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Expected CO_2-induced ocean acidification modulates copper toxicity in the green tide alga *Ulva prolifera*

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ABSTRACT

Cu is considered to be toxic to macroalgae at higher levels. Ocean acidification can also alter the physiological performances of macroalgae. However, little is known regarding the interactive effects of Cu and ocean acidification on macroalgae. In this study, a green tide macroalga, *Ulva prolifera*, was cultured at the conditions of three levels of Cu (control, 0.5 μM, and 2 μM) and pCO_2 (ambient, 1000 μatm, and 1400 μatm) to investigate the responses of *U. prolifera* to interaction of Cu exposure and ocean acidification. The relative growth rate of thalli decreased with the rise of Cu for all pCO_2 conditions except the 1000 μatm pCO_2. Compared with the control, 2 μM Cu reduced the net photosynthetic rate for all pCO_2 conditions while 0.5 μM Cu only reduced it at 1400 μatm pCO_2. The inhibition rate of Cu on the relative growth rate and net photosynthetic rate was reduced at 1000 μatm pCO_2 but was magnified at 1400 μatm pCO_2. Contrary to growth, the dark respiration rate was enhanced by 0.5 μM Cu at ambient pCO_2 and by 2 μM Cu at ambient and 1000 μatm pCO_2, although it was reduced by 2 μM Cu at 1400 μatm pCO_2 compared to the control. The 0.5 μM Cu did not affect the relative electron transport rate (rETR) for any pCO_2 condition but 2 μM Cu decreased it for all pCO_2 conditions except 1000 μatm pCO_2. The mute effect of 0.5 μM Cu on the net photosynthetic rate and rETR at ambient pCO_2 may be due to more Chl *a* and Chl *b* being synthesized. In addition, 2 μM Cu and 1400 μatm pCO_2 led to branched thalli, which may be a defense mechanism against the stress of high Cu and pCO_2. Our data, for the first time, demonstrate that a modest increase of pCO_2 can alleviate the toxicity of Cu to *U. prolifera* whilst a further increase exacerbates it. *U. prolifera* can respond to the stress of Cu pollution and ocean acidification via physiological and morphological alterations.

1. Introduction

Heavy metals, generally defined as metals or metal elements with a specific density more than 5 g cm^{-3} (Jarup, 2003), e.g., copper (Cu), chromium (Cr), cadmium (Cd), zinc (Zn), lead (Pb) and mercury (Hg), are one of the most common types of estuarine and coastal contaminant (Bryan and Langston, 1992; Milan et al., 2016). They have been posing a huge threat to fauna and flora (Kumar et al., 2014; Lewis et al., 2016; Roberts et al., 2013). Among them, Cu, which is abundant in wastewaters due to domestic and industrial activities and has commonly displayed high levels in coastal waters, is one of the most studied metals (Moenne et al., 2016). It has been generally considered that photosynthetic apparatus is the primary target of damage caused by Cu for plants (Kumar et al., 2014; Rocchetta and Kupper, 2009). Copper can damage both electron donors and acceptors of photosynthetic electron transport and thus inhibit the primary photochemical reaction (Küpper et al., 2002; Patsikka et al., 1998). Therefore, copper can generally reduce algal growth (Moenne et al., 2016 and the reference therein). In addition, copper can inhibit the synthesis of D1 protein in PSII, thereby hindering the recovery from photoinhibition (Vavilin et al., 1995).

The atmospheric concentration of carbon dioxide increased by 40% to 391 ppm between 1750 and 2011 due to human activity; a rate of increase that is unprecedented within at least the last 800,000 years (IPCC, 2013). When CO_2 dissolves in seawater, it forms carbonic acid and as more CO_2 is taken up by the ocean's

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surface, the pH decreases, moving towards a less alkaline and therefore more acidic state, termed ocean acidification. The mean surface ocean pH has already decreased by 0.1 units since the beginning of the industrial era, corresponding to a 26% increase in hydrogen ion concentration (IPCC, 2013).

Ocean uptake of anthropogenic CO_2 will continue, leading to the increase of ocean acidification (IPCC, 2013). Maroalgae have developed multiple strategies in inorganic carbon (Ci) acquisition, with different carboxylation efficiencies associated with different photosynthetic affinities for Ci (Giordano et al., 2005; Zou and Gao, 2010). Most macroalgae can take up HCO_3^- and/or CO_2 by active transport (termed carbon concentrating mechanisms, CCMs), while a few red and green macroalgae acquire Ci solely by diffusion of dissolved CO_2 (Raven et al., 1995; Hurd et al., 2009). Therefore, the influence of ocean acidification and a changed seawater carbonate system on macroaglae is species-specific. For instance, increasing atmospheric CO_2 concentrations have been shown to promote the growth of the red algae *Porphyra yezoensis* (Gao et al., 1991), *U. prolifera* (Xu and Gao, 2012) and *Lomentaria articulata* (Kübler et al., 1999). However, neutral effects of ocean acidification on photosynthesis and growth in *U. rigida* (Rautenberger et al., 2015) and a giant kelp *Macrocystis pyrifera* (Fernández et al., 2015) were also reported. Furthermore, reduced growth rates under high CO_2 concentrations were observed in *Gracilaria tenuistipitata* (García-Sánchez et al., 1994), *Porphyra linearis* (Mercado et al., 1999), and *Fucus vesiculosus* (Gutow et al., 2014).

Neither ocean acidification nor heavy mental pollution occurs in isolation; rather they are proceeding simultaneously, especially in coastal areas. Furthermore, ocean acidification not only alters the seawater carbon chemistry but also the bioavailability of water-borne metals. For instance, the inorganic speciation of copper is dominated by the complexation to $CO_3^{2-}$and OH^- which will be reduced by decreased pH and ocean acidification (Byrne et al., 1988; Richards et al., 2011). Consequently, the toxic free-ion concentration of copper is predicted to increase by as much as 115% in coastal waters over the next 100 years due to the projected decline in pH (Richards et al., 2011). Therefore, greater metal toxicity in organisms has been predicted in the context of ocean acidification and this hypothesis is supported by the findings in increased bioaccumulation of metals in the squid *Loligo vulgari* (Lacoue-Labarthe et al., 2011) and increased toxicity of metals to amphipod *Corophium volutator* (Roberts et al., 2013) and the green algae *Chlorella vulgaris* (Král'Ová et al., 2004; Rai et al., 1981) under conditions of elevated pCO_2. On the other hand, increased pCO_2 and decreased pH could also alleviate the toxicity of heavy metals to algae (Franklin et al., 2000; Peterson et al., 1984). For instance, the toxicity of Cd to phosphate uptake in *Scenedesmus quadricaerda* decreased strongly with declining pH over a range of 5.5–8.5 and Cu toxicity also decreased when the pH changed from 6.5 to 5.8 (Peterson et al., 1984). The concentrations of copper and uranium required to inhibit growth rate of *Chlorella* sp. by 50% increased from 1.5 to 35 $\mu g\,L^{-1}$ and 44 to 78 $\mu g\,L^{-1}$ respectively as the pH decreased from 6.5 to 5.7 (Franklin et al., 2000). The different effects of ocean acidification on the toxicology of heavy metal to organisms remain unknown.

Marine macroalgae, mainly inhabiting the intertidal and subtidal zones of coastal waters, are economically significant in the industries of food, pharmaceutical, chemical, etc. As an important part of global primary producers, they also play a key role in the marine biological CO_2 pump and the coastal carbon cycle (Ji et al., 2016). Among them, *Ulva* is cosmopolitan and the only genus causing green tides (Fletcher, 1996; Smetacek and Zingone, 2013). Therefore, the effects of copper on *Ulva* have been widely studied. Maximal chlorophyll fluorescence (Fv/Fm) and maximum electron transport rate (ETR_{max}) in *U. pertusa* decreased with the increase of Cu concentration (0.125–1 $mg\,L^{-1}$) (Kumar et al., 2009). The gross photosynthetic rate in *U. flexuosa* also decreased from 23.68 ± 4.95 to -0.99 ± 0.25 mg O_2 $mg\,FW^{-1}$ h^{-1} when the Cu concentration increased from 50 to 250 $\mu g\,L^{-1}$ (Andrade et al., 2004). Han et al. (2008) compared copper (Cu) toxicity to *U. pertusa* and *U. armoricana* and found the maximum efficiency of photosystem II, maximum electron transport rate and non-photochemical quenching in *U. armoricana* were not affected by Cu except at the highest concentration (250 $\mu g\,L^{-1}$) whilst *U. pertusa* showed a noticeable decrease in those parameters at much lower Cu concentrations. Baumann et al. (2009) investigated the effects of five metals, copper (Cu), chromium (Cr), Zinc (Zn), cadmium (Cd) and lead (Pb), on photosynthetic activity in seven species of green, red and brown macroalgae, in which *U. intestinalis* accumulated the highest amounts of all metals, and the metals were accumulated in the order of Cu > Pb > Zn > Cr > Cd.

A few studies have been conducted to investigate the effects of ocean acidification on the physiological traits of *Ulva* species, such as photosynthesis and growth (Xu and Gao, 2012; Rautenberger et al., 2015). Growth of *U. prolifera* was significantly enhanced by high pCO_2 (1000 μatm) after the thalli had acclimatized to the pCO_2 levels for more than 50 days (Xu and Gao, 2012). Meanwhile, neutral effects of elevated pCO_2 on *U. rigida* were reported (Rautenberger et al., 2015). The photosynthesis and growth of *U. rigida* were not stimulated by increased pCO_2 as *U. rigida* was inorganic carbon saturated (Rautenberger et al., 2015).

The studies in regard to the combined effects of copper and ocean acidification have focused on marine animals and microalgae. To the best of our knowledge, none of the previous studies has referred to macroalgae. Based on the previous studies, we hypothesized that the response of macroaglae to Cu is pCO_2-dependent. Specifically, moderate increase of pCO_2 can alleviate the toxicity of Cu because of the competition mechanism between H^+ and Cu^{2+} at the cell surface but further increase of pCO_2 would magnify the toxicity of Cu due to greater Cu^{2+} availability in the seawater and also the harm caused by decreased pH. To test the hypothesis, the physiological and morphological performances of a green tide alga *U. prolifera* cultured under the conditions of three levels of Cu and pCO_2 were examined. Our findings could provide insight into how simultaneous changes in two of the most environmentally significant factors influence this ecologically important marine macroalga and hence the evolution of green tides.

2. Materials and methods

2.1. Thalli collection and culture conditions

The *U. prolifera* that caused the largest macroalgal bloom in the world (Liu et al., 2013) were collected from the coastal water of Lianyungang (119.3°E, 34.5°N), Jiangsu province, China, in March 2013. The healthy thalli were selected, transported to the lab in a cooling box (4–6 °C) within one hour and rinsed with 0.2 μm filtered and autoclaved seawater to remove sediments, epiphytes and small grazers. The thalli were pre-incubated in an intelligent illumination incubator (Jiangnan GXZ-300C, Ningbo, China) for three days before the experiment. The temperature in the incubator was set as 20 °C with a 12 h: 12 h (light/dark) photoperiod of 240 μmol photons $m^{-2}\,s^{-1}$ photosynthetically active radiation (PAR). The temperature and light dose were close to the conditions at the sampling site.

After the pre-incubation, the thalli were placed in 500 ml polycarbonate flasks (one thallus per flask) and cultured at fully crossed three total Cu (control, LCu; 0.5 μM, MCu; 2 μM, HCu) and three pCO_2 (390 μatm, LC; 1000 μatm, MC;1400 μatm, HC) levels with continuous aeration for two weeks. The 1000 μatm pCO_2 is already a high level and 1400 μatm is a very high level. Here, we

used MC and HC to respectively represent them only for a good correspondence between the pCO_2 and Cu. Media were made from natural seawater with the addition of 60 μM $NaNO_3$ and 8 μM KH_2PO_4. The natural seawater without the addition of $Cu_2SO_4 \cdot H_2O$ (Sigma) was regarded as the control (LCu) and the Cu concentration was 0.02 μM in the natural seawater. The Cu concentrations in seawater for control and treatments were measured by an atomic absorption spectrophotometer (AA240, VARIAN, USA).The media were replaced every two days. The procedures of Cu test conformed to the trace metal clean protocols (Leal et al., 2016). The 390 μatm pCO_2 was achieved by bubbling ambient air, and the higher pCO_2 levels were made by CO_2 plant chambers (HP1000 G-D, Wuhan Ruihua Instrument & Equipment Ltd, China) with the variation of CO_2 less than 5%. The corresponding pH in seawater for three levels of pCO_2 (390 μatm, LC; 1000 μatm, MC; 1400 μatm, HC) were 8.16, 7.80, and 7.69 respectively, measured by a pH meter (pH 700, Eutech Instruments, Singapore). The temperature and light conditions were consistent with the temporary conditions above. Each treatment had three replicates.

2.2. Measurement of growth

The growth of *U. prolifera* was determined by weighing fresh thalli. The thalli of *U. prolifera* were blotted gently with tissue paper to remove water on the surface of the thalli before weighing. The relative growth rate (RGR) was estimated as follows: $RGR = (\ln W_{t2} - \ln Wt1)/t \times 100$, where W_{t1} is the initial fresh weight (FW) and W_{t2} is the weight after t days culture. The inhibition of Cu on RGR was calculated as: Inhibition of Cu = $(RGRc - RGRt)/RGRc \times 100$, where RGRc is the RGR at LCu-LC and RGRt is the RGR at MCu or HCu treatments.

2.3. Assessment of photosynthesis and respiration

The net photosynthetic rate of thalli was measured by a Clark-type oxygen electrode (YSI model 5300A). Approximately 0.02 g segments of fresh weight algae were harvested from the culture flask and incubated in growth media for 1 h to minimize the cutting damage. The segments were then transferred to the oxygen electrode cuvette containing 2 ml sterilized medium, and the media were stirred. The irradiance and temperature conditions were set the same as those in the growth incubators. The increase of the oxygen content in the seawater within five minutes was defined as the net photosynthetic rate and the decrease of the oxygen content in the seawater in darkness within ten minutes was defined as the respiration rate. The net photosynthetic rate (NPR) and the respiration rate were presented as μmol $O_2\, g^{-1}\, FW\, h^{-1}$. The inhibition of Cu on NPR was calculated as: Inhibition of Cu = $(NPRc - NPRt)/NPRc \times 100$, where NPRc is the NPR at LCu-LC and NPR t is the NPRt at MCu or HCu treatments.

2.4. Chlorophyll fluorescence measurement

A pulse modulation fluorometer (Water-PAM, Walz, Germany) was used to measure the chlorophyll fluorescence parameters. The actinic light was set as 240 μmol photons $m^{-2}\, s^{-1}$ to be consistent with the culture light intensity and the saturating pulse was 5000 μmol photons $m^{-2}\, s^{-1}$ (0.8 s). The relative ETR (rETR) was calculated as follows (Genty et al., 1989): rETR (μmol $e^-\, m^{-2}\, s^{-1}$) = $Fv'/Fm' \times 0.5 \times PFD$, where Fv'/Fm' represents the effective PSII quantum yield and PFD is the photosynthetically active photon flux density. The rapid light curves for rETR were measured under nine different PAR levels with every measurement lasting 10 s. The parameters of maximum rETR ($rETR_{max}$), efficiency of electron transport (α) and saturating irradiance (I_k) were calculated from the rETR curves following the model (Eilers and Peeters, 1988): $rETR = I/(a \times I^2 + b \times I + c)$, $I_k = (c/a)^{1/2}$, $\alpha = 1/c$, $rETR_{max} = 1/[b + 2 \times (a \times c)^{1/2}]$, where I is the incident irradiance; a, b, and c are the adjustment parameters. The nonphotochemical quenching (NPQ) was calculated as follows: $NPQ = (Fm - Fm')/Fm'$, where Fm is the maximum fluorescence yield after 15 min dark adaptation and Fm' is the maximum fluorescence yield under actinic light.

2.5. Determination of photosynthetic pigments

Approximately 15 mg of fresh weight thalli was exacted by 5 ml methanol at 4 °C for 24 h in darkness. Then the absorbance values of samples at 470 (A470), 653 (A653) and 666 (A666) nm were read with a UV/Visible spectrophotometer (Ultrospect 3300 pro, Amersham Bioscience, Sweden). The contents of pigments were estimated according to Wellburn (1994).

2.6. Estimate of carbonate system parameters

The seawater pH was monitored with a pH meter (pH 700, Eutech Instruments, Singapore) and total alkalinity (TA) was calculated by titrations. Other carbonate system parameters, which were not directly measured, were calculated via CO2SYS (Pierrot et al., 2006), using the equilibrium constants of K_1 and K_2 for carbonic acid dissociation (Roy et al., 1993).

2.7. Data analysis

Results were expressed as means of replicates ± standard deviation. Data were analyzed using the software SPSS v.21. 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 ANOVA was conducted to assess the effects of Cu and pCO_2 on relative growth rate, net photosynthesis rate, respiration rate, rETR, NPQ, ETR_{max}, α, I_k, Chl *a*, Chl *b*, and carotenoids. One-way ANOVA was conducted to compare the inhibition rates of Cu on relative growth rate and net photosynthesis rate at different pCO_2 conditions. Tukey HSD was conducted for *post hoc* investigation. A confidence interval of 95% was set for all tests.

3. Results

Both Elevated pCO_2 and Cu altered carbonate parameters in seawater (Tables 1 and 2). Moderate increase in pCO_2 level (MC) led to a decrease of 4.4% in pH and 50.8% in CO_3^{2-}, but an increase of 7.5% in DIC, 11.9% in HCO_3^-, 154.4% in CO_2 compared to LC. High pCO_2 level (HC) resulted in a further decrease of 1.8% in pH and 25.8% in CO_3^{2-}, while a further increase of 2.4% in DIC, 2.9% in HCO_3^-, 42.7% in CO_2. Neither MC nor HC affected TA. HCu reduced TA by 1.2%, pH by 0.2%, CO_3^{2-} by 4.0% compared to LCu.

The effects of Cu and pCO_2 on the growth of *U. prolifera* were investigated (Fig. 1A). The two-way ANOVA showed that Cu and pCO_2 had an interactive effect, and both Cu and pCO_2 had a main effect on the RGR of *U. prolifera* (Table 3). *Post hoc* Tukey HSD comparison ($P = 0.05$) showed that RGR decreased with the rise of Cu level at all conditions except MC-MCu. The RGR increased from 34.5 ± 3.1% (LC) to 42.2 ± 3.1% (MC) and then decreased to 35.3 ± 2.8% (HC) with the rise of pCO_2 at LCu. The MC increased the RGR to 35.9 ± 3.7% and HC decreased it to 19.7 ± 1.8% compared to LC (26.6 ± 2.0%) at MCu. A similar trend was found at HCu, with highest RGR (7.1 ± 0.7%) at MC and the lowest (2.5 ± 0.6%) at HC. To explain the interactive effects of Cu and pCO_2, the inhibition rates of Cu on relative growth rate based on the value at LCu-LC were calculated (Fig. 1B). The inhibition rate of MCu on growth decreased from 23.0 ± 5.9% to −3.8 ± 10.7% and then increased to 43.0 ± 5.2% with the increase of pCO_2. A similar pattern was

Table 1
Parameters of the seawater carbonate system in different cultures. LCu, control; MCu, 0.5 μM; HCu, 2 μM; LC, 390 μatm; MC, 1000 μatm; HC, 1400 μatm. DIC = dissolved inorganic carbon, TA = total alkalinity. Data are the means ± SD (n = 3).

Treatment	pH	pCO_2 (μatm)	DIC (μmol kg^{-1})	HCO_3^- (μmol kg^{-1})	CO_3^{2-} (μmol kg^{-1})	CO_2 (μmol kg^{-1})	TA (μmol kg^{-1})
LC-LCu	8.20 ± 0.01	377.9 ± 6.5	1996.6 ± 21.9	1810.7 ± 19.7	173.4 ± 3.0	12.5 ± 0.2	2246.7 ± 24.1
LC-MCu	8.20 ± 0.01	382.9 ± 2.0	1990.1 ± 18.0	1807.0 ± 14.5	170.4 ± 3.6	12.6 ± 0.1	2236.0 ± 22.5
LC—HCu	8.19 ± 0.01	390.1 ± 9.3	1977.1 ± 19.7	1798.5 ± 19.6	165.7 ± 0.9	12.9 ± 0.3	2216.7 ± 18.5
MC-LCu	7.84 ± 0.01	973.0 ± 19.5	2152.3 ± 21.6	2035.1 ± 20.7	85.1 ± 1.1	32.1 ± 0.6	2253.7 ± 21.4
MC-MCu	7.84 ± 0.01	969.6 ± 25.7	2127.7 ± 16.4	2012.2 ± 15.8	83.5 ± 1.8	32.0 ± 0.8	2227.3 ± 16.2
MC—HCu	7.83 ± 0.01	985.4 ± 15.1	2129.0 ± 15.6	2014.2 ± 14.8	82.3 ± 1.2	32.5 ± 0.5	2226.3 ± 16.2
HC-LCu	7.70 ± 0.01	1376.2 ± 16.0	2193.8 ± 15.4	2085.3 ± 14.6	63.1 ± 1.1	45.4 ± 0.5	2250.0 ± 16.5
HC-MCu	7.70 ± 0.01	1395.2 ± 18.1	2190.0 ± 9.8	2081.8 ± 9.2	62.1 ± 0.9	46.0 ± 0.6	2244.0 ± 10.4
HC—HCu	7.69 ± 0.01	1408.0 ± 25.9	2176.1 ± 13.7	2068.9 ± 12.8	60.8 ± 1.7	46.5 ± 0.9	2228.0 ± 16.5

Table 2
Two-way analysis of variance for the effects of Cu and pCO_2 on pH, dissolved inorganic carbon (DIC), HCO_3^-, CO_3^{2-}, CO_2, total alkalinity (TA) in the seawater. Cu*pCO_2 means the interactive effect of Cu and pCO_2, df means degree of freedom, F means the value of F statistic, and Sig. means *p*-value.

Source		pH		DIC		HCO_3^-		CO_3^{2-}		CO_2		TA	
	df	F	Sig.	F	Sig.	F	Sig.	F	Sig.	F	Sig.	F	Sig.
Cu	2	8.385	0.003	3.091	0.070	2.396	0.119	11.019	0.001	2.756	0.090	4.630	0.024
pCO_2	2	>1000	<0.001	321.172	<0.001	716.233	<0.001	>1000	<0.001	>1000	<0.001	0.388	0.684
Cu*pCO_2	4	0.192	0.939	0.346	0.843	0.388	0.815	1.807	0.172	0.465	0.761	0.331	0.854
Error	18												

observed at HCu, which indicates that MC alleviated the toxicity of Cu to growth while HC increased it at both Cu levels.

Both Cu and pCO_2 had a main effect on NPR of *U. prolifera* while there was no interactive effect between these two factors (Table 3 and Fig. 2A). *Post hoc* Tukey HSD comparison ($P = 0.05$) demonstrated that the NPR decreased with a rise of Cu at all conditions except MCu-LC and MCu-MC. MC increased NPR to 127.2 ± 10.8 μmol O_2 g^{-1} FW h^{-1} compared to LC (103.4 ± 6.7 μmol O_2 g^{-1} FW h^{-1}) while the difference between HC (109.4 ± 8.2 μmol O_2 g^{-1} FW h^{-1}) and MC or HC and LC was not significant at LCu. Neither MC (115.4 ± 11.4 μmol O_2 g^{-1} FW h^{-1}) nor HC (83.5 ± 9.0 μmol O_2 g^{-1} FW h^{-1}) changed the NPR compared to LC (94.1 ± 8.6 μmol O_2 g^{-1} FW h^{-1}) but the NPR at HC was significantly lower than MC at MCu. MC increased NPR to 54.9 ± 7.0 μmol O_2 g^{-1} FW h^{-1} while HC reduced it to 16.4 ± 4.2 μmol O_2 g^{-1} FW h^{-1} compared with LC (33.8 ± 6.1 μmol O_2 g^{-1} FW h^{-1}) at HCu. In terms of inhibition rates of Cu on NPR (Fig. 2B), they decreased to −11.5 ± 3.8% and 47.1 ± 3.3% at MC but increased to 19.3 ± 3.5% and 84.3 ± 3.1% at HC compared with LC (9.1 ± 2.4% at MCu, 67.5 ± 3.8% at HCu).

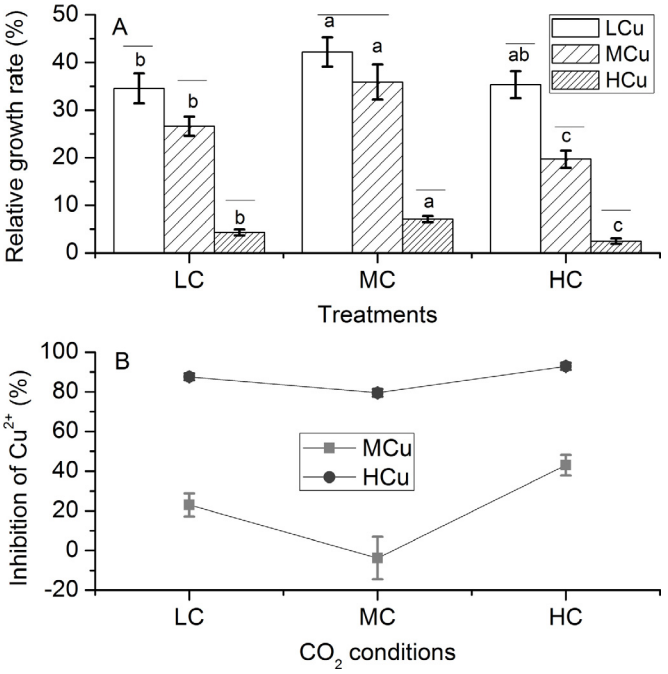

Fig. 1. RGR (A) and inhibition rate of Cu (B) on growth in *U. prolifera* cultured at different Cu and pCO_2 conditions. LCu, control; MCu, 0.5 μM; HCu, 2 μM; LC, 390 μatm; MC, 1000 μatm; HC, 1400 μatm. The error bars indicate the standard deviations (n = 3). Horizontal lines represent the significant difference ($P < 0.05$) among the Cu concentrations at the same pCO_2 concentration. Different letters represent the significant difference ($P < 0.05$) among the pCO_2 concentrations at the same Cu concentration.

Cu and pCO_2 had an interactive effect and Cu had a main effect on the respiration rate of *U. prolifera*, which indicates that the effects of Cu on the respiration rate are different at various pCO_2 levels (Fig. 3 and Table 4). For instance, MCu increased the respiration rate to 46.3 ± 4.0 μmol O_2 g^{-1} FW h^{-1} compared with LCu (34.2 ± 2.8 μmol O_2 g^{-1} FW h^{-1}) at LC while the differences between MCu and LCu at MC or HC were not significant. Compared to LCu, HCu increased the respiration rate to 44.3 ± 3.0 μmol O_2 g^{-1} FW h^{-1} at LC and 48.8 ± 4.2 μmol O_2 g^{-1} FW h^{-1} at MC while it decreased the respiration rate to 21.1 ± 2.3 μmol O_2 g^{-1} FW h^{-1} at HC.

Cu and pCO_2 had an interactive effect and each had a main effect on rETR (Table 4 and Fig. 4A). *Post hoc* Tukey HSD comparison ($P = 0.05$) showed that MCu did not affect rETR at any pCO_2 conditions. In contrast, HCu reduced the rETR by 65.3% and 80.7% at LC and HC respectively compared to the control, although it still did

Table 3
Two-way analysis of variance for the effects of Cu and pCO_2 on relative growth rate and net photosynthetic rate of *U. prolifera*. Cu*pCO_2 means the interactive effect of Cu and pCO_2, df means degree of freedom, F means the value of F statistic, and Sig. means *p*-value.

Source	Relative growth rate			Net photosynthetic rate		
	df	F	Sig.	df	F	Sig.
Cu	2	461.299	<0.001	2	225.343	<0.001
pCO_2	2	36.906	<0.001	2	30.721	<0.001
Cu*pCO_2	4	5.853	0.003	4	1.883	0.157
Error	18			18		

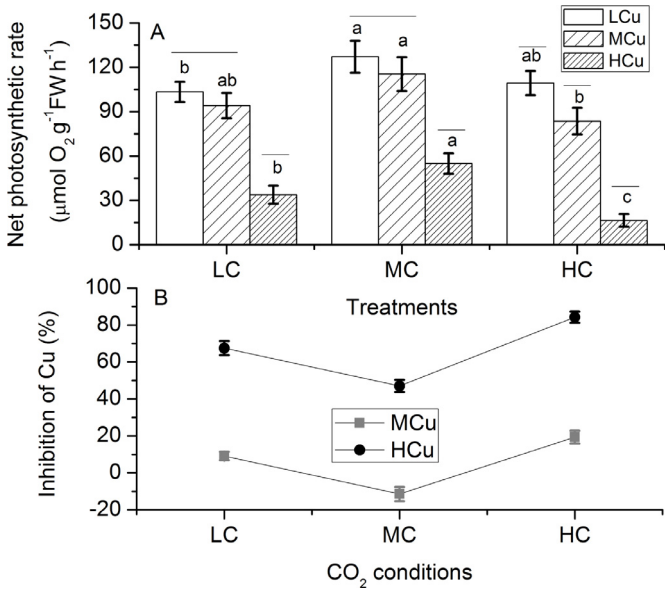

Fig. 2. Net photosynthetic rate (A) and inhibition rate of Cu (B) in *U. prolifera* cultured at different Cu and pCO_2 conditions. LCu, control; MCu, 0.5 μM; HCu, 2 μM; LC, 390 μatm; MC, 1000 μatm; HC, 1400 μatm. The error bars indicate the standard deviations (n = 3). Horizontal lines represent the significant difference ($P < 0.05$) among the Cu concentrations at the same pCO_2 concentration. Different letters represent the significant difference ($P < 0.05$) among the pCO_2 concentrations at the same Cu concentration.

not cause a decline at MC. In regard to the effect of pCO_2, rETR increased to 68.4 ± 4.0 μmol $e^- m^{-2} s^{-1}$ at MC compared with LC (55.5 ± 2.2 μmol $e^- m^{-2} s^{-1}$) and then decreased to 49.4 ± 5.3 μmol $e^- m^{-2} s^{-1}$ at HC compared to MC, with the insignificant difference between LC and HC at LCu. At MCu, the difference in rETR between any two pCO_2 conditions was significant and the order was MC (70.7 ± 4.9 μmol $e^- m^{-2} s^{-1}$) > LC (56.9 ± 4.3 μmol $e^- m^{-2} s^{-1}$) > HC (44.0 ± 7.5 μmol $e^- m^{-2} s^{-1}$). The pattern at HCu was similar to that at MCu, with the highest rETR (64.3 ± 3.9 μmol $e^- m^{-2} s^{-1}$) at MC and the lowest (9.5 ± 2.8 μmol $e^- m^{-2} s^{-1}$) at HC.

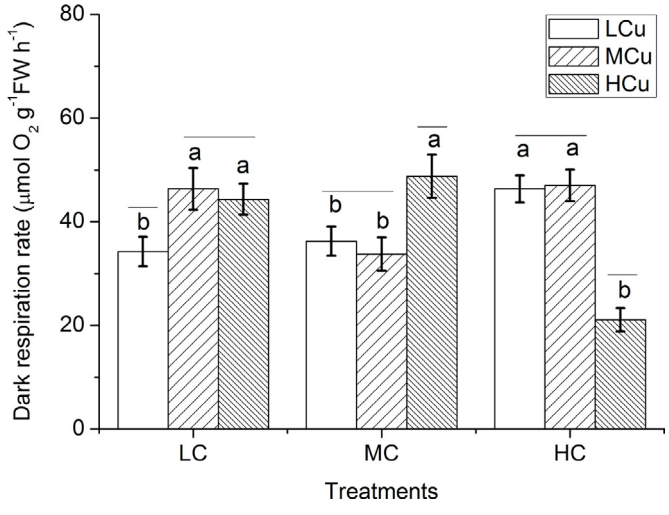

Fig. 3. The dark respiration rate of *U. prolifera* cultured at different Cu and pCO_2 conditions. LCu, control; MCu, 0.5 μM; HCu, 2 μM; LC, 390 μatm; MC, 1000 μatm; HC, 1400 μatm. The error bars indicate the standard deviations (n = 3). Horizontal lines represent the significant difference ($P < 0.05$) among the Cu concentrations at the same pCO_2 concentration. Different letters represent the significant difference ($P < 0.05$) among the pCO_2 concentrations at the same Cu concentration.

Table 4
Two-way analysis of variance for the effects of Cu and pCO_2 on dark respiration rate, rETR, and NPQ of *U. prolifera*. Cu*pCO_2 means the interactive effect of Cu and pCO_2, df means degree of freedom, F means the value of F statistic, and Sig. means *p*-value.

Source	Dark respiration rate			rETR			NPQ		
	df	F	Sig.	df	F	Sig.	df	F	Sig.
Cu	2	4.670	0.023	2	108.837	<0.001	2	1.429	0.265
pCO_2	2	2.771	0.089	2	139.293	<0.001	2	4.493	0.026
Cu*pCO_2	4	46.467	<0.001	4	18.133	<0.001	4	20.204	<0.001
Error	18			18			18		

Cu and pCO_2 also had an interactive effect on NPQ. pCO_2 had a main effect whilst Cu did not significantly alter NPQ (Table 4 and Fig. 4B). *Post hoc* Tukey HSD comparison ($P = 0.05$) showed that MC (0.14 ± 0.03) did not alter NPQ but HC increased it to 0.31 ± 0.05 compared with LC (0.14 ± 0.02) at LCu. The values of NPQ at all three pCO_2 condition were not significantly different at MCu whereas HC reduced NPQ to 0.11 ± 0.02 compared to LC (0.24 ± 0.03) with an insignificant difference between MC (0.16 ± 0.04) and LC or between MC and HC at HCu.

The fluorescence parameters of efficiency of electron transport (α), maximum rETR ($rETR_{max}$), and saturating irradiance (I_k) were obtained from the rETR-irradiance curves (Fig. 5). Cu and pCO_2 had an interactive effect on α and either had a main effect (Fig. 6A and Table 5), which indicates that the effects of Cu on α were different at different pCO_2 levels and vice versa. For instance, α decreased from 0.24 ± 0.00 to 0.18 ± 0.01 and further to $0.13 \pm 0.01$when Cu rose from control to 2 μM at LC. In contrary, α was not affected by changes of Cu at MC and only HCu reduced α at HC.

Cu and pCO_2 interacted on $rETR_{max}$ and each had a main effect (Fig. 6B and Table 5), which indicates the effect of Cu on $rETR_{max}$ altered with the concentration of pCO_2 and vice versa. For instance, MCu did not significantly affect $rETR_{max}$ at LC but it significantly reduced $rETR_{max}$ at MC or HC. HCu reduced $rETR_{max}$ at all pCO_2 conditions. As for the effect of pCO_2, although MC increased rETRmax and HC reduced it at all Cu levels, the extents of change were different. *Post hoc* Tukey HSD comparison ($P = 0.05$) showed that MC increased $rETR_{max}$ by 44.3%, 24.3%, and 71.6% at LCu, MCu, and HCu respectively. The biggest inhibitory effect of HC was found at HCu (74.0%), followed by MCu (41.5%) and LCu (18.2%).

Cu and pCO_2 interacted on I_k and each had a main effect (Fig. 6C and Table 5). I_k decreased with the increase of Cu at all pCO_2 conditions while the extents of the decrease were different. For instance, MCu decreased it by 23.1%, 34.8%, and 21.9% at LC, MC, and HC respectively. The inhibitory effect of HCu increased with the pCO_2 level with the biggest of 62.8% at HC and the smallest of 36.4% at LC. In terms of the effect of pCO_2, MC increased I_k and HC decreased it at all Cu conditions except that at MCu where HC did not affect I_k.

Cu and pCO_2 had an interactive effect on Chl *a* and each had a main effect (Fig. 7A and Table 6). *Post hoc* Tukey HSD comparison ($P = 0.05$) showed both MCu and HCu increased Chl *a* at LC compared to LCu while only HCu promoted it at MC. On the contrary, HCu decreased Chl *a* at HC compared to LCu. In regard to the effect of pCO_2, MC (0.35 ± 0.03 mg g^{-1} FW) did not change Chl *a* but HC increased it to 0.58 ± 0.07 mg g^{-1} FW compared to LC (0.38 ± 0.03 mg g^{-1} FW) at LCu. MC decreased Chl *a* to 0.39 ± 0.04 mg g^{-1} FW while HC (0.53 ± 0.05 mg g^{-1} FW) did not affect it compared to LC (0.61 ± 0.07 mg g^{-1} FW) at MCu. MC (0.62 ± 0.07 mg g^{-1} FW) did not alter Chl *a* while HC reduced it to 0.30 ± 0.05 mg g^{-1} FW compared to LC (0.59 ± 0.05 mg g^{-1} FW) at HCu. The effects of Cu and pCO_2 on Chl *b* (Fig. 7B) and carotenoids (Fig. 7C) were similar to Chl *a*.

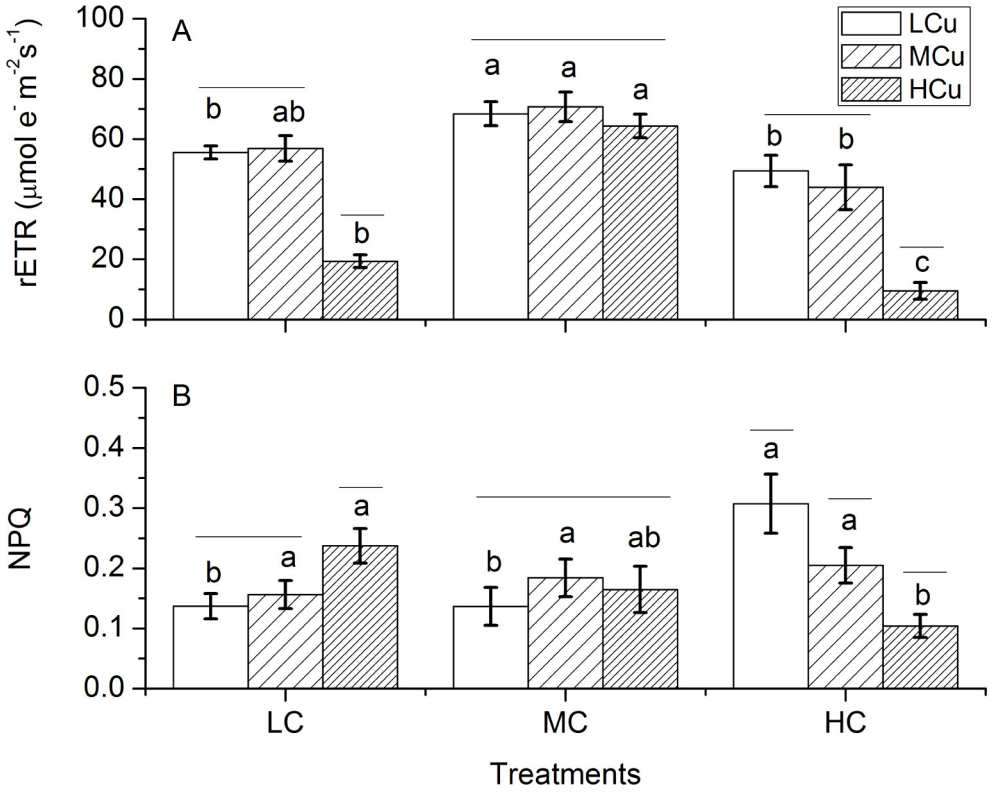

Fig. 4. The rETR (A) and NPQ (B) of *U. prolifera* cultured at different Cu and pCO_2 conditions. LCu, control; MCu, 0.5 μM; HCu, 2 μM; LC, 390 μatm; MC, 1000 μatm; HC, 1400 μatm. The actinic light was 240 μmol photons $m^{-2} s^{-1}$ and the the saturating pulse was 5000 μmol photons $m^{-2} s^{-1}$ (0.8 s). The error bars indicate the standard deviations (n = 3). Horizontal lines represent the significant difference ($P < 0.05$) among the Cu concentrations at the same pCO_2 concentration. Different letters represent the significant difference ($P < 0.05$) among the pCO_2 concentrations at the same Cu concentration.

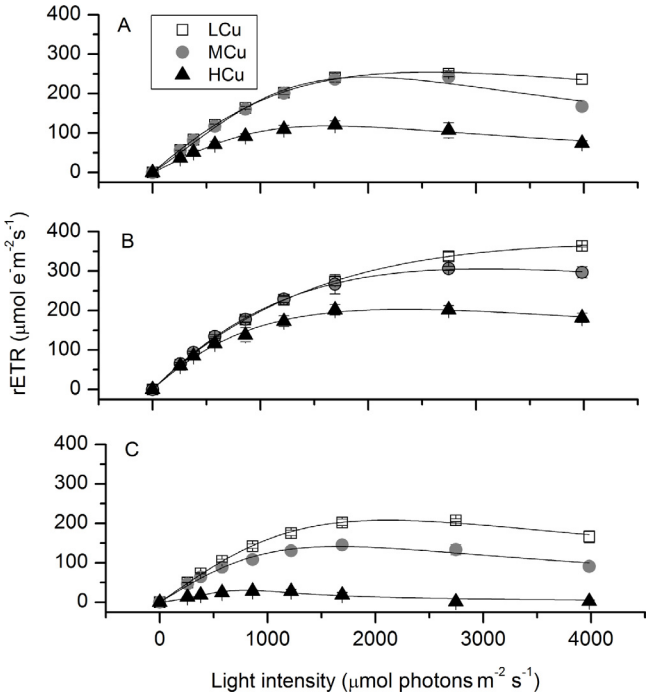

Fig. 5. Rapid light curve of *U. prolifera* cultured at different Cu and pCO_2 conditions. A, LC (390 μatm); B, MC (1000 μatm); C, HC (1400 μatm). LCu, control; MCu, 0.5 μM; HCu, 2 μM. The error bars indicate the standard deviations (n = 3).

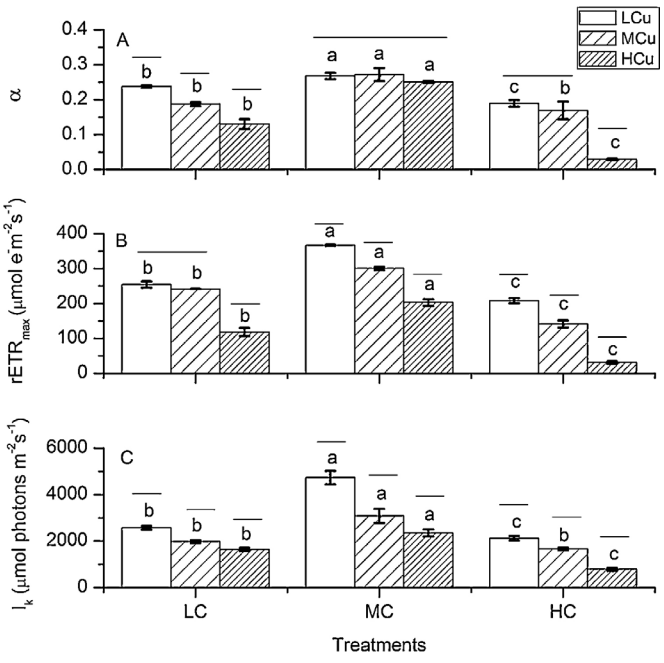

Fig. 6. The electron transport efficiency (α), maximum rETR ($rETR_{max}$), and saturating irradiance (I_k) of *U. prolifera* cultured at different Cu and pCO_2 conditions. LCu, control; MCu, 0.5 μM; HCu, 2 μM; LC, 390 μatm; MC, 1000 μatm; HC, 1400 μatm. The error bars indicate the standard deviations (n = 3). Horizontal lines represent the significant difference ($P < 0.05$) among the Cu concentrations at the same pCO_2 concentration. Different letters represent the significant difference ($P < 0.05$) among the pCO_2 concentrations at the same Cu concentration.

Table 5
Two-way analysis of variance for the effects of Cu and pCO_2 on efficiency of electron transport (α), maximum rETR ($rETR_{max}$), and saturating irradiance (I_k) of *U. prolifera*. Cu*pCO_2 means the interactive effect of Cu and pCO_2, df means degree of freedom, F means the value of F statistic, and Sig. means *p*-value.

Source	α			$rETR_{max}$			I_k		
	df	F	Sig.	df	F	Sig.	df	F	Sig.
Cu	2	143.309	<0.001	2	1073.241	<0.001	2	219.353	<0.001
pCO_2	2	263.174	<0.001	2	1076.433	<0.001	2	333.875	<0.001
Cu*pCO_2	4	31.084	<0.001	4	14.173	<0.001	4	21.148	<0.001
Error	18			18			18		

The changes of morphology in *U. prolifera* grown under different Cu and pCO_2 conditions were observed (Fig. 8). HC and HCu induced branches of thalli (Fig. 8C, F, G, and H) and the combination of HC and HCu led to a noticeable shrinkage of thalli (Fig. 8I).

4. Discussion

4.1. The main effects of Cu and pCO_2

The addition of 0.5 μM Cu did not significantly affect the net photosynthetic rate or rETR of *U. prolifera* at LC in the present study. A possible reason might be that the concentration of 0.5 μM is not enough to harm photosynthesis of *U. prolifera*. This can be supported by the fact that the concentration of 0.8 μM Cu did not affect the photosynthetic rate of *U. flexuosa* (Andrade et al., 2004) and the concentration of 1 μM Cu did not reduce the quantum yield of PSII *U. intestinalis* (Baumann et al., 2009). On the other hand, the 0.4 μM Cu reduced the maximum electron transport rate of PSII in *U. pertusa* (Han et al., 2008), which indicates 0.5 μM Cu might be harmful for photosynthesis of *U. prolifera*. Then the mute effect of 0.5 μM Cu on photosynthesis might be due to the working of protective mechanisms. Macroalgae have evolved multiple mechanisms to defend against the toxicity of heavy metals, such as metal-exclusion mechanisms, metal-chelating mechanisms, ion exchange mechanisms, etc. (Baumann et al., 2009; Mata et al., 2009). In the present study, more photosynthetic pigments (Chl *a*, Chl *b*, and carotenoids) were synthesized at 0.5 μM Cu concentration. The increased pigments may play an important role in exchanging intercellular Cu^{2+} with the Mg^{2+} in Chl *a* and Chl *b* to counteract the toxicity of Cu^{2+}, since Mg^{2+} compounds in a blue–green alga *Spirulina* sp. could exchange Cu^{2+} with their own Mg^{2+} (Chojnacka et al., 2005). In addition, chlorophyll in *Koeleria splendens* was also enhanced by a moderate increase of Cu^{2+}, which was deemed as an adaptive mechanism to Cu^{2+}(Ouzounidou, 1995). This hypothesis that Chl *a* and Chl *b* could reduce the toxicity of Cu through ion exchange mechanisms needs further study to test. Although the 0.5 μM Cu concentration did not decrease the net photosynthetic rate or rETR of *U. prolifera*, it did reduce the growth of thalli at LC. The decreased growth could be attributed to the additional energy requirement for synthesizing pigments or other Cu chelating compounds, which can be confirmed by the increased respiration rate at MCu-LC. The dark respiration rate in *S. incrassatulus* was also enhanced when exposed to Cu (0.629–1.887 μM), which was considered a sign of increased energy demand to defend against Cu (Perales-Vela et al., 2007). In the present study a further increase of Cu (2 μM) reduced growth, the net photosynthetic rate, and the rETR although there were still higher pigment contents and respiration rate compared to control, which indicates that the protective mechanisms were beaten by the harm of excess Cu.

MC (1000 μatm) increased the growth rate of *U. prolifera* at LCu in the present study, which can be attributed to the increased photosynthesis at MC. The promoting effect of increased pCO_2 (1000 μatm) on the growth of *U. prolifera* was also reported in Xu and Gao's study (2012) and in other macroalgae species, such as *Porphyra yezoensis* (Gao et al., 1991), *Hizikia fusiforme* (Zou, 2005), and *Neosiphonia harveyi* (Olischläger and Wiencke, 2013). On the other hand, the further increase of pCO_2 (1400 μatm) did not affect

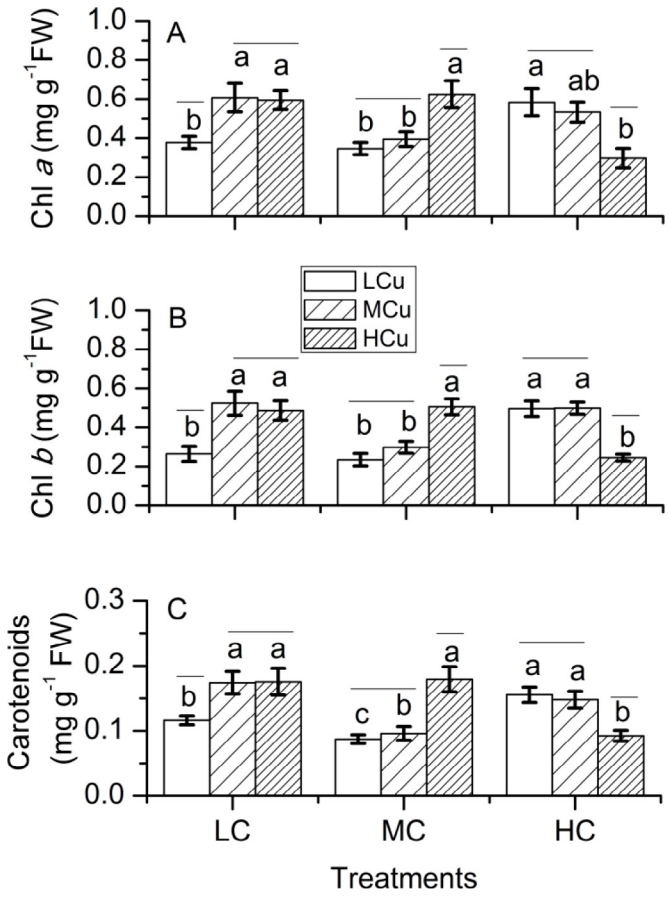

Fig. 7. The pigment content (A: Chl *a*, B: Chl *b*, C: Carotenoids) of *U. prolifera* cultured at different Cu and pCO_2 conditions. LCu, control; MCu, 0.5 μM; HCu, 2 μM; LC, 390 μatm; MC, 1000 μatm; HC, 1400 μatm. The error bars indicate the standard deviations (n = 3). Horizontal lines represent the significant difference ($P < 0.05$) among the Cu concentrations at the same pCO_2 concentration. Different letters represent the significant difference ($P < 0.05$) among the pCO_2 concentrations at the same Cu concentration.

Table 6
Two-way analysis of variance for the effects of Cu and pCO_2 on Chl *a*, Chl *b*, and carotenoids of *U. prolifera*. Cu*pCO_2 means the interactive effect of Cu and pCO_2, df means degree of freedom, F means the value of F statistic, and Sig. means *p*-value.

Source	Chl *a*			Chl *b*			Carotenoids		
	df	F	Sig.	df	F	Sig.	df	F	Sig.
Cu	2	5.524	0.013	2	18.313	<0.001	2	10.901	0.001
pCO_2	2	4.383	0.028	2	10.293	0.001	2	14.971	<0.001
Cu*pCO_2	4	29.597	<0.001	4	49.163	<0.001	4	34.302	<0.001
Error	18			18			18		

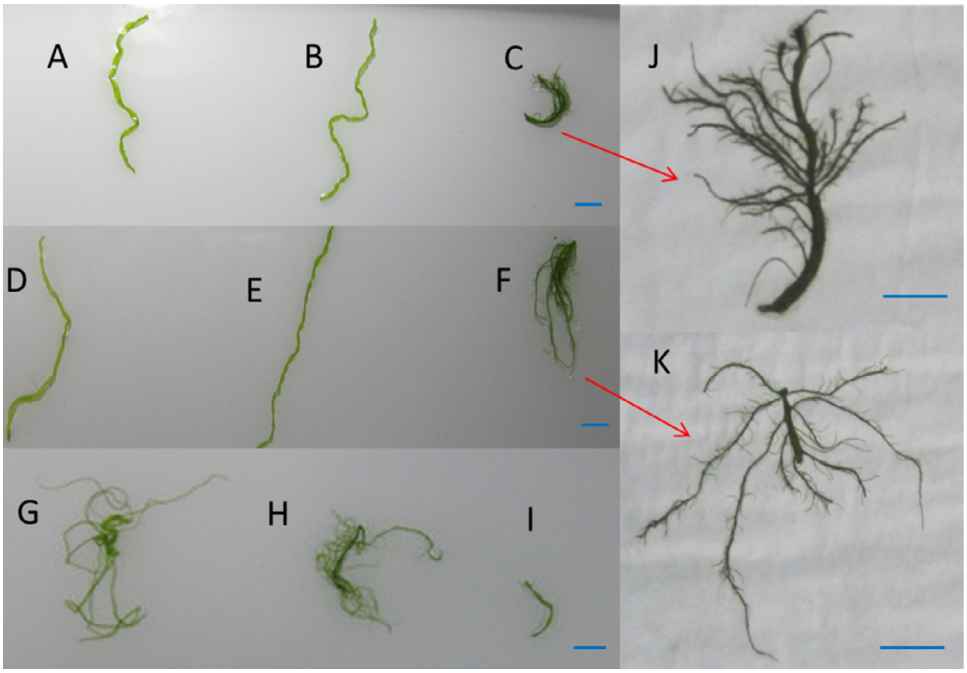

Fig. 8. Morphological variation of *U. prolifera* cultured at different Cu and pCO_2 conditions. A, LC-LCu; B, LC-MCu; C, LC-HCu; D, MC-LCu; E, MC-MCu; F, MC-HCu; G, HC-LCu, H, HC- MCu, I, HC-HCu. J and K are specimens made from C and F respectively. The blue scale bars represent the length of 1 cm (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

the growth rate or net photosynthesis rate in *U. prolifera* compared to the LC in the present study. One possible reason is that the higher dark respiration rate was induced at HC. The decreased pH and increased acidity of seawater associated with the increased pCO_2 could disturb the pH of the cell surface (Flynn et al., 2012). Accordingly, algae may need to allocate additional energy to transport ions against the acid–base perturbation, which leads to increased respiration and decreased growth. The increased respiration rate at higher pCO_2 and decreased pH was also found in macroaglae *Hizikia fusiformis* (Zou et al., 2011) and microalgae *Phaeodactylum tricornutum* (Wu et al., 2010) and *Emiliania huxleyi* (Jin et al., 2015).

4.2. Interactive effects of pCO_2 and Cu

MCu and HCu reduced the growth rate of *U. prolifera* at all pCO_2 conditions, and the inhibitory effect of Cu was decreased at MC but increased at HC compared with LC in the present study. The decrease in pH caused by increased pCO_2 would result in a decline in the levels of both $CO_3^{2-}$and OH^- in natural waters. Consequently, Cu will have a higher fraction in its free-ion form at lower pH given its dominant complexes with $CO_3^{2-}$and OH^-. In addition, the decreased pH can enhance free Cu^{2+} by reducing its organic ligand in natural waters (Millero, 2009). The increased the free-ion concentration of Cu may lead to a rising toxicity to organisms (Richards et al., 2011). For instance, the toxic effect of metals to DNA and survival in amphipod *Corophium volutator* was more significant at higher pCO_2 conditions (Roberts et al., 2013). Although no studies regarding the increased toxicity of Cu to algae at reduced pH have been reported, the increased toxicity of Zn^{2+} at lower pH were found in *Chlorella vulgaris* (Král'Ová et al., 2004; Rai et al., 1981). Conversely, the decreased toxicity of Cu at lower pH was found in *S. quadricaerda* (Peterson et al., 1984) and *Chlorella* sp. (Franklin et al., 2000). It has been proposed that hydrogen ions may decrease the toxicity of metals by competitively excluding them from binding to ligands at the cell surface (Franklin et al., 2000; Parent and Campbell, 1994). Our results support this hypothesis in an indirect way. The reduced respiration rate at MCu-MC compared with MCu-LC indicates that pigment ion exchange mechanisms or metal-chelating mechanism did not work as the synthesis of pigments and metal-chelating compounds need respiration-driven energy. On the other hand, the competition mechanism between H^+ and Cu^{2+} at the cell surface does not require energy and thereby may function in this case. Contrary to the increased toxicity of metals with the decrease of pH in the previous study (Král'Ová et al., 2004; Rai et al., 1981), the decreased inhibitory effect of Cu on growth and photosynthesis in the present study at MC indicates competition mechanism between H^+ and Cu at the cell surface outcompetes the increased availability of Cu caused by reduced pH in *U. prolifera* and therefore moderate increase of pCO_2 alleviates the toxicity of Cu.

On the other hand, the inhibitory effect of Cu was magnified at HC compared to MC or LC. The reasons behind that may be twofold. Firstly, the further decline of pH at HC leads to more free-ion Cu in the seawater, promoting its toxicity to cells which cannot be counteracted by the competition mechanism between H^+ and Cu^{2+} at the cell surface. Secondly, the further decline of pH at HC exacerbates acid–base perturbation at the cell surface which may make cells more vulnerable to environmental stress. This hypothesis can be partially supported by the decreased efficiency of electron transport, $rETR_{max}$ and I_k at HC-LCu compared to LC-LCu. Our study demonstrates the interactive effects of pCO_2 and Cu depend on the pH of the seawater: the moderate decrease of pH (7.80, corresponding to 1000 μatm pCO_2) can alleviate the toxicity of Cu, whereas a further decrease of pH (7.69, corresponding to 1400 μatm pCO_2) exacerbates it compared to the control (8.16, corresponding to 390 μatm). This finding is different from previous studies referring to freshwater algae, in which the toxicity of Cu decreased continuously with pH even if the pH was very low (Franklin et al., 2000; Peterson et al., 1984). For instance, the toxicity of Cd to phosphate uptake in the freshwater green alga *S. quadricaerda* decreased continuously with the decline of pH from 8.5 to 5.5 and the Cu concentration causing 50% inhibition of phosphate uptake in *S. quadricaerda* also decreased with the

increase of pH over the range of 5.5–8.5 (Peterson et al., 1984). Seawater has a higher pH in comparison with freshwater and thus marine macroalgae that acclimatize to a higher pH might not have the same tolerance as freshwater algae to a decrease of pH. Our study displays the differential responses between marine algae and freshwater algae to the interaction of Cu and pCO_2.

4.3. Morphological changes caused by high Cu and pCO_2

An interesting finding in this study is that the thalli became smaller at HCu (2 μM Cu) and branched at HCu and HC except HCu-HC (Fig. 8). The shrinkage of thalli was also found in *U. pertusa* and *U. armoricana*, when they were exposed to 1.6 μM and 3.9 μM Cu respectively (Han et al., 2008). The shrinkage may be related to the loss of membrane integrity (Brown and Newman, 2003), which can harm the cells. This can be seen from a decreased growth rate, net photosynthetic rate, and rETR in *U. prolifera* at HCu in the present study. Meanwhile, branches were induced at HCu and HC except for HCu-HC. These branches might be a defense strategy of *U. prolifera* against high Cu and pCO_2 because both high Cu and pCO_2 could be stressful to cells. *U. flexuosa* was reported to form a thicker cell wall to attempt to prevent Cu reaching the cytoplasm (Andrade et al., 2004). The shrank and branched thalli in the present study might play a similar role to the thickened cell wall in preventing the membranal penetration of Cu and pCO_2. But this aggregated form of thalli may receive less light due to shading. Therefore, thalli synthesized more pigments to capture more light at HCu and HC except HCu-HC (Fig. 7). This morphological acclimation may have alleviated the harm of Cu to some content. However, when the highest Cu and pCO_2 were combined, *U. prolifera* seems to be incapable of conducting morphological acclimation (Fig. 8I). The severe shrinkage and thus loss of membrane integrity led to seriously damaged physiological performances (RGR, NPR, rETR, NPQ, α, $rETR_{max}$, I_k, pigments, etc.) at HCu-HC.

4.4. The sensitivity of physiological traits to Cu and pCO_2

The order of sensitivity in *U. prolifera* to Cu, based on the level of Cu at which the traits altered at LC, was: growth = respiration = pigments > photosynthetic O_2 evolution = rETR = morphology. Growth along with pigments and respiration were the most sensitive to Cu. This may be explained by saying that cells synthesized compounds, eg. pigments, to defend against Cu, which led to increased respiration. This strategy can protect the photosynthetic apparatus from harm of Cu but sacrifices growth. The phenomena that growth is most and morphology is least sensitive to Cu was also found in red macroalga *Gracilariopsis longissimi* (Brown and Newman, 2003). It seems that macroalgae tend to protect photosynthesis at a cost of growth, while alteration of morphology is their last weapon to defend against metals. The order of sensitivity in *U. prolifera* to pCO_2, based on the level of pCO_2 at which the traits altered at LCu, was growth = photosynthetic O_2 evolution = rETR = pigment > respiration = morphology. This order is similar to Xu and Gao's study (2012) on *U. prolifera*. These findings indicate the photosynthetic apparatus of *U. prolifera* are more sensitive than the respiratory apparatus to changes of pCO_2. The order of sensitivity in *U. prolifera* to HCu-HC, based on the inhibition rate of HCu- HC, was growth (92.9%) > photosynthesis (84.2%) > rETR (82.9%) > respiration (38.5%) > pigments (none). At the condition of HC-HCu, photosynthesis and ETR were hugely harmed by the combination of HC and HCu and respiration was also inhibited, which led to growth almost stopping. The reason that pigment is the least sensitive parameter to HC-Cu could be the balance between the negative effect of high Cu on pigment content and the induction of pigment synthesis due to the shrinkage of thalli at HCu-HC.

5. Conclusion

The present study investigated the interactive effects of Cu and pCO_2 on *U. prolifera* and displayed that medium pCO_2 (1000 μatm) could reduce the toxicity of Cu while a further increase of pCO_2 (1400 μatm) magnified the harm of Cu. *U. prolifera* could respond to the changes of Cu and CO_2 by physiological alteration firstly and thus by morphological acclimation. This is the first report regarding the responses of macroalgae to the interaction of Cu and pCO_2. The future ocean would continue to absorb CO_2 from the atmosphere and experience further decreases of pH. This may impose a positive effect on *U. prolifera* that experiences copper pollution by 2100 since 1000 ppm is the projected CO_2 level (Representative Concentration Pathway 8.5) in atmosphere at the end of this century (IPCC, 2013). But when the pH of seawater decreases further, it will magnify the toxicity of Cu and cause a significant decrease of *U. prolifera*'s growth, which may hinder the occurrence of green tides, considering that *U. prolifera* is the dominant species in green tides.

Acknowledgments

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