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Diurnal pH fluctuations of seawater influence the responses of an economic red macroalga *Gracilaria lemaneiformis* to future CO_2-induced seawater acidification

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ABSTRACT

The diurnal fluctuation of pH in costal seawater in sea-farming areas is larger than that the pH reduction induced by progressive seawater acidification by the end of this century. To best understand how seawater acidification influences the primary productivity of macroalgae in coastal waters under the pH fluctuation conditions, *Gracilaria lemaneiformis*, an important economical red macroalgae, was selected in our study. pH fluctuation (pH-F, high density cultivation) and pH stabilization (pH-S, low density cultivation) conditions were set to study the effects of seawater acidification on the production of *G. lemaneiformis* experiencing different pH fluctuation conditions. The results showed that seawater acidification significantly decreased the relative growth rate (RGR) of *G. lemaneiformis* grown at pH-S condition, but the enhancement of RGR was found in the pH-F-grown thalli. The similar trends were showed in the net photosynthetic rates of *G. lemaneiformis*. The different responses of RGR and net photosynthetic rates to seawater acidification between pH-S and pH-F conditions might be attributed to different daily pH variations. Under pH-S treatment, pH values varied <0.2 units with all values <8.3, indicating a relative sufficient CO_2 supply. Therefore, the effect of seawater acidification was more determined by the negative effect of elevated acidity rather than positive effect of the increased CO_2 supply. While under pH-F level, pH values varied >0.6 units and it could reach 9.09 in the LC-grown thalli, suggesting limited CO_2 supply as CO_2 level decreases about 14.1 $\mu mol\ kg^{-1}$ (from 15.8 to 0.7 $\mu mol\ kg^{-1}$) with the increase of pH (from 8.11 to 9.09). Increased CO_2 at the seawater acidification condition could significantly relieve this carbon resource limitation, resulting in the enhancement of RGR of HC-grown plants.

Statement of relevance: It can be predicted that the carbon resource limitation for high-density cultivation of *G. lemaneiformis* will be significantly relieved by coastal ocean acidification, resulting in the production increases in the future ocean, which can provide some reference values for the aquaculture of macroalgae.

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1. Introduction

The oceans have absorbed approximately one third of the human emitted CO_2 since the beginning of the Industrial Revolution, leading to ocean acidification. Based on RCP8.5 scenario (Gattuso et al., 2015), atmospheric CO_2 concentration will reach about 1000 ppmv (parts per million by volume) by the end of this century resulting in a further decline (~0.4 units) in pH (Doney et al., 2009; Gattuso et al., 2015). The decreasing pH would alter the carbonate chemistry of seawater (Millero et al., 2009). Compared with pelagic waters, coastal waters is suggested to induce faster pH decrease due to natural pH fluctuations especially by biological activity (Cornwall et al., 2013) or eutrophication and deoxygenation owing to human's large-scale agriculture and inputs of nutrients to coastal waters (Cai et al., 2011).

Marine macroalgae, the major producers especially in temperate coastal area, play an important role in the coastal ecosystems (Koch et al., 2013). As C_3 plants, photosynthesis of most macroalgae are not saturated with current dissolved inorganic carbon concentration [DIC] though most of them can utilize HCO_3^- (Koch et al., 2013). Therefore, elevated CO_2 concentration could increase the growth of many macroalgae, such as red macroalgae *Gracilaria lemaneiformis*, *Pyropia haitanensis*, *Phaeocystis globosa* and *Neosiphonia harveyi* (Chen et al., 2015; Xu and Gao, 2015; Chen and Gao, 2011; Olischläger and Wiencke, 2013), brown alga *Alaria esculenta*, *Ecklonia radiata*, *Hizikia fusiforme* (Iñiguez et al., 2016; Britton et al., 2016; Zou, 2005) and green alga *Ulva lactuca* (Chen et al., 2015; Olischläger et al., 2013).

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However, increasing CO_2 induced ocean acidification also decreased the growth of *Gracilaria tenuistipitata* (García-Sánchez et al., 1994), *Porphyra leucosticta* (Mercado et al., 1999). The different responses of macroalgae to elevated CO_2 concentration might be attributed to the interspecific differences in responding to the positive effect of CO_2 increase and negative effect of pH decrease (Xu and Gao, 2012; Xu et al., 2017).

Compared with open ocean, macroalgae live in an environment with the highly diurnal fluctuation of pH that can exceed 1 unit (Middleboe and Hansen, 2007). The pH fluctuation is mostly induced by physiological activities of primary producers such as photosynthesis in daytime and respiration at night (Cornwall et al., 2013). pH shifts within farm area of macroalgae might be very high due to highly cultivated densities and the carbonate equilibrium was altered by the changes of pH, which can influence the marine ecosystems (Hofmann et al., 2010). However, it is seldom known regarded how diurnal pH fluctuations in costal ecosystem affect the physiology of macroalgae especially under the seawater acidification condition.

Gracilaria lemaneiformis, an important economical crop, distributes worldwide and have been farmed for years in coastal surface waters in China. This species is economically important mainly due to the production of phycocolloids that are widely used as natural food colorants, fluorescence probes, photosensitizers and in tumor photodynamic therapy (Troell et al., 1999; Wang, 2002; Zhou et al., 2013). It is also suggested to be a kind of suitable species for alleviating coastal eutrophication in China (Fei, 2004). In the process of cultivation, the pH of seawater increases with biomass density due to more CO_2 being removed by photosynthesis of the plants. In recent years, many researches focused on the physiological responses of macroalgae to seawater acidification, but most of them ignored the effect of pH fluctuations under different cultivated densities. In the present study, we selected this economic red macroalga *Gracilaria lemaneiformis* to investigate how pH fluctuations influence the production of this alga under the seawater acidification in the future.

2. Material and methods

2.1. Materials and culture condition

G. lemaneiformis was collected from the cultivated areas of Gaogong island (119.3°E, 34.5^0N), Lianyungang, Jiangsu province of China. Selected healthy thalli were cleaned with filtered natural seawater to remove epiphytes and then pre-cultured in the laboratory at 200 μmol photons m^{-2} s^{-1} (12 L:12D) and 20 °C. After 3 days, *G. lemaneiformis's* thalli were cut into small segments (approximately 1 cm length) and cultured in 500 mL glass bottles filled with filtered natural seawater (salinity 31‰, enriched with 60 μM NO_3^- and 8 μM PO_4^{3-}). The seawater medium was aerated vigorously and changed every other day. The light and temperature were set as the same as the pre-culture condition. pH fluctuation (pH-F, pH varied >0.61) and pH stabilization (pH-S, pH varied <0.2) conditions were defined by setting high and low cultivated densities: Low density (LD, about 0.15 g/L; pH-S), High density (HD, about 0.8 g/L; pH-F), only 1.5% and 4% light were cut off by self-shading for LD and HD, respectively. CO_2 levels were set up as follows: atmospheric CO_2 level (LC, 400 μ atm) and elevated CO_2 level (HC, 1000 μatm). That is, the treatments were LC-S, LC-F, HC-S, and HC-F. To maintain constant range of biomass density under different culture conditions, we adjusted the algal fresh weights for every treatment to the initial values every other day. Different CO_2 levels were attained by bubbling ambient air or enriched CO_2 from a CO_2 plant chamber (HP1000G-D, Ruihua Instruments, Wuhan, China).

2.2. Daily variation of pH

The daily pH variations of seawater under different treatments were recorded with a pH meter (pH 700, Oakton Instruments, Vernon Hills, USA) every 2 h from 8:00 am to 8:00 am on next day after the thalli acclimated to different treatments for 2 weeks.

2.3. Seawater carbonate system

To check the effect of pH fluctuation on the seawater chemistry, the concentrations of total alkalinity (TA, the equivalent sum of the bases that are titratable with strong acid) and pH changes were measured at the beginning and end of light periods, and then other parameters of the carbonate system were obtained with CO2SYS software (Lewis and Wallace, 1998) based on the known values of TA, pH, salinity, nutrients, the equilibrium constants K_1 and K_2 for carbonic acid dissociation (Roy et al., 1993), and K_B for boric acid (Dickson, 1990).

2.4. Measurement of growth

After acclimating to different treatments for 2 weeks, the relative growth rates (RGR) were attained by measuring the changes of fresh weights of the thalli in 2 days between day 14 and day 16. The RGR was calculated using the follow: $RGR = 100 \times (lnN_t - lnN_0) / t$, where N_0 is the fresh weight on day 14 and N_t is that on day 16.

2.5. Measurement of daily net photosynthetic rate

Daily net photosynthetic rates of *G. lemaneiformis* under different conditions were measured by a Clark-type oxygen electrode (YSI Model 5300, USA). The thalli were cut into about 1 cm length segments and placed in growth condition for at least 1 h to avoid the effect of the cutting damage (Zhou et al., 2016). About 0.05 g fresh weight thalli were transferred to the photosynthetic chamber containing 8 mL filtered natural seawater. The temperature was controlled at 20 °C and the irradiance was set as 200 μmol photons m^{-2} s^{-1}. The net photosynthetic rates were measured every 2 h from 9:00 am to 7:00 pm.

2.6. Chlorophyll fluorescence measurements

The rapid light curves for relative electron transport rate (rETR) were measured with a Water-PAM (Walz, Germany) at 8 different light intensities. The incubation at each light intensity was 10 s. The actinic light was set at 168 μmol photons m^{-2} s^{-1} and the saturating pulse was 3000 μmol photons m^{-2} s^{-1}. The parameters were calculated according to modified function from Jassby and Platt (1976):

$$\text{rETR}\ (\mu\text{mol e}\ \ m^{-2}\ \ s^{-1}) = Fv'/Fm' \times 0.5 \times \text{PFD}$$

where Fv′/Fm′ represents the effective *PSI*I quantum yield, PFD is the photosynthetically active photon flux density.

$$\text{rETR} = \text{rETR}_{\text{max}} \times \tanh(\alpha \times \text{PAR}/\text{rETR}_{\text{max}})$$

where rETR_{max} is the light-saturated rETR and α is the efficiency of the electron transport.

2.7. Data analysis

The data are shown as the means ± SD (standard deviation) of three replicates. The experimental data was conducted using the Origin 9.0 software, and statistical significance of the data was tested with one-way or two-way ANOVA (Tukey's post-hoc test). The significance level was set at 0.05.

3. Result

3.1. Carbonate system

Under the simulated condition for ocean acidification, the carbonate system in.

high CO_2 bubbled cultures differed significantly from that of atmospheric CO_2 treatment ($P < 0.05$) (Table 1), that is, CO_2 concentration in seawater sharply decreased with the increase of pH. Under LC-F treatment, CO_2 concentration in the end of light period was only 1/20 of that at the beginning of light period. For HC-F treatment, CO_2 concentration decreased by 80% at the end of light period. The changes of total alkalinity of all treatments were not significant ($P > 0.05$).

3.2. Daily variations of pH

pH of all treatments followed an obvious diurnal variation pattern, increasing at daytime and then declining at night. Under the pH-F condition, pH values increased sharply at daytime and showed bigger pH fluctuations than pH-S condition. At daytime, the pH of *G. lemaneiformis* cultured under pH-S condition varied <0.2 units, with the values maintaining at about 8.25 and 7.95 for the LC and HC-grown thalli, respectively. While the pH varied greatly at pH-F condition, 0.61 units change was found in HC culture and even about 1 unit was showed in LC culture (Fig. 1).

3.3. Growth

The relative growth rate (RGR) of HC-grown thalli was significantly (by 36% $P < 0.05$) lower compared with the LC-grown thaill under pH-S condition. While in the pH-F level, the HC-grown ones showed higher relative growth rate (by 17% $P < 0.05$) than the LC-grown thalli. Under LC condition, pH-F significantly decreased the relative growth rate ($P < 0.05$), but it had no significant effect on the RGR of thalli grown in HC level ($P > 0.05$). There were significant interactions between CO_2 and pH fluctuation ($P < 0.05$) (Fig. 2).

3.4. Photosynthetic performance

Under pH-S condition, HC significantly decreased the net photosynthetic rate of *G. lemaneiformis* at daytime ($P < 0.05$). While it showed the different pattern when the thalli cultured in pH-F level, and HC condition reduced the net photosynthetic rate in the early morning but this trend reversed with increased illumination time ($P < 0.05$). *G. lemaneiformis* grown under pH-F conditions showed the lower net photosynthetic rate for both HC and LC treatments compared to pH-S conditions ($P < 0.05$) (Fig. 3).

When the thalli of *G. lemaneiformis* was cultured under LC-F condition, CO_2 concentrations of the seawater medium changed greatly, from 15.8 to 0.7 μmol kg^{-1}, with the pH varying up to 0.98 units (Fig. 4). The net photosynthetic rates significantly decreased from 20.3 ± 0.9 to 8.8 ± 1.3 μmol O_2 gFW^{-1} h^{-1} with the increase of CO_2 concentrations ($P < 0.05$), which followed Michaelis-Menten kinetics. While CO_2 concentrations of the seawater medium under HC-F treatment changed from 32.2 to 6.6 μmol kg^{-1} and pH varied about 0.61 units. The net photosynthetic rates increased with the increase of CO_2 concentrations but significantly decreased when the CO_2 concentration exceed 8.8 μmol kg^{-1} ($P < 0.05$) (Fig. 4).

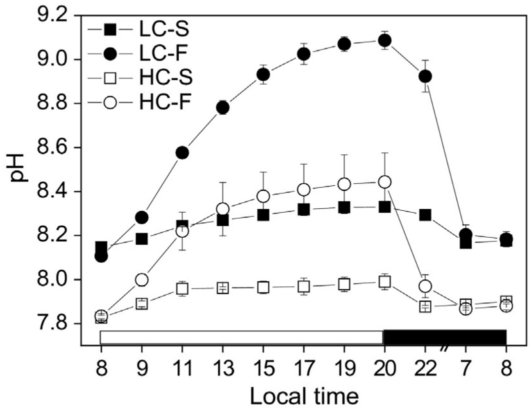

Fig. 1. Daily pH changes of *G. lemaneiformis* grown at different pH fluctuations (pH fluctuations, pH-F; pH stabilization, pH-S) and different CO_2 conditions (ambient CO_2 concentration, LC, high CO_2 concentration, HC).

When the thalli of *G. lemaneiformis* were cultured under low densities (pH-S) conditions, pH changed <0.2 units and the CO_2 concentrations varied from 14.4 to 8.6 μmol kg^{-1} for LC-grown thalli and from 32.3 to 21.7 μmol kg^{-1} for HC-grown thalli, respectively. There was a slight decrease of net photosynthetic rate (from 33.4 ± 1.7 to 22.9 ± 3.1 μmol O_2 gFW^{-1} h^{-1}) for LC-grown plant with the increase of CO_2 concentrations, but no significant differences between the net photosynthetic rate in HC-grown plant were found ($P > 0.05$).

For the light-saturated electron transport rate ($rETR_{max}$), HC also showed a significant inhibitive effect when the thalli were grown in pH-S level, but the positive effects of HC were found in pH-F-grown thalli ($P < 0.05$) (Fig. 5, Table 2). There were no significant differences between the different pH fluctuations in the LC level ($P > 0.05$), but pH-F treatment significantly enhanced the $rETR_{max}$ when the thalli were cultured under HC condition ($P < 0.05$). There were no significant differences of electron transport efficiency (α) or the light saturation point (E_k) among all the treatments ($P > 0.05$) (Table 2).

4. Discussion

The results of this study showed that CO_2-induced seawater acidification decreased the *G. lemaneiformis's* relative growth rate under pH-S condition (Fig. 2). This phenomenon might be attributed to the change of carbonate system in seawater medium (Table 1). In the condition of

Table 1
Parameters of the seawater carbonate system under the ambient (LC) and enriched CO_2 (HC) levels at the beginning and end of light periods. Total alkalinity (TA), pH_{NBS}, salinity, nutrient concentration, and temperature were used to derive all other parameters using a CO_2 system analyzing software (CO2SYS). Data are the means ± SD of 3 measurements. S and F are stable and fluctuant pH treatments, respectively; and the number 1 and 2 represent the beginning and end of light periods, respectively. Different letters represent significant difference ($P < 0.05$) among the treatments.

	pCO_2 (μatm)	pH_{NBS}	DIC (μmol kg^{-1})	HCO_3^- (μmol kg^{-1})	CO_3^{2-} (μmol kg^{-1})	CO_2 (μmol kg^{-1})	Total alkalinity (μmol kg^{-1})
LC-S1	436 ± 13^a	8.15 ± 0.01^a	2003 ± 40^{ab}	1835 ± 38^a	154.2 ± 2.6^a	14.4 ± 0.4^a	2224 ± 41^a
LC-S2	260 ± 14^b	8.33 ± 0.01 ^{b}	1890 ± 57^b	1668 ± 54^b	213.7 ± 2.6^b	8.6 ± 0.5^b	2205 ± 57^a
LC-F1	480 ± 9^c	8.11 ± 0.01^c	1997 ± 20^{ab}	1840 ± 19^a	141.0 ± 2.2^c	15.8 ± 0.3^c	2198 ± 22^a
LC-F2	22 ± 4^d	9.09 ± 0.04^d	1376 ± 46^c	794 ± 56^c	580.6 ± 14.8^d	0.7 ± 0.1^d	2248 ± 31^a
HC-S1	980 ± 44^e	7.83 ± 0.01^e	2082 ± 42^a	1971 ± 41^d	79.3 ± 1.2^e	32.3 ± 1.4^e	2167 ± 40^a
HC-S2	658 ± 67^f	8.0 ± 0.04^f	2056 ± 38^a	1922 ± 42^{ad}	112.7 ± 7.3^f	21.7 ± 2.2^f	2209 ± 28^a
HC-F1	974 ± 40^e	7.83 ± 0.01^e	2104 ± 32^a	1991 ± 31^d	81.3 ± 1.4^e	32.2 ± 1.3^e	2201 ± 30^a
HC-F2	201 ± 82^b	8.44 ± 0.10 ^{b}	1863 ± 96^b	1588 ± 145^b	267.7 ± 56.4^b	6.6 ± 2.7^b	2256 ± 42^a

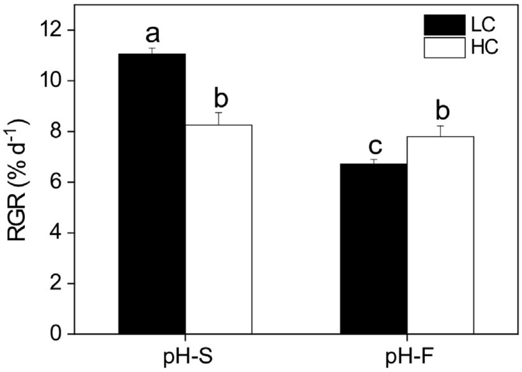

Fig. 2. Changes of relative growth rates of *G. lemaneiformis* grown at different pH fluctuations (pH fluctuations, pH-F; pH stabilization, pH-S) and different CO_2 conditions (ambient CO_2 concentration, LC, high CO_2 concentration, HC). Different letters represent the significant differences ($P < 0.05$) among the treatments.

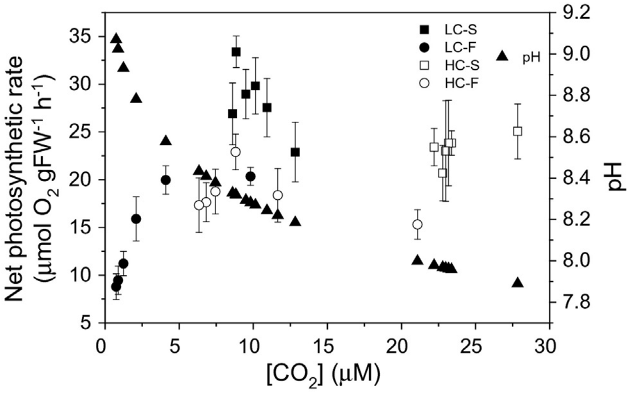

Fig. 4. The changes of pH and net photosynthetic rates of *G. lemaneiformis* grown at different pH fluctuations (pH fluctuations, pH-F; pH stabilization, pH-S) and different CO_2 conditions (ambient CO_2 concentration, LC, high CO_2 concentration, HC) with the CO_2 concentrations in the seawater medium, which derived from Fig. 1 and Fig. 3.

low densities culture, the daily pH values varied <0.2 units and thus carbonate system of seawater medium kept relatively constant. The effects of seawater acidification on the growth rate of *G. lemaneiformis* depended on the balance between acidity of decreased pH and supply of increased carbon resource. For *G. lemaneiformis*, photosynthetic rate became fully saturated in the normal natural seawater (Zou et al., 2004), so the effect of seawater acidification was almost attributed to the negative effect of decreased pH. It has been demonstrated that decreased pH can disturb the acid–base balance on the cell surface (Flynn et al., 2012) and cells trend to allocate additional energy to combat this perturbation, leading to the negative effect on growth (Gao et al., 2017; Xu et al., 2017). The negative effects of decreased pH on growth rates were also found in other red macroalgae, such as *Gracilaria tenuistipitata*, *Porphyra linearis* and *P. leucosticta* (Garcı́a-Sánchez et al., 1994; Israel et al., 1999; Mercado et al., 1999). The negative effect of seawater acidification on the *G. lemaneiformis's* relative growth rate in this study should be due to the decreased daily photosynthetic rate under HC and pH-S conditions (Figs. 3, 4). The increased CO_2 also reduced the photosynthetic rate in *Chlamydomonas acidophila*, which was attributed to reduced intracellular Ci (dissolved inorganic carbon) pools at higher CO_2 conditions (Spijkerman, 2011; Raven et al., 2012).

In the condition of pH-F culture, the daily pH value varied in the range of 0.61–0.99 units, which is greater than the variability of pH in coastal oceans (about 0.3–0.5 units) (Hofmann et al., 2011; Mercado and Gordillo, 2011; Duarte et al., 2013). Such a large range of pH can result from the CO_2 removal by photosynthesis of *G. lemaneiformis* during the daytime and CO_2 release by dark respiration, which was termed metabolic signal (Duarte et al., 2013). *G. lemaneiformis* that possesses an indirect HCO_3^- use mechanism (Zou et al., 2004) showed high photosynthetic rate in normal seawater (pH around 8.2) but the photosynthetic rate sharply decreased with increase pH due to poor function of external CA at high pH (Axelsson et al., 1995; Larsson et al., 1997). The photosynthetic rate in HC-grown thalli with high densities increased with pH but it decreased with the increase of pH in the LC-grown plants with high densities (Fig. 3). This different response of photosynthesis to the increased pH might be the result of different range of pH changes. For LC-grown thalli, pH of seawater medium varied from normal pH to high pH (from 8.11 to 9.09), leading to dramatic decline of CO_2 (Fig. 4), and therefore the photosynthetic rate was reduced. While pH changed from low pH to normal pH (from 7.83 to 8.44) in the seawater medium of HC-grown thalli, the decrease of CO_2 was relatively small and the negative effect of acidity on photosynthetic rate became low resulting in increased photosynthetic rate. Our findings indicate that the effect of seawater acidification on photosynthesis of *G. lemaneiformis* under high cultivated density depends on the levels of pH variability.

Similar to the trend of growth under different treatments, the higher pCO_2 treatment decreased the alga's $rETR_{max}$ at pH-S condition, but increased it at pH-F condition. Elevated levels of pCO_2 are known to down-regulate algal CCMs and thereby save the operational energy (Zou et al., 2011; Gao et al., 2016; Xu et al., 2017). At high light intensity, the saved energy combined with excess light energy could impair the PS II and the relative electron transport rate (Gao et al., 2012). This could

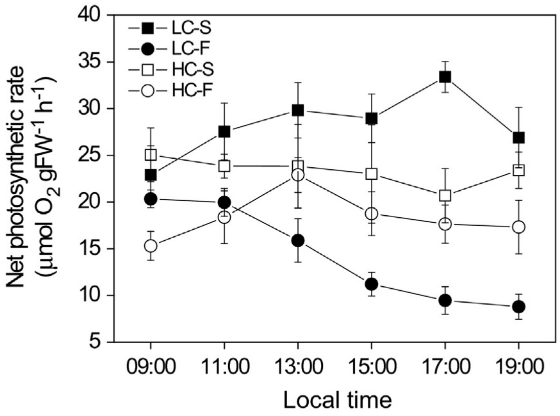

Fig. 3. Daily changes of net photosynthetic rate of *G. lemaneiformis* grown at different pH fluctuations (pH fluctuations, pH-F; pH stabilization, pH-S) and different CO_2 conditions (ambient CO_2 concentration, LC, high CO_2 concentration, HC).

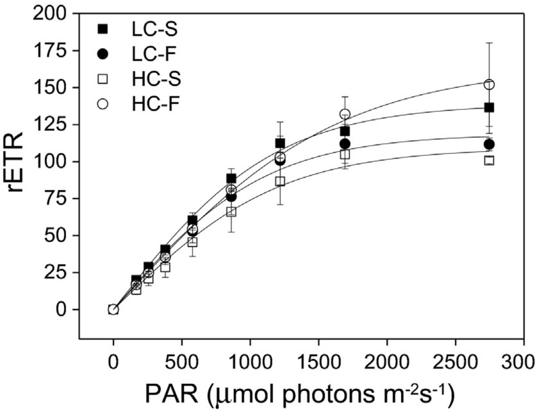

Fig. 5. The relative electron transport rate (rETR) of PAR in the *G. lemaneiformis* grown at different pH fluctuations (pH fluctuations, pH-F; pH stabilization, pH-S) and different CO_2 conditions (ambient CO_2 concentration, LC, high CO_2 concentration, HC).

Table 2

Photosynthetic parameters of rETR as a function of PAR (Fig. 4) in *G. lemaneiformis* grown at different pH fluctuations (pH fluctuations, pH-F; pH stabilization, pH-S) and different CO_2 conditions (ambient CO_2 concentration, LC, high CO_2 concentration, HC). Data are the means ± SD, different letters represent the significant differences ($P < 0.05$) among all the treatments.

Treatments	rETR $_{max}$	α	E_K (μmol photons m^{-2} s^{-1})
LC-S	140.1 ± 19.6^{ab}	0.118 ± 0.01^{a}	1196.3 ± 147.8^{a}
HC-S	111.1 ± 1.5^{c}	0.09 ± 0.02^{a}	1277.5 ± 309.9^{a}
LC-F	119.6 ± 7.4^{bc}	0.106 ± 0.02^{a}	1146.6 ± 173.4^{a}
HC-F	169.3 ± 37.2^{a}	0.1 ± 0.005^{a}	1676.1 ± 444.1^{a}

explain the decreased $rETR_{max}$ caused by elevated pCO_2 at pH-S condition since the $rETR_{max}$ was obtained at high light intensity. The reason that elevated pCO_2 increased $rETR_{max}$ at pH-F condition could be that the saved energy from down-regulated CCMs was used to increase CO_2 or bicarbonate acquisition at higher pH during daytime. On the other hand, elevated pCO_2 did not affect α or E_k, indicating the effect of elevated pCO_2 on photochemical parameters may not be significant at lower light intensity.

Cultivated density plays a very important role in the production of economic macroalgae (Nagler et al., 2003), and our results showed that high density significantly decreased the growth rate of *G. lemaneiformis* under LC treatment (Fig. 2), which can be attributed to the self-shading, carbon resource or nutrients supply limitation. But when the thalli were cultivated in high CO_2 level, this inhibition of high density was not found. That is, the negative impact of high cultivated density might be relieved by increased CO_2 supply. That resulted in a kind of dynamic balance in *G. lemaneiformis* grown in HC level, which was shown as relatively stable growth rates regardless of cultivated densities. Predictably this balance might be destroyed if the cultivated densities were further enhanced.

In the natural condition, increasing atmospheric CO_2 might decrease the production of *G. lemaneiformis* in low stocking density at early period of cultivation, but the thalli can benefit from CO_2 rise with the increasing weight. Other marine environment factors can also affect the production of *G. lemaneiformis*, such as depths (Xu and Gao, 2008), nutrients (Xu et al., 2010; Yang et al., 2006), solar UV radiation (Zheng and Gao, 2009; Xu and Gao, 2010; Gao and Zheng, 2010). To further understand how seawater acidification affects the production of *G. lemaneiformis* in the future ocean, field studies with a more extended study period should be conducted.

Author contributions

L.Q. J.S. and X.L. contributed to carry out the experiments. J.X. and K.G. contributed to experimental designs, data analysis and writing the paper.

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