***	**Sig.**	***F***	**Sig.**	***F***	**Sig.**	***F***	**Sig.**	***F***	**Sig.**
Salinity	2	116.706	<0.001	142.283	<0.001	124.388	<0.001	382.769	<0.001	98.153	<0.001	186.113	<0.001
CO_2	1	240.941	<0.001	1.374	0.264	3.176	0.100	135.759	<0.001	301.313	<0.001	0.114	0.742
Salinity*CO_2	2	5.882	0.017	0.140	0.871	0.672	0.529	36.593	<0.001	5.423	0.021	0.092	0.913
Error	12												

Salinity*CO_2 means the interactive effect of salinity and CO_2, df means degree of freedom, *F* means the value of *F* statistic, and Sig. means *p*-value.

Generation span and growth

The generation spans of *U. linza* grown under various salinity and CO_2 conditions were recorded (Figure 1). Both salinity and CO_2 affected generation span and they had an interactive effect (Supplementary Table S1). Decreased salinity lengthened generation span at LC but it did not affect generation span at HC. High CO_2 reduced generation span although the decline at CS was not statistically significant. It is worth noting that *U. linza* did not complete its life cycle in the LS condition (10 PSU), and remained at the juvenile stage until the thalli died. Therefore, there was no data shown for LS in Figure 1.

The SGRs of *U. linza* at juvenile and adult stages under different culture conditions are shown in Figure 2. Both salinity and CO_2 had a significant effect on the SGR of juvenile *Ulva* (Supplementary Table S1). Decreased salinity noticeably reduced SGR of juvenile *Ulva*, particularly for the salinity of 10 PSU that led to a 51% decline for LC and a 42% decline for HC compared to the control salinity condition. However, high CO_2 increased SGR of juvenile thalli and the increases were 14, 14, and 35% for CS, MS, and LC, respectively. Salinity interacted with CO_2 to affect adult growth (Supplementary Table S1). Decreased salinity reduced the SGR of adult thalli in ambient air but did not affect it at elevated concentrations of CO_2.

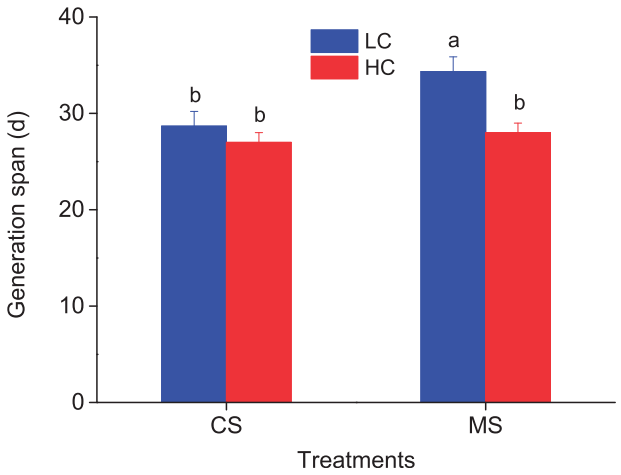

Figure 1. Generation span of *U. linza* at two levels of salinity (CS, 30 PSU; MS, 20 PSU) and CO_2 (LC, 400 ppmv; HC, 1000 ppmv). Significant differences ($p < 0.05$) among the treatments are indicated by different lowercase letters.

Fluorescence parameters

The rETR and NPQ of *U. linza* at adult stage cultured at different salinity and CO_2 conditions were measured (Figure 3). Both salinity and CO_2 affect rETR (Supplementary Table S2). Decreased salinity reduced rETR at LC but did not affect it at HC. Elevated CO_2 increased rETR at each salinity level. Salinity and CO_2 interacted on NPQ (Supplementary Table S2). Elevated CO_2 did not affect NPQ at CS but increased it at MS. The measurement of rETR under various light intensities was conducted to obtain the electron transport efficiency (α), light-saturated relative ETR ($rETR_{max}$) and saturating irradiance (Figures 4 and 5). Electron transport efficiency was not affected by salinity or CO_2 (Supplementary Table S2). $rETR_{max}$ was reduced by decreased salinity but enhanced by elevated CO_2 (Supplementary Table S3). The pattern of saturating irradiance under changing salinity and CO_2 was similar to rETR although the decrease in saturating irradiance caused by decreased salinity at HC was not statistically significant (Supplementary Table S3).

Photosynthetic and respiratory performance

The NPR and R_d of *U. linza* under different salinity and CO_2 conditions were also measured (Figure 6). Salinity and CO_2 had an interactive effect on NPR and each factor had a main effect (Supplementary Table S3). Decreased salinity reduced NPR at each CO_2 level and the inhibitory effect was more significant at LC. Salinity and CO_2 also interacted on R_d (Supplementary Table S4). Low salinity decreased R_d at LC but increased it at HC.

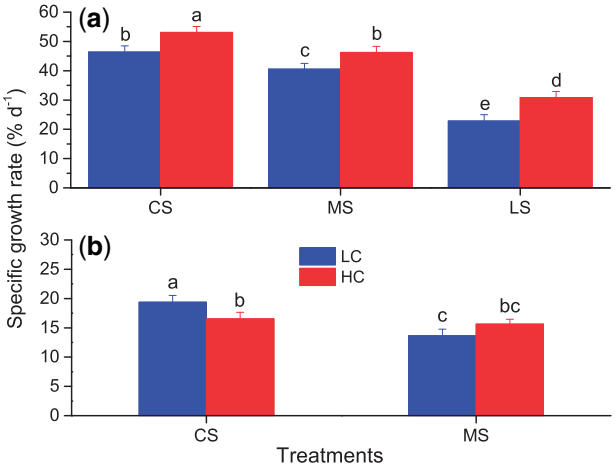

Figure 2. Specific growth rate of juvenile (a) and adult (b) *U. linza* at two levels of salinity (CS, 30 PSU; MS, 20 PSU) and CO_2 (LC, 400 ppmv; HC, 1000 ppmv). Significant differences ($p < 0.05$) among the treatments are indicated by different lowercase letters.

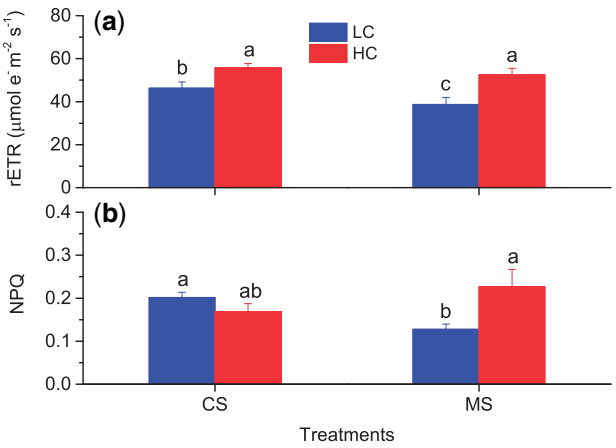

Figure 3. Relative electron transport rate (a) and NPQ (b) of *U. linza* at two levels of salinity (CS, 30 PSU; MS, 20 PSU) and CO_2 (LC, 400 ppmv; HC, 1000 ppmv). Significant differences ($p < 0.05$) among the treatments are indicated by different lowercase letters.

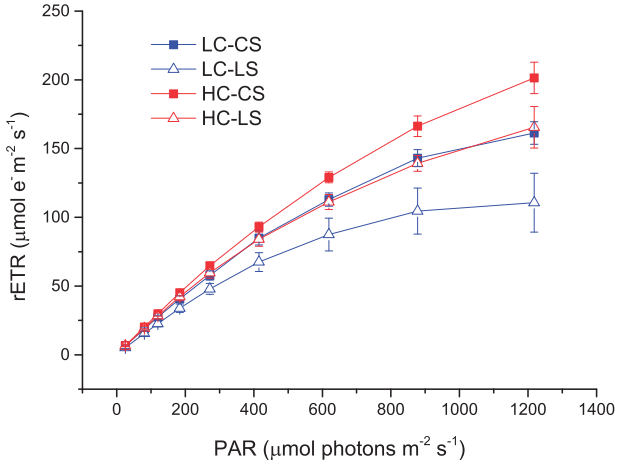

Figure 4. The rETR as a function of PAR in *U. linza* at two levels of salinity (CS, 30 PSU; MS, 20 PSU) and CO_2 (LC, 400 ppmv; HC, 1000 ppmv).

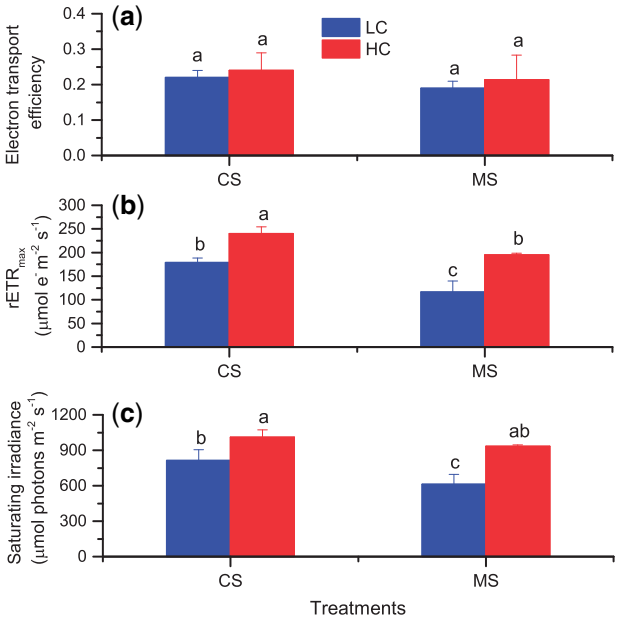

Figure 5. Electron transport efficiency (a), $rETR_{max}$ (b), and saturating irradiance (c) of *U. linza* at two levels of salinity (CS, 30 PSU; MS, 20 PSU) and CO_2 (LC, 400 ppmv; HC, 1000 ppmv). Significant differences ($p < 0.05$) among the treatments are indicated by different lowercase letters.

Pigment content

The levels of photosynthetic pigments under different culture conditions were measured (Figure 7). Salinity and CO_2 had an interactive effect on Chl *a*, Chl *b*, and carotenoid and each factor had a main effect on them (Supplementary Tables S4 and S5). Elevated CO_2 did not affect the content of Chl *a*, Chl *b*, or carotenoid in the CS condition, but increased them by 54.0, 66.2, and 54.6%, respectively in MS condition. In the LC condition, the lower salinity decreased the contents of Chl *a*, Chl *b*, and carotenoids by 50.3, 64.1, and 49.1%, respectively, while in the HC condition, the decreased salinity increased content of Chl *a* by 8% and carotenoid by 12%, with insignificant effect on the content of Chl *b*.

Nitrate reductase activity

The nitrate reductase activity of *U. linza* grown in different culture conditions is shown in Figure 8. Both salinity and CO_2 have a main effect on it (Supplementary Table S5). In the LC and HC conditions, the lower salinity significantly decreased the nitrate reductase activity by 37.5 and 34.8%, respectively. Under the CS condition, the elevated CO_2 concentration increased the nitrate reductase activity from 0.16 ± 0.013 to 0.23 ± 0.016 mmol NO_2^- g FW^{-1} h^{-1}. Under the MS condition, higher CO_2 concentration enhanced the nitrate reductase activity by 33.3%.

Discussion

Growth and generation span

Previous studies have investigated the effect of salinity (Lin *et al.*, 2011; Yamochi, 2013; Rybak, 2018) or ocean acidification

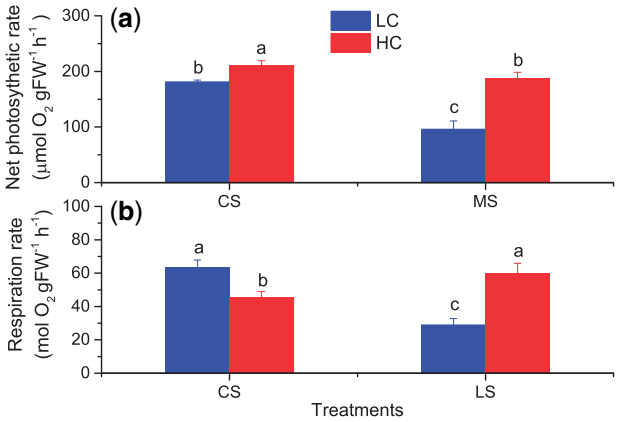

Figure 6. Net photosynthetic rate (a) and R_d (b) of *U. linza* at two levels of salinity (CS, 30 PSU; MS, 20 PSU) and CO_2 (LC, 400 ppmv; HC, 1000 ppmv). Significant differences ($p < 0.05$) among the treatments are indicated by different lowercase letters.

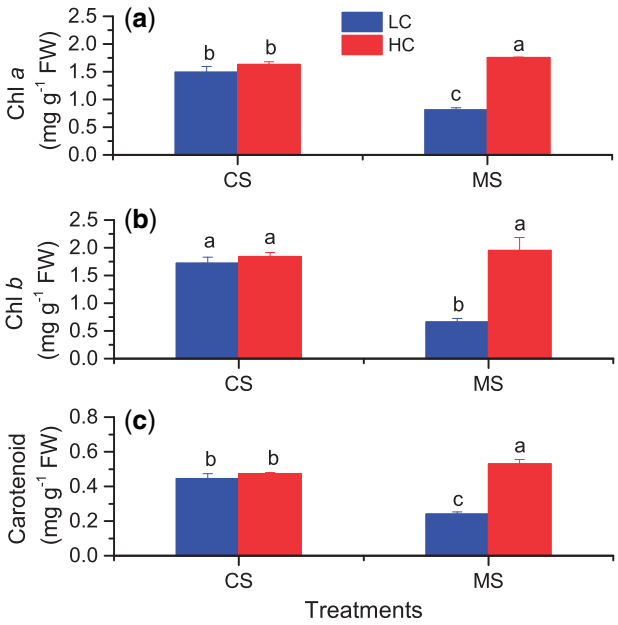

Figure 7. Chl *a* (a), Chl *b* (b), and carotenoid (c) of *U. linza* at two levels of salinity (CS, 30 PSU; MS, 20 PSU) and CO_2 (LC, 400 ppmv; HC, 1000 ppmv). Significant differences ($p < 0.05$) among the treatments are indicated by different lowercase letters.

on physiological performance in *Ulva* species (Xu and Gao, 2012; Gao *et al.*, 2016a) but little is known about how salinity affects *Ulva* species while experiencing ocean acidification. Our results demonstrate that decreased salinity reduced adult growth at ambient CO_2 conditions but did not affect it under elevated CO_2 condition. In terms of juvenile *U. linza*, decreased salinity decreased its growth at each CO_2 condition, which indicates that juvenile *U. linza* is more sensitive to decreased salinity and the stimulatory effect of elevated CO_2 cannot completely offset the negative effect of salinity. Furthermore, juvenile *U. linza* growing at low salinity (10 PSU) could not mature into the adult stage and complete its whole life cycle. This suggests that *U. linza* at very low salinity

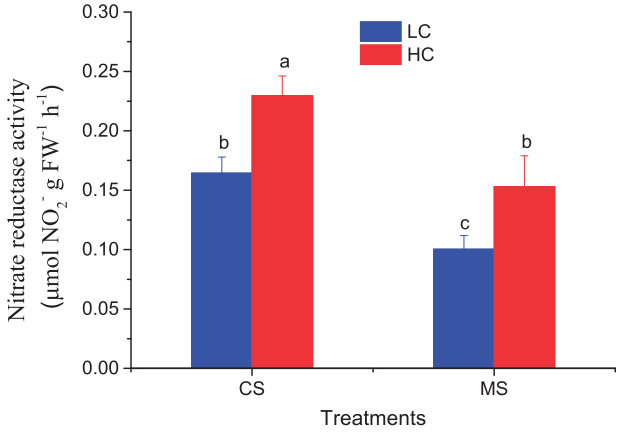

Figure 8. Nitrate reductase activity of *U. linza* at two levels of salinity (CS, 30 PSU; MS, 20 PSU) and CO_2 (LC, 400 ppmv; HC, 1000 ppmv). Significant differences ($p < 0.05$) among the treatments are indicated by different lowercase letters.

cannot proliferate by sporulation but only by asexual fragmentation.

The effect of salinity on physiological performance in *Ulva* species has been intensively investigated (Wang *et al.*, 2007; Turner *et al.*, 2008; Ichihara *et al.*, 2013; Yamochi, 2013). However, little is known on how salinity affects generation span of *Ulva* species. Our study showed that lower salinity decreased the generation span in ambient air conditions. This could be attributed to reduced carbon and nitrogen assimilation and growth at lower salinity. A longer generation span is unfavourable for rapid development of green tides as reproduction and following germling growth is an essential phase of green tides (Wang *et al.*, 2016). However, increased CO_2 reduced the generation span of *U. linza*, which is more significant under lower salinities. A shorter generation span suggests that more generations would adapt to environmental stress and may lead to a more solid dominance in coastal macroalgae communities under future predicted ocean acidification conditions (Kang and Chung, 2017; Gao *et al.*, 2018). *Ulva prolifera* is considered to be more tolerant to low salinity than *U. linza* (Ichihara *et al.*, 2013; Rybak, 2018), which may explain why *U. prolifera* can outcompete *U. linza* in the development of green tides in the western Yellow Sea. Our study shows that elevated CO_2 can offset or alleviate the negative effect of low salinity on *U. linza* and shorten its generation span. These findings indicate that the competitive situation between *U. linza* and *U. prolifera* may change in future CO_2 enriched oceans.

Carbon assimilation

The photosynthetic apparatus is considered to be one of the main targets affected during salinity stress (Gill *et al.*, 2013; Liang *et al.*, 2018). In this study, we also found that lower salinity decreased $rETR_{max}$, α, I_k, and the NPR of *U. linza*. This may be caused by less pigment synthesis at lower salinity condition, including the Chl *a*, Chl *b*, and carotenoids which serve an important role in the electron transport chain in both photosystems I and II. The damage caused by low salinity to photosynthesis is probably responsible for the decreased growth observed. In contrast to *U. linza*, it has been shown that lower salinity enhanced the growth of *U. prolifera* (Lin *et al.*, 2011; Li *et al.*, 2017). This species specific difference in responding to lower salinity may allow *U.*

prolifera to outcompete *U. linza* during the emergence of green tides in the western Yellow Sea, as the coast of Jiangsu experiences high freshwater inflows from Changjiang and the effect of this freshwater inflow often impacts hundreds of kilometres offshore (Chen *et al.*, 2008).

Little information is available on the interaction between salinity and CO_2 on physiological performance of macroalgae. Studies on higher plant show that elevated CO_2 did not significantly improve salt-induced reductions in growth or net gas exchange in olive trees although elevated CO_2 increased water use efficiency in salinized leaves and decreased salt ion uptake (Melgar *et al.*, 2008). However, decreased salinity did not affect the growth of adult *U. linza* at elevated CO_2 levels in this study, indicating that elevated CO_2 increased the tolerance of *U. linza* to low salinity. Increased CO_2 can down-regulate algal CCMs (Cornwall *et al.*, 2017) and the energy saved could be used to counteract low salinity induced stress, for example, by maintaining ion homeostasis and running antioxidant systems (Liang *et al.*, 2018). This is also supported by the dramatically enhanced NPQ and R_d s at the elevated CO_2 and decreased salinity, since NPQ and respiration are considered to be indicators of plant defence against environmental stress (Deng *et al.*, 2015; Xu *et al.*, 2015). Although the specific mechanisms regarding the enhanced tolerance to low salinity under elevated CO_2 remain unclear, our study demonstrates that elevated CO_2 increased photosynthesis, including rETR and NPR. The increased ATP generation from the linear electron flow involving PSII plus PSI combined with the energy saved from down-regulated expression of CCMs could contribute to the alleviating effect of elevated CO_2 on low salinity induced stress. Another interesting phenomenon is that elevated CO_2 did not affect photosynthetic pigment at CS but dramatically increased it at MS. Elevated CO_2 usually reduces the photosynthetic pigments of algae (Gao *et al.*, 2016a, 2018; Jiang *et al.*, 2016), termed pigment economy (Gao *et al.*, 2016a). Enhanced pigment synthesis caused by elevated CO_2 could be related to the low salinity stress response, which also contributed to increased rETR and NPR.

Nitrogen assimilation

In addition to photosynthesis, salinity also reduced the nitrate reductase activity of *U. linza* in this study. Nitrate reductase catalyses the conversion of nitrate to nitrite NO_2^-, playing an essential role in nitrogen assimilation (McCarthy *et al.*, 2017). Nitrate reductase activity was also reported to decrease with a decline in salinity (from 37 to 29 PSU) in the seagrass *Zostera marina* (Touchette and Burkholder, 2001). This was considered to be related to a Na^+-dependent NO_3^- transport system. Lower salinity could reduce the uptake of NO_3^- through the Na^+-dependent NO_3^- transport system, resulting in lower cellular NO_3^- concentration and thus lower nitrate reductase activity within cells (Touchette, 2007). However, the nitrate reductase activity in *U. prolifera* did not change when salinity decreased from 30 to 10 PSU (Gao *et al.*, 2016b), which further confirms the tolerance of *U. prolifera* to low salinity. Meanwhile, elevated CO_2 increased nitrate reductase activity, which appeared to counteract the decline caused by decreased salinity. The positive effect of CO_2 on nitrate reductase activity is related to increased nitrate reductase transcription triggered by soluble sugar produced under elevated CO_2 (Fonseca *et al.*, 1997). This is supported by our observation that elevated CO_2 enhanced carbon assimilation in *U. linza*. In addition to *U. linza*, elevated CO_2 also increased nitrate reductase activity in *U. rigida* (Gordillo *et al.*, 2001), *Hizikia fusiforme* (Zou, 2005), *Pyropia haitanensis* (Liu and Zou, 2015), *Sargassum muticum* (Xu *et al.*, 2017), and *Corallina officinalis* (Hofmann *et al.*, 2013). These results, taken together suggest a common response to elevated CO_2 of nitrate reductase activity in macroalgae.

It is worth noting that significant changes in the carbonate system also occurred along with the decline of salinity. Among these changes, elevated CO_2 and decreased pH should be the most significant parameters which affect the physiological performance of non-calcifying algae (Moss, 1972; Raven *et al.*, 2012; McMinn *et al.*, 2014). CO_2 is the only carbon substrate for RuBisCO-mediated carbon fixation and therefore elevated CO_2 usually has positive effects for algal physiological performance; however, the decreased pH usually has a negative effect on algal physiology (Goldman *et al.*, 2017; Hong *et al.*, 2017; Shi *et al.*, 2019). It is likely that the combined effect of elevated CO_2 and decreased pH can be neutral (Hong *et al.*, 2017; Gao *et al.*, 2018; Shi *et al.*, 2019), though further studies are needed to confirm this. Even if there is a net effect after combining CO_2 and pH, based on the previous studies, the effect of such small changes in CO_2 and pH can be negligible for *U. linza* compared to the dramatic decrease in salinity (Ichihara *et al.*, 2013; Gao, 2016; Gao *et al.*, 2018).

Conclusions

Overall, our study demonstrates that low salinity can reduce growth, carbon and nitrogen assimilation of *U. linza* but elevated CO_2 can offset or alleviate the negative effect of low salinity by enhancing carbon and nitrogen assimilation. Furthermore, elevated CO_2 can shorten the generation span of *U. linza*. These findings indicate that ocean acidification processes may increase the resilience of *U. linza* to low salinity and supply thalli with more opportunities to adapt to this environmental stress via the changes of genotype and phenotype. This study also suggests that there could be more *U. linza* dominated green tides in future CO_2-enriched oceans.

Supplementary data

Supplementary material is available at the *ICESJMS* online version of the manuscript.

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