Regulation of inorganic carbon acquisition in a red tide alga (*Skeletonema costatum*): the importance of phosphorus availability

Guang Gao[a,b]，Jianrong Xia[a*], Jinlan Yu[a], Jiale Fan[b], Xiaopeng Zeng[a]

[a]School of Environmental Science and Engineering, Guangzhou University, Guangzhou, 510006, China

[b]Marine Resources Development Institute of Jiangsu, Huaihai Institute of Technology, Lianyungang, 222005, China

[*]Corresponding author, Email: jrxia@gzhu.edu.cn; Phone: +86 (0)20 39366941; Fax: +86 (0)20 39366949

Abstract:

Skeletonema costatum is a common bloom-forming diatom and encounters eutrophication and severe carbon dioxide (CO_2) limitation during red tides. However, little is known regarding the role of phosphorus (P) in modulating inorganic carbon acquisition in *S. costatum*，particularly under CO_2 limitation conditions. We cultured *S. costatum* under five phosphate levels (0.05, 0.25, 1, 4, 10 $\mu mol\ L^{-1}$) and then treated it with two CO_2 conditions (2.8 and 12.6 $\mu mol\ L^{-1}$) for two hours. The lower CO_2 reduced net photosynthetic rate at lower phosphate levels ($< 4\ \mu mol\ L^{-1}$) but did not affect it at higher phosphate levels (4 and 10 $\mu mol\ L^{-1}$). In contrast, the lower CO_2 induced higher dark respiration rate at lower phosphate levels (0.05 and 0.25 $\mu mol\ L^{-1}$) and did not affect it at higher phosphate levels ($> 1\ \mu mol\ L^{-1}$). The lower CO_2 did not change relative electron transport rate (rETR) at lower phosphate levels (0.05 and 0.25 $\mu mol\ L^{-1}$) and increased it at higher phosphate levels ($> 1\ \mu mol\ L^{-1}$). Photosynthetic CO_2 affinity ($1/K_{0.5}$) increased with phosphate levels. The lower CO_2 did not affect photosynthetic CO_2 affinity at 0.05 $\mu mol\ L^{-1}$ phosphate but enhanced it at the other phosphate levels. Activity of extracellular carbonic anhydrase was dramatically induced by the lower CO_2 at phosphate replete conditions ($> 0.25\ \mu mol\ L^{-1}$) and the same pattern also occurred for redox activity of plasma membrane. Direct bicarbonate (HCO_3^-) use was induced when phosphate concentration was more than 1 $\mu mol\ L^{-1}$. These findings indicate P enrichment could enhance inorganic carbon acquisition and thus maintain the photosynthesis rate in *S. costatum* grown under CO_2 limiting conditions via increasing activity of extracellular carbonic anhydrase and facilitating direct HCO_3^- use. This study sheds light on how bloom-forming algae cope with carbon limitation during the development of red tides.

23   **Keywords:** carbonic anhydrase; CO_2 concentrating mechanisms; pH compensation point;

24   photosynthesis; redox activity; respiration

1. Introduction

Diatoms are unicellular photosynthetic microalgae that can be found worldwide in freshwater and oceans. Marine diatoms account for 75% of the primary productivity for coastal and other nutrient-rich zones and approximately 20% of global primary production (Field *et al.*, 1998; Falkowski, 2012), hence playing a vital role in the marine biological carbon pump as well as the biogeochemical cycling of important nutrients, such as nitrogen and silicon (Nelson *et al.*, 1995; Moore *et al.*, 2013; Young & Morel, 2015). Diatoms usually dominate the phytoplankton communities and form large-scale blooms in nutrient–rich zones and upwelling regions (Bruland *et al.*, 2001; Anderson *et al.*, 2008; Barton *et al.*, 2016). Nutrient enrichment is considered as a key factor that triggers algal blooms albeit the occurrence of diatom blooms may be modulated by other environmental factors, such as temperature, light intensity, salinity, and so forth (Smetacek & Zingone, 2013; Jeong *et al.*, 2015). When inorganic nitrogen and phosphorus are replete, diatoms can out-compete chrysophytes, raphidophytes and dinoflagellates (Berg *et al.*, 1997; Jeong *et al.*, 2015; Barton *et al.*, 2016) and dominate algal blooms due to their quicker nutrient uptake and growth rate.

In normal natural seawater (pH 8.1, salinity 35), HCO_3^- is the majority (~90%) of total dissolved inorganic carbon (DIC, 2.0–2.2 mM). CO_2 (1%, 10–15 μM), which is the only direct carbon source that can be assimilated by all photosynthetic organisms, only accounts for 1% of total dissolved inorganic carbon. Diatoms' ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco), catalyzing the primary chemical reaction by which CO_2 is transformed into organic carbon, has a relatively low affinity for CO_2 and is commonly less than half saturated under current CO_2 levels in seawater (Hopkinson & Morel, 2011),

47   suggesting that CO_2 is limiting for marine diatoms' carbon fixation. To cope with the CO_2

48   limitation in seawater and maintain a high carbon fixation rate under the low CO_2 conditions,

49   diatoms have evolved various inorganic carbon acquisition pathways and CO_2 concentrating

50   mechanisms (CCMs), for instance, active transport of HCO_3^-, the passive influx of CO_2,

51   multiple carbonic anhydrase (including both common (α, β, γ) and unusual (δ, ζ) families that

52   carries out the fast interconversion of CO_2 and HCO_3^-), assumed C4-type pathway (using

53   phosphoenolpyruvate to capture more CO_2 in the periplastidal compartment), to increase the

54   concentration at the location of Rubisco and thus the carbon fixation. (Hopkinson & Morel,

55   2011; Hopkinson *et al.*, 2016). *Skeletonema costatum* is a worldwide diatom species that can

56   be found from equatorial to polar waters. It usually dominates large-scale algal blooms in

57   eutrophic seawaters (Wang, 2002; Li *et al.*, 2011). When blooms occur, seawater pH increases

58   and CO_2 decreases because the dissolution rate of CO_2 from the atmosphere cannot catch up

59   with its removal rate caused by intensive photosynthesis of algae. For instance, pH level in

60   the surface waters of the eutrophic Mariager Fjord, Denmark, could be up to 9.75 during algal

61   blooms (Hansen, 2002). Consequently, *S. costatum* experiences very severe CO_2 limitation

62   when blooms occur. To deal with it, *S. costatum* has developed multiple CCMs (Nimer *et al.*,

63   1998; Rost *et al.*, 2003). However, contrasting findings were reported. Nimer *et al.* (1998)

64   documented that extracellular carbonic anhydrase activity in *S. costatum* was only induced

65   when CO_2 concentration was less than 5 μmol L^{-1} while Rost *et al.* (2003) reported that

66   activity of extracellular carbonic anhydrase could be detected even when CO_2 concentration

67   was 27 μmol L^{-1}. Chen and Gao (2004) showed that in *S. costatum* had little capacity in direct

68   HCO_3^- utilization. On the other hand, Rost *et al.* (2003) demonstrated that this species could

69   take up CO_2 and HCO_3^- simultaneously.

70   Phosphorus (P) is an indispensable element for all living organisms, serving as an integral

71   component of lipids, nucleic acids, ATP and a diverse range of other metabolites. Levels of

72   bioavailable phosphorus are very low in many ocean environments and phosphorus

73   enrichment can commonly increase algal growth and marine primary productivity in the

74   worldwide oceans (Davies & Sleep, 1989; Müller & Mitrovic, 2015; Lin *et al.*, 2016). Due to

75   the essential role of phosphorus, extensive studies have been conducted to investigate the

76   effect of phosphorus on photosynthetic performances (Geider *et al.*, 1998; Liu *et al.*, 2012;

77   Beamud *et al.*, 2016), growth (Jiang *et al.*, 2016; Reed *et al.*, 2016; Mccall *et al.*, 2017),

78   phosphorus acquisition, utilization and storage (Lin *et al.*, 2016; Gao et al., 2018a). Some

79   studies show the essential role of phosphorus in regulating inorganic carbon acquisition in

80   green algae (Beardall *et al.*, 2005; Hu & Zhou, 2010). In terms of *S. costatum*, studies

81   regarding the inorganic carbon acquisition in *S. costatum* focus on its response to variation of

82   CO_2 availability. The role of phosphorus in *S. costatum*'s CCMs remains unknown. Based on

83   the connection between phosphorus and carbon metabolism in diatoms (Brembu *et al.*, 2017),

84   we hypothesize that phosphorus enrichment could enhance inorganic carbon utilization and

85   hence maintain high rates of photosynthesis and growth in *S. costatum* under CO_2 limitation

86   conditions. In the present study, we aimed to test this hypothesis by investigating the variation

87   of CCMs (including active transport of HCO_3^- and carbonic anhydrase activity) and

88   photosynthetic rate under five levels of phosphate and two levels of CO_2 conditions. We also

89   measured redox activity of plasma membrane as it is deemed to be critical to activate carbonic

90   anhydrase (Nimer *et al.*, 1998). Our study would provide helpful insights into how

91   bloom-forming diatoms overcome CO_2 limitation to maintain a quick growth rate during red

92   tides.

2. Materials and Methods

94   *2.1. Culture conditions*

95   *Skeletonema costatum* (Grev.) Cleve from Jinan University, China, was cultured in f/2

96   artificial seawater with five phosphate levels (0.05, 0.25, 1, 4, 10 µmol L^{-1}) by adding

97   different amounts of NaH_2PO_4 $2H_2O$. The cultures were carried out semi-continuously at

98   20^oC for seven days. The light irradiance was set 200 µmol photons m^{-2} s^{-1}, with a light and

99   dark period of 12: 12. The cultures were aerated with ambient air (0.3 L min^{-1}) to maintain the

100   pH around 8.2. The cells during exponential phase were collected and rinsed twice with

101   DIC-free seawater that was made according to Xu *et al.* (2017). Afterwards, cells were

102   resuspended in fresh media with two levels of pH (8.20 and 8.70, respectively corresponding

103   to ambient CO_2 (12.6 µmol L^{-1}, AC) and low CO_2 (2.8 µmol L^{-1}, LC) under corresponding

104   phosphate levels for two hours before the following measurements, with a cell density of 1.0

105   $\times 10^6$ mL^{-1}. Cell density was determined by direct counting with an improved Neubauer

106   haemocytometer (XB-K-25, Qiu Jing, Shanghai, China). This transfer aimed to investigate the

107   effects of phosphate on DIC acquisition under a CO_2 limitation condition. The pH of 8.70 was

108   chosen considering that it is commonly used as a CO_2 limitation condition (Nimer *et al.*, 1998;

109   Chen & Gao, 2004) and also occurs during algal bloom (Hansen, 2002). Two hours should be

110   enough to activate CCMs in *S. costatum* (Nimer *et al.*, 1998). The cell density did not vary

111   during the two hours of pH treatment. All experiments were conducted in triplicates.

112   *2.2. Manipulation of seawater carbonate system*

113   The two levels of pH (8.20 and 8.70) were obtained by aerating the ambient air and pure

114   nitrogen (99.999%) till the target value, and were then maintained with a buffer of 50 mM tris

115   (hydroxymethyl) aminomethane-HCl. The cultures were open to the ambient atmosphere and

116   the rise of culture pH was below 0.02 unites (corresponding to the rise of CO_2 less than 0.7

117   and 0.2 μmol L^{-1} for pH 8.20 and 8.70 treatments respectively) during the two hours of pH

118   treatment. CO_2 level in seawater was calculated via CO2SYS (Pierrot *et al.*, 2006) based on

119   measured pH and TAlk, using the equilibrium constants of K1 and K2 for carbonic acid

120   dissociation (Roy *et al.*, 1993) and the KSO_4^- dissociation constant from Dickson (1990).

121   *2.3.*The pH_{NBS} was measured by a pH meter (pH 700, Eutech Instruments, Singapore) that

122   was equipped with an Orion$^®$ 8102BN Ross combination electrode (Thermo Electron Co.,

123   USA) and calibrated with standard National Bureau of Standards (NBS) buffers (pH =

124   4.01, 7.00, and 10.01 at 25.0 ^{o}C; Thermo Fisher Scientific Inc., USA). Total alkalinity

125   (TAlk) was determined at 25.0 ^{o}C by Gran acidimetric titration on a 25-ml sample with a

126   TAlk analyzer (AS-ALK1, Apollo SciTech, USA), using the precision pH meter and an

127   Orion$^®$ 8102BN Ross electrode for detection. To ensure the accuracy of TAlk, the TAlk

128   analyser was regularly calibrated with certified reference materials from Andrew G.

129   Dickson's laboratory (Scripps Institute of Oceanography, U.S.A.) at a precision of ± 2

130   μmol kg^{-1}. CO_2 level in seawater was calculated via CO2SYS (Pierrot *et al.*, 2006) based

131   on measured pH and TAlk, using the equilibrium constants of K1 and K2 for carbonic acid

132   dissociation (Roy *et al.*, 1993) and the KSO_4^- dissociation constant from Dickson (1990).

133   *Chlorophyll fluorescence measurement*

134   Chlorophyll   fluorescence   was   measured   with   a   pulse   modulation   fluorometer

135 (PAM-2100, Walz, Germany) to assess electron transport in photosystem II and the possible

136 connection between electron transport and redox activity of the plasma membrane. The

137 measuring light and actinic light were 0.01 and 200 μmol photons m^{-2} s^{-1}, respectively. The

138 saturating pulse was set 4,000 μmol photons m^{-2} s^{-1} (0.8 s). Relative electron transport in

139 photosystem II (rETR, μmol e^- m^{-2} s^{-1}) = (F_M' − F_t) / F_M' × 0.5 × PFD (Gao et al., 2018),

140 where F_M' is the maximal fluorescence levels from algae in the actinic light after application a

141 saturating pulse, Ft is the fluorescence at an excitation level and PFD is the actinic light

142 density.

143 *2.4. Estimation of photosynthetic oxygen evolution and respiration*

144 The net photosynthetic and respiration rates of *S. costatum* were measured using a

145 Clark-type oxygen electrode (YSI Model 5300, USA) that was held in a circulating water bath

146 (Cooling Circulator; Cole Parmer, Chicago, IL, USA) to keep the setting temperature (20°C).

147 Five mL of samples were transferred to the oxygen electrode cuvette and were stirred during

148 measurement. The light intensity and temperature were maintained as the same as that in the

149 growth condition. The illumination was provided by a halogen lamp. The increase of oxygen

150 content in seawater within five minutes was defined as net photosynthetic rate. To measure

151 dark respiration rate, the samples were placed in darkness and the decrease of oxygen content

152 within ten minutes was defined as dark respiration rate given the slower oxygen variation rate

153 for dark respiration. Net photosynthetic rate and dark respiration rate were presented as μmol

154 O_2 (10^9 cells)$^{-1}$ h^{-1}.

155 To obtain the curve of net photosynthetic rate versus DIC, seven levels of DIC (0, 0.1, 0.2,

156 0.5, 1, 2, and 4 mM) were made by adding different amounts of $NaHCO_3$ to the Tris buffered

157   DIC-free seawater (pH 8.20). The algal samples were washed twice with DIC-free seawater

158   before transferring to the various DIC solutions. Photosynthetic rates at different DIC levels

159   were measured under saturating irradiance of 400 μmol photons m^{-2} s^{-1} and growth

160   temperature. The algal samples were allowed to equilibrate for 2–3 min at each DIC level

161   during which period a linear change in oxygen concentration was obtained and recorded. The

162   parameter, photosynthetic half saturation constant ($K_{0.5}$, i.e., the DIC concentration required to

163   give half of DIC-saturated maximum rate of photosynthetic O_2 evolution), was calculated

164   from the Michaelis-Menten kinetics equation (Caemmerer and Farquhar 1981): $V = V_{max} \times [S]$

165    $/ (K_{0.5} + [S])$, where V is the real-time photosynthetic rate, V_{max} is maximum photosynthetic

166   rate and [S] is the DIC concentration. The value of $1/ K_{0.5}$ represents photosynthetic DIC

167   affinity. $K_{0.5}$ for CO_2 was calculated via CO2SYS (Pierrot *et al.*, 2006) based on pH and TA,

168   using the equilibrium constants of K1 and K2 for carbonic acid dissociation (Roy *et al.*, 1993)

169   and the KSO_4^- dissociation constant from Dickson (1990).

170   *2.5. Measurement of photosynthetic pigment*

171   To determine the photosynthetic pigment (Chl *a*) content, 50 mL of culture were filtered

172   on a Whatman GF/F filter, extracted in 5 mL of 90% acetone for 12 h at $4^{o}C$, and centrifuged

173   (3, 000 *g*, 5 min). The optical density of the supernatant was scanned from 200 to 700 nm

174   with a UV-VIS spectrophotometer (Shimadzu UV-1800, Kyoto, Japan). The concentration of

175   Chl *a* was calculated based on the optical density at 630 and 664 nm: Chl *a* $=11.47 \times OD_{664} -$

176    $0.40 \times OD_{630}$, Chl *c* $=24.36 \times OD_{630} - 3.73 \times OD_{664}$ (Gao et al., 2018b), and was normalized

177   to pg cells^{-1}.

178   *2.6. Measurement of extracellular carbonic anhydrase activity*

179   Carbonic anhydrase activity was assessed using the electrometric method (Gao *et al.*,

180   2009). Cells were harvested by centrifugation at 4, 000 *g* for five minutes at $20^{\circ}C$, washed

181   once and resuspended in 8 mL Na-barbital buffer (20 mM, pH 8.2). Five mL CO_2-saturated

182   icy distilled water was injected into the cell suspension, and the time required for a pH

183   decrease from 8.2 to 7.2 at $4^{\circ}C$ was recorded. Extracellular carbonic anhydrase (CA_{ext})

184   activity was measured using intact cells. CA activity (E.U.) was calculated using the

185   following formula: E.U. $= 10 \times (T_0 / T - 1)$, where T_0 and T represent the time required for the

186   pH change in the absence or presence of the cells, respectively.

187   *2.7. Measurement of redox activity in the plasma membrane*

188   The redox activity of plasma membrane was assayed by monitoring the change in

189     $K_3Fe(CN)_6$ concentration that accompanied reduction of the ferricyanide to ferrocyanide. The

190   ferricyanide [$K_3Fe(CN)_6$] cannot penetrate intact cells and has been used as an external

191   electron acceptor (Nimer *et al.*, 1998; Wu & Gao, 2009). Stock solutions of $K_3Fe(CN)_6$ were

192   freshly prepared before use. Five mL of samples were taken after two hours of incubation

193   with 500 μmol $K_3Fe(CN)_6$ and centrifuged at 4000 g for 10 min ($20^{\circ}C$). The concentration of

194     $K_3Fe(CN)_6$ in the supernatant was measured spectrophotometrically at 420 nm (Shimadzu

195   UV-1800, Kyoto, Japan). The decrease of $K_3Fe(CN)_6$ during the two hours of incubation

196   was used to assess the rate of extracellular ferricyanide reduction that was presented as μmol

197     $(10^6 \, \text{cells})^{-1} \, h^{-1}$ (Nimer et al., 1998).

198   *2.8. Cell-driving pH drift experiment*

199   To obtain the pH compensation point, the cells were transferred to sealed glass vials

200   containing fresh medium (pH 8.2) with corresponding phosphate levels. The cell

201   concentration for all treatments was 5.0×10^5 mL^{-1}. The pH drift of the suspension was

202   monitored at 20°C and 200 μmol photons m^{-2} s^{-1} light level. The pH compensation point was

203   obtained when there was no a further increase in pH.

204   *2.9. Statistical analysis*

205   Results were expressed as means of replicates $\pm$ standard deviation and data were

206   analyzed using the software SPSS v.21. The data from each treatment conformed to a normal

207   distribution (Shapiro-Wilk, $P > 0.05$) and the variances could be considered equal (Levene's

208   test, $P > 0.05$). Two-way ANOVAs were conducted to assess the effects of CO_2 and phosphate

209   on net photosynthetic rate, dark respiration rate, ratio of net photosynthetic rate to dark

210   respiration rate, rETR, Chl *a*, $K_{0.5}$, CA$_{ext}$, reduction rate of ferricyanide, and pH compensation

211   point. Least Significant Difference (LSD) was conducted for *post hoc* investigation. Repeated

212   measures ANOVAs were conducted to analyze the effects of DIC on net photosynthetic rate

213   and the effect of incubation time on media pH in a closed system. Bonferroni was conducted

214   for *post hoc* investigation as it is the best reliable *post hoc* test for repeated measures ANOVA

215   (Ennos, 2007). The threshold value for determining statistical significance was $P < 0.05$.

216   **3. Results**

217   *3.1. Effects of CO_2 and phosphate on photosynthetic and respiratory performances*

218   The net photosynthetic rate and dark respiration rate in *S. costatum* grown at various CO_2

219   and phosphate concentrations were first investigated (Fig. 1). CO_2 interacted with phosphate

220   on net photosynthetic rate, with each factor having a main effect (Table 1). *Post hoc* LSD

221   comparison ($P = 0.05$) showed that LC reduced net photosynthetic rate when the phosphate

222   levels was below 4 μmol L^{-1} but did not affect it at the higher phosphate levels. Under AC, net

223 photosynthetic rate increased with phosphate level and reached the plateau (100.51 $\pm$ 9.59

224 μmol O_2 $(10^9$ cells$)^{-1}$ h^{-1}) at 1 μmol L^{-1} phosphate. Under LC, net photosynthetic rate also

225 increased with phosphate level but did not hit the peak (101.46 $\pm$ 9.19 μmol O_2 $(10^9$ cells$)^{-1}$ h^{-1})

226 until 4 μmol L^{-1} phosphate. In terms of dark respiration rate (Fig. 1b), phosphate had a main

227 effect on it and it interacted with CO_2 (Table 1). Specifically, LC increased dark respiration

228 rate at 0.05 and 0.25 μmol L^{-1} phosphate levels, but did not affect it when phosphate level was

229 above 1 μmol L^{-1} (LSD, $P < 0.05$). Regardless of CO_2 level, respiration rate increased with

230 phosphate availability and stopped at 1 μmol L^{-1}.

231   The ratio of respiration to photosynthesis ranged from 0.23 to 0.40 (Fig. 2). Both CO_2 and

232 phosphate had a main effect, and they interacted on the ratio of respiration to photosynthesis

233 (Table 1). LC increased the ratio when phosphate was lower than 4 μmol L^{-1} but did not affect

234 it when phosphate levels were 4 or 10 μmol L^{-1}.

235   Both CO_2 and phosphate affected rETR and they also showed an interactive effect (Fig. 3

236 & Table 2). For instance, *post hoc* LSD comparison showed that LC did not affect rETR at

237 lower phosphate levels (0.05 and 0.25 μmol L^{-1}) but increased it at higher phosphate levels

238 (1–10 μmol L^{-1}). Regardless of CO_2 treatment, rETR increased with phosphate level (0.05–4

239 μmol L^{-1}) but the highest phosphate concentration did not result in a further increase in rETR

240 (LSD, $P > 0.05$).

241   The content of Chl *a* was measured to investigate the effects of CO_2 and phosphate on

242 photosynthetic pigment in *S. costatum* (Fig. 4). Both CO_2 and phosphate affected the

243 synthesis of Chl *a* and they had an interactive effect (Table 2). *Post hoc* LSD comparison ($P =$

244 0.05) showed that LC did not affect Chl *a* at 0.05 or 0.25 μmol L^{-1} phosphate but stimulated

245 Chl *a* synthesis at higher phosphate levels (1–10 μmol L^{-1}). Irrespective of CO_2 treatment, Chl

246 *a* content increased with phosphate level and reached the plateau (0.19 ±0.01 pg cell^{-1} for AC

247 and 0.23 ±0.01 pg cell^{-1} for LC) at 4 μmol L^{-1} phosphate.

248   To assess the effects of CO_2 and phosphate on photosynthetic CO_2 affinity in *S. costatum*,

249 the net photosynthetic rates of cells exposure to seven levels of DIC were measured (Fig. 5).

250 After curve fitting, the values of $K_{0.5}$ for CO_2 were calculated (Fig. 6). CO_2 and phosphate

251 interplayed on $K_{0.5}$ and each had a main effect (Table 2). LC did not affect $K_{0.5}$ at the lowest

252 phosphate level but reduced it at the other phosphate levels. Under AC, higher phosphate

253 levels (0.25–4 μmol L^{-1}) reduced $K_{0.5}$ and the highest phosphate level led to a further decrease

254 to 2.59 ±0.29 μmol kg^{-1} seawater compared to the value of 4.00 ±0.30 μmol kg^{-1} seawater at

255 0.05 μmol L^{-1} phosphate. The pattern with phosphate under LC was the same as the AC.

256 *3.3. The effects of CO_2 and phosphate on inorganic carbon acquisition*

257   To investigate the potential mechanisms that cells overcame CO_2 limitation during algal

258 blooms, the activity of CA_{ext}, a CCM related enzyme, was estimated under various CO_2 and

259 phosphate conditions (Fig. 7a). Both CO_2 and phosphate had a main effect and they interacted

260 on CA_{ext} activity (Table 3). *Post hoc* LSD comparison ($P = 0.05$) showed that LC induced

261 more CA_{ext} activity under all phosphate conditions except for 0.05 μmol L^{-1} levels, compared

262 to AC. Under AC, CA_{ext} activity increased (0.04–0.10 EU (10^6 cells)$^{-1}$) with phosphate level

263 and stopped increasing at 1 μmol L^{-1} phosphate. Under LC, CA_{ext} activity also increased

264 (0.04–0.35 EU (10^6 cells)$^{-1}$) with phosphate level but reached the peak at 4 μmol L^{-1}

265 phosphate. The redox activity of plasma membrane was also assessed to investigate the

266 factors that modulate CA_{ext} activity (Fig. 7b). The pattern of redox activity of plasma

267   membrane under various CO_2 and phosphate conditions was the same as that of CA_{ext} activity.

268   That is, CO_2 and phosphate had an interactive effect on redox activity of plasma membrane,

269   each having a main effect (Table 3).

270   To test cells' tolerance to high pH and obtain pH compensation points in *S. costatum*

271   grown under various CO_2 and phosphate levels, changes of media pH in a closed system were

272   monitored (Fig. 8). The media pH under all phosphate conditions increased with incubation

273   time (Table 4). Specifically speaking, there was a steep increase in pH during the first three

274   hours, afterwards the increase became slower and it reached a plateau in six hours (Bonferroni,

275     $P < 0.05$). Phosphate had an interactive effect with incubation time (Table 4). For instance,

276   there was no significant difference in media pH among phosphate levels during first two

277   hours of incubation but then divergence occurred and they stopped at different points.

278   Two-way ANOVA analysis showed that CO_2 treatment did not affect pH compensation point

279   but phosphate had a main effect (Table 3). Under each CO_2 treatment, pH compensation point

280   increased with phosphate level, with lowest of 9.03 ± 0.03 at 0.05 µmol L^{-1} and highest of

281   9.36 ± 0.04 at 10 µmol L^{-1} phosphate.

282   **4. Discussion**

283   *4.1. Photosynthetic performances under various CO_2 and phosphate conditions*

284   The lower CO_2 availability reduced the net photosynthetic rate of *S. costatum* grown at

285   the lower phosphate levels in the present study. However, Nimer *et al.* (1998) demonstrated

286   that the increase in pH (8.3−9.5) did not reduce photosynthetic CO_2 fixation of *S. costatum*

287   and Chen and Gao (2004) reported that a higher pH (8.7) even stimulated the photosynthetic

288   rate of *S. costatum* compared to the control (pH 8.2). The divergence between our and the

289   previous studies may be due to different nutrient supply. Both Nimer *et al.* (1998) and Chen

290   and Gao (2004) used f/2 media to grow algae. The phosphate concentration in f/2 media is

291   ~36 μmol L^{-1}, which is replete for physiological activities in *S. costatum*. *Skeletonema*

292   *costatum* grown at higher phosphate levels (4 and 10 μmol L^{-1}) also showed similar

293   photosynthetic rates for the lower and higher CO_2 treatments. Our finding combined with the

294   previous studies indicates phosphorus plays an important role in dealing with low CO_2

295   availability for photosynthesis in *S. costatum*.

296   Different from net photosynthetic rate, LC did not affect rETR at lower phosphate levels

297   (0.05 and 0.25 μmol L^{-1}) and stimulated it at higher phosphate levels (1−10 μmol L^{-1}). This

298   interactive effect of CO_2 and phosphate may be due to their effects on Chl *a*. LC induced

299   more synthesis of Chl *a* at higher phosphate levels (1−10 μmol L^{-1}). This induction of LC on

300   photosynthetic pigment is also reported in green algae (Gao *et al.*, 2016). More energy is

301   required under LC to address the more severe CO_2 limitation and thus more Chl *a* are

302   synthesized to capture more light energy, particularly when phosphate was replete. Although P

303   is not an integral component for chlorophyll, it plays an important role in cell energetics

304   through high-energy phosphate bonds, i.e. ATP, which could support chlorophyll synthesis.

305   The stimulating effect of P enrichment on photosynthetic pigment is also found in green alga

306   *Dunaliella tertiolecta* (Geider *et al.*, 1998) and brown alga *Sargassum muticum* (Xu et al.,

307   2017). The increased photosynthetic pigment in *S. costatum* could partially explain the

308   increased rETR and photosynthetic rate under the higher P conditions.

309   *4.6. Ratio of respiration to photosynthesis*

310   The ratio of respiration to photosynthesis in algae indicates carbon balance in cells and

311 carbon flux in marine ecosystems as well (Zou & Gao, 2013). LC increased this ratio in *S.*

312 *costatum* grown at the lower P conditions but did not affect it under the higher P conditions,

313 indicating that P enrichment can offset the carbon loss caused by carbon limitation. To cope

314 with CO_2 limitation, cells might have to obtain energy from dark respiration under lower P

315 conditions as it seems infeasible to acquire energy from the low rETR, which led to the

316 increased dark respiration. However, LC induced higher rETR under P replete conditions and

317 energy used for inorganic carbon (CO_2 and HCO_3^-) acquisition could be from the increased

318 rETR. Therefore, additional dark respiration was not triggered, avoiding carbon loss. Most

319 studies regarding the effect of CO_2 on ratio of respiration to photosynthesis focus on higher

320 plants (Gifford, 1995; Ziska & Bunce, 1998; Cheng *et al.*, 2010; Smith & Dukes, 2013), little

321 is known on phytoplankton. Our study suggests that CO_2 limitation may lead to carbon loss in

322 phytoplankton but P enrichment could alter this trend, regulating carbon balance in

323 phytoplankton and thus their capacity in carbon sequestration.

324 *4.3. Inorganic carbon acquisition under CO_2 limitation and phosphate enrichment*

325   Decreased CO_2 can usually induce higher inorganic carbon affinity in algae (Raven *et al.*,

326 2012; Wu *et al.*, 2012; Raven *et al.*, 2017; Xu *et al.*, 2017). In the present study, the lower

327 CO_2 did increase inorganic carbon affinity when P level was higher than 0.25 $\mu mol\ L^{-1}$ but did

328 not affect it when P was 0.05 $\mu mol\ L^{-1}$, indicating the important role of P in regulating cells'

329 CCMs in response to environmental CO_2 changes. LC induced larger CA activity when P was

330 above 0.25 $\mu mol\ L^{-1}$ but did not increase it at 0.05 $\mu mol\ L^{-1}$ of P, which could explain the

331 interactive effect of P and CO_2 on inorganic carbon affinity as CA can accelerate the

332 equilibrium between HCO_3^- and CO_2 and increase inorganic carbon affinity. Regardless of

333   CO_2, P enrichment alone increased CA activity and inorganic carbon affinity. P enrichment

334   may stimulate the synthesis of CA by supplying required ATP. In addition, P enrichment

335   increased the redox activity of plasma membrane in this study. It has been proposed that redox

336   activity of plasma membrane could induce extracellular CA activity via protonation extrusion

337   of its active center (Nimer *et al.*, 1998). Our result that the pattern of CA is exactly same as

338   that of redox activity of plasma membrane shows a compelling correlation between CA and

339   redox activity of plasma membrane. The stimulating effect of P on redox activity of plasma

340   membrane may be due to its effect on rETR. The increased rETR could generate excess

341   reducing equivalents, particularly under CO_2 limiting conditions. These excess reducing

342   equivalents would be transported from the chloroplast into the cytosol (Heber, 1974),

343   supporting the redox chain in the plasma membrane (Rubinstein & Luster, 1993; Nimer *et al.*,

344   1999) and triggering CA activity.

345   *4.4. Direct HCO_3^- utilization due to phosphate enrichment*

346   A pH compensation point over 9.2 has been considered a sign of direct HCO_3^- use for

347   algae (Axelsson & Uusitalo, 1988) as the CO_2 concentration is nearly zero at pH above 9.2.

348   This criterion has been justified based on the experiments for both micro and macro-algae.

349   For instance, the marine diatom *Phaeodactylum tricornutum*, with a strong capacity for direct

350   HCO_3^- utilization, has a higher pH compensation point of 10.3 (Chen *et al.*, 2006). In contrast,

351   the red macroalgae, *Lomentaria articulata* and *Phycodrys rubens* that cannot utilize HCO_3^-

352   directly and photosynthesis only depends on CO_2 diffusion, have pH compensation points of

353   less than 9.2 (Maberly, 1990). In terms of *S. costatum*, it has been reported to have a pH

354   compensation point of 9.12, indicating a very weak capacity in direct HCO_3^- utilization (Chen

355 & Gao, 2004). Our study demonstrates that the pH compensation point of *S. costatum* varies

356 with the availability of P. It is lower than 9.2 under P limiting conditions but higher than 9.2

357 under P replete conditions, suggesting that the capacity of direct HCO_3^- utilization is regulated

358 by P availability. Contrary to CO_2 passive diffusion, the direct use of HCO_3^- depends on

359 positive transport that requires energy (Hopkinson & Morel, 2011). P enrichment increased

360 rETR in the present study and the ATP produced during the process of electron transport could

361 be used to support HCO_3^- positive transport. In addition, the increased respiration at higher P

362 levels can also generate ATP to help HCO_3^- positive transport. Our study indicates that P

363 enrichment could trigger HCO_3^- direct utilization and hence increase inorganic acquisition

364 capacity of *S. costatum* to cope with CO_2 limitation.

365 *4.5. CCMs and red tides*

366 With the development of red tides, the pH in seawater could be very high along with

367 extremely low CO_2 availability due to intensive photosynthesis (Hansen, 2002; Hinga, 2002).

368 For instance, pH level in the surface waters of the eutrophic Mariager Fjord, Denmark, is

369 often above 9 during dinoflagellate blooms (Hansen, 2002). Diatoms are the casautive species

370 for red tides and *S. costatum* could outcompete other bloom algae (dinoflagellates

371 *Prorocentrum minimum* and *Alexandrium tamarense*) under nutrient replete conditions (Hu *et*

372 *al.*, 2011). However, the potential mechanisms are poorly understood. Our study demonstrates

373 *S. costatum* has multiple CCMs to cope with CO_2 limitation and the operation of CCMs is

374 regulated by P availability. The CCMs of *S. costatum* are hampered under P limiting

375 conditions and only function when P is replete. Therefore, P enrichment would be critical for

376 *S. costatum* to overcome carbon limitation during algal bloom and to dominate red tides.

5. Conclusions

The present study investigated the role of P in regulating inorganic carbon acquisition and CO_2 concentrating mechanisms in diatoms for the first time. The intensive photosynthesis and quick growth during algal blooms usually result in noticeable increase of pH and decrease of CO_2. Our study demonstrates that P enrichment could induce activity of extracellular carbonic anhydrase and direct utilization of HCO_3^- in *S. costatum* to help overcome the CO_2 limitation, as well as increasing photosynthetic pigment content and rETR to provide required energy. This study provides important insight into the connection of phosphorus and carbon acquisition in diatoms and the mechanisms that *S. costatum* dominates algal blooms.

Author contribution

JX and GG designed the experiments, and GG, JY, JF and XZ carried them out. GG prepared the manuscript with contributions from all co-authors.

Acknowledgements

References

Anderson DM, Burkholder JM, Cochlan WP, Glibert PM, Gobler CJ, Heil CA, Kudela R, Parsons ML, Rensel JE, Townsend DW. 2008. Harmful algal blooms and

399   eutrophication: Examining linkages from selected coastal regions of the United States.

400   *Harmful Algae* **8**: 39-53.

401   **Axelsson L, Uusitalo J. 1988.** Carbon acquisition strategies for marine macroalgae. *Marine*

402   *Biology* **97**: 295-300.

403   **Barton AD, Irwin AJ, Finkel ZV, Stock CA. 2016.** Anthropogenic climate change drives

404   shift and shuffle in North Atlantic phytoplankton communities. *Proceedings of the*

405   *National Academy of Sciences, USA* **113**: 2964-2969.

406   **Beamud SG, Baffico GD, Reid B, Torres R, Gonzalez-Polo M, Pedrozo F, Diaz M. 2016.**

407   Photosynthetic performance associated with phosphorus availability in mats of

408   *Didymosphenia geminata* (Bacillariophyceae) from Patagonia (Argentina and Chile).

409   *Phycologia* **55**: 118-125.

410   **Beardall J, Roberts S, Raven JA. 2005.** Regulation of inorganic carbon acquisition by

411   phosphorus limitation in the green alga *Chlorella emersonii*. *Canadian Journal of*

412   *Botany* **83**: 859-864.

413   **Berg GM, Glibert PM, Lomas MW, Burford MA. 1997.** Organic nitrogen uptake and

414   growth by the chrysophyte *Aureococcus anophagefferens* during a brown tide event.

415   *Marine Biology* **129**: 377-387.

416   **Brembu T, Mühlroth A, Alipanah L, Bones AM. 2017.** The effects of phosphorus limitation

417   on carbon metabolism in diatoms. *Philosophical Transactions of the Royal Society B*

418   *Biological Sciences* **372**: 20160406.

419   **Bruland KW, Rue EL, Smith GJ. 2001.** Iron and macronutrients in california coastal

420   upwelling regimes: implications for diatom blooms. *Limnology and Oceanography* **46**:

421   1661-1674.

422 **Caemmerer SV, Farquhar GD. 1981.** Some relationships between the biochemistry of

423   photosynthesis and the gas exchange of leaves. *Planta* 153: 376-387.

424 **Chen X, Gao K. 2004.** Photosynthetic utilisation of inorganic carbon and its regulation in the

425   marine diatom *Skeletonema costatum. Functional Plant Biology* **31**: 1027-1033.

426 **Chen X, Qiu CE, Shao JZ. 2006.** Evidence for K^+-dependent HCO_3^- utilization in the marine

427   diatom *Phaeodactylum tricornutum. Plant Physiology* **141**: 731-736.

428 **Cheng W, Sims DA, Luo Y, Coleman JS, Johnson DW. 2010.** Photosynthesis, respiration,

429   and net primary production of sunflower stands in ambient and elevated atmospheric

430   CO_2 concentrations: an invariant NPP:GPP ratio? *Global Change Biology* **6**: 931-941.

431 **Davies AG, Sleep JA. 1989.** The photosynthetic response of nutrient-depleted dilute cultures

432   of *Skeletonema costatum* to pulses of ammonium and nitrate; the importance of

433   phosphate. *Journal of Plankton Research* **11**: 141-164.

434 **Dickson AG. 1990.** Standard potential of the reaction: AgCl(s) + 12H2(g) = Ag(s) + HCl(aq),

435   and the standard acidity constant of the ion HSO_4^- in synthetic sea water from 273.15

436   to 318.15 K. *Journal of Chemical Thermodynamics* **22**: 113-127.

437 **Ennos AR. 2017.** Statistical and Data Handling Skills in Biology. Pearson Education, p.96.

438 **Falkowski P. 2012.** Ocean Science: The power of plankton. *Nature* **483**: 17-20.

439 **Field CB, Behrenfeld MJ, Randerson JT, Falkowski P. 1998.** Primary production of the

440   biosphere: integrating terrestrial and oceanic components. *Science* **281**: 237-240.

441 **Gao G, Clare AS, Rose C, Caldwell GS. 2018a.** *Ulva rigida* in the future ocean: potential

442   for carbon capture, bioremediation, and biomethane production. *Global Change*

443   *Biology Bioenergy* **10:** 39-51.

444 **Gao G, Gao K, Giordano M. 2009.** Responses to solar UV radiation of the diatom

445   *Skeletonema costatum* (Bacillariophyceae) grown at different Zn^{2+} concentrations

446   *Journal of Phycology* **45**: 119-129.

447 **Gao G, Liu Y, Li X, Feng Z, Xu J. 2016.** An ocean acidification acclimatised green tide alga

448   Is robust to changes of seawater carbon chemistry but vulnerable to light stress. *PloS*

449   *One* **11**: e0169040.

450 **Gao G, Xia J, Yu J, Zeng X. 2018b.** Physiological response of a red tide alga (*Skeletonema*

451   *costatum*) to nitrate enrichment, with special reference to inorganic carbon acquisition.

452   *Marine Environmental Research*, **133:** 15-23.

453 **Geider RJ, Macintyre HL, Graziano LM, McKay RML. 1998.** Responses of the

454   photosynthetic apparatus of *Dunaliella tertiolecta* (Chlorophyceae) to nitrogen and

455   phosphorus limitation. *European Journal of Phycology* **33**: 315-332.

456 **Gifford RM. 1995.** Whole plant respiration and photosynthesis of wheat under increased CO_2

457   concentration and temperature: long-term vs. short-term distinctions for modelling.

458   *Global Change Biology* **1**: 385–396.

459 **Hansen PJ. 2002.** Effect of high pH on the growth and survival of marine phytoplankton:

460   implications for species succession. *Aquatic Microbial Ecology* **28**: 279-288.

461 **Heber U. 1974.** Metabolite exchange between chloroplasts and cytoplasm. *Annual Review of*

462   *Plant Physiology* **25**: 393-421.

463 **Hinga KR. 2002.** Effects of pH on coastal marine phytoplankton. *Marine Ecology Progress*

464   *Series* **238**: 281-300.

465 **Hopkinson BM, Dupont CL, Matsuda Y. 2016.** The physiology and genetics of CO_2

466   concentrating mechanisms in model diatoms. *Current Opinion in Plant Biology* **31**:

467   51-57.

468   **Hopkinson BM, Morel FMM. 2011.** Efficiency of the CO_2-concentrating mechanism of

469   diatoms. *Proceedings of the National Academy of Sciences, USA* **108**: 3830-3837.

470   **Hu H, Zhang J, Chen W. 2011.** Competition of bloom-forming marine phytoplankton at low

471   nutrient concentrations. *Journal of Environmental Sciences* **23**: 656-663.

472   **Hu H, Zhou Q. 2010.** Regulation of inorganic carbon acquisition by nitrogen and phosphorus

473   levels in the *Nannochloropsis* sp. *World Journal of Microbiology & Biotechnology* **26**:

474   957-961.

475   **Jeong HJ, An SL, Franks PJS, Lee KH, Ji HK, Kang NS, Lee MJ, Jang SH, Lee SY,**

476   **Yoon EY. 2015.** A hierarchy of conceptual models of red-tide generation: Nutrition,

477   behavior, and biological interactions. *Harmful Algae* **47**: 97-115.

478   **Jiang X, Han Q, Gao X, Gao G. 2016.** Conditions optimising on the yield of biomass, total

479   lipid, and valuable fatty acids in two strains of *Skeletonema menzelii*. *Food Chemistry*

480   **194**: 723-732.

481   **Li G, Gao K, Yuan D, Zheng Y, Yang G. 2011.** Relationship of photosynthetic carbon

482   fixation with environmental changes in the Jiulong River estuary of the South China

483   Sea, with special reference to the effects of solar UV radiation. *Marine Pollution*

484   *Bulletin* **62**: 1852-1858.

485   **Lin S, Litaker RW, Sunda WG. 2016.** Phosphorus physiological ecology and molecular

486   mechanisms in marine phytoplankton. *Journal of Phycology* **52**: 10.

487   **Liu Y, Song X, Cao X, Yu Z. 2012.** Responses of photosynthetic characters of *Skeletonema*

488   *costatum* to different nutrient conditions. *Journal of Plankton Research* **35**: 165-176.

489   **Maberly SC. 1990.** Exogenous sources of inorganic carbon for photosynthesis by marine

490   macroalgae. *Journal of Phycology* **26**: 439-449.

491   **Mccall SJ, Hale MS, Smith JT, Read DS, Bowes MJ. 2017.** Impacts of phosphorus

492   concentration and light intensity on river periphyton biomass and community structure.

493   *Hydrobiologia* **792**: 315-330.

494   **Moore CM, Mills MM, Arrigo KR, Bermanfrank I, Bopp L, Boyd PW, Galbraith ED,**

495   **Geider RJ, Guieu C, Jaccard SL. 2013.** Processes and patterns of oceanic nutrient

496   limitation. *Nature Geoscience* **6**: 701-710.

497   **Müller S, Mitrovic SM. 2015.** Phytoplankton co-limitation by nitrogen and phosphorus in a

498   shallow reservoir: progressing from the phosphorus limitation paradigm.

499   *Hydrobiologia* **744**: 255-269.

500   **Nelson DM, Tréguer P, Brzezinski MA, Leynaert A, Quéguiner B. 1995.** Production and

501   dissolution of biogenic silica in the ocean: Revised global estimates, comparison with

502   regional data and relationship to biogenic sedimentation. *Global Biogeochemical*

503   *Cycles* **9**: 359-372.

504   **Nimer NA, Ling MX, Brownlee C, Merrett MJ. 1999.** Inorganic carbon limitation,

505   exofacial carbonic anhydrase activity, and plasma membrane redox activity in marine

506   phytoplankton species. *Journal of Phycology* **35**: 1200-1205.

507   **Nimer NA, Warren M, Merrett MJ. 1998.** The regulation of photosynthetic rate and

508   activation of extracellular carbonic anhydrase under CO_2-limiting conditions in the

509   marine diatom *Skeletonema costatum*. *Plant Cell and Environment* **21**: 805–812.

510   **Pierrot D, Lewis E, Wallace DWR. 2006.** MS Excel program developed for CO_2 system

511   calculations. *ORNL/CDIAC-105a. Carbon Dioxide Information Analysis Center, Oak*

512   *Ridge National Laboratory, US Department of Energy, Oak Ridge, Tennessee.*

513   **Raven JA, Beardall J, Sánchez-Baracaldo P. 2017.** The possible evolution, and future, of

514   CO2-concentrating mechanisms. *Journal of Experimental Botany.*

515   **Raven JA, Giordano M, Beardall J, Maberly SC. 2012.** Algal evolution in relation to

516   atmospheric CO2: carboxylases, carbon-concentrating mechanisms and carbon

517   oxidation cycles. *Phil. Trans. R. Soc. B* **367**: 493-507.

518   **Reed ML, Pinckney JL, Keppler CJ, Brock LM, Hogan SB, Greenfield DI. 2016.** The

519   influence of nitrogen and phosphorus on phytoplankton growth and assemblage

520   composition in four coastal, southeastern USA systems. *Estuarine Coastal & Shelf*

521   *Science* **177**: 71-82.

522   **Rost B, Riebesell U, Burkhardt S, Sültemeyer D. 2003.** Carbon acquisition of

523   bloom-forming marine phytoplankton. *Limnology and Oceanography* **48**: 55-67.

524   **Roy RN, Roy LN, Vogel KM, Porter-Moore C, Pearson T, Good CE, Millero FJ,**

525   **Campbell DM. 1993.** The dissociation constants of carbonic acid in seawater at

526   salinities 5 to 45 and temperatures 0 to 45°C. *Marine Chemistry* **44**: 249-267.

527   **Rubinstein B, Luster DG. 1993.** Plasma membrane redox activity: components and role in

528   plant processes. *Annual Review of Plant Biology* **44**: 131-155.

529   **Smetacek V, Zingone A. 2013.** Green and golden seaweed tides on the rise. *Nature* **504**:

530   84-88.

531   **Smith NG, Dukes JS. 2013.** Plant respiration and photosynthesis in global-scale models:

532   incorporating acclimation to temperature and CO_2. *Global Change Biology* **19**: 45-63.

533   **Wang J. 2002.** Phytoplankton communities in three distinct ecotypes of the Changjiang

534   estuary. *Journal of Ocean University of China* **32**: 422-428.

535   **Wu H, Gao K. 2009.** Ultraviolet radiation stimulated activity of extracellular carbonic

536   anhydrase in the marine diatom *Skeletonema costatum*. *Functional Plant Biology* **36**:

537   137-143.

538   **Wu X, Gao G, Giordano M, Gao K. 2012.** Growth and photosynthesis of a diatom grown

539   under elevated CO_2 in the presence of solar UV radiation. *Fundamental and Applied*

540   *Limnology* **180**: 279-290.

541   **Xu Z, Gao G, Xu J, Wu H. 2017.** Physiological response of a golden tide alga (*Sargassum*

542   *muticum*) to the interaction of ocean acidification and phosphorus enrichment.

543   *Biogeosciences* **14**: 671-681.

544   **Young JN, Morel FMM. 2015.** Biological oceanography: The CO_2 switch in diatoms. *Nature*

545   *Climate Change* **5**: 722-723.

546   **Ziska LH, Bunce JA. 1998.** The influence of increasing growth temperature and CO_2

547   concentration on the ratio of respiration to photosynthesis in soybean seedlings.

548   *Global Change Biology* **4**: 637-643.

549   **Zou D, Gao K. 2013.** Thermal acclimation of respiration and photosynthesis in the marine

550   macroalga *Gracilaria lemaneiformis* (Gracilariales, Rhodophyta). *Journal of*

551   *Phycology* **49**: 61–68.

552

553

554 Table 1 Two-way analysis of variance for the effects of CO_2 and phosphate on net

555 photosynthetic rate, dark respiration rate and ratio of respiration to photosynthesis of *S.*

556 *costatum*. CO_2*phosphate means the interactive effect of CO_2 and phosphate, df means degree

557 of freedom, F means the value of F statistic, and Sig. means p-value.

558

Source	Net photosynthetic rate			Dark respiration rate			Ratio of respiration to photosynthesis		
	df	F	Sig.	df	F	Sig.	df	F	Sig.
CO_2	1	11.286	0.003	1	1.262	0.275	1	32.443	<0.001
Phosphate	4	157.925	<0.001	4	169.050	<0.001	4	7.081	0.001
CO_2*phosphate	4	3.662	0.021	4	3.226	0.034	4	8.299	<0.001
Error	20			20			20		

559 Table 2 Two-way analysis of variance for the effects of CO_2 and phosphate on relative

560 electron transport rate (rETR), Chl *a*, and CO_2 level required to give half of DIC-saturated

561 maximum rate of photosynthetic O_2 evolution ($K_{0.5}$) of *S. costatum*. CO_2*phosphate means the

562 interactive effect of CO_2 and phosphate, df means degree of freedom, F means the value of F

563 statistic, and Sig. means p-value.

564

Source	rETR			Chl *a*			$K_{0.5}$		
	df	F	Sig.	df	F	Sig.	df	F	Sig.
CO_2	1	28.717	<0.001	1	32.963	<0.001	1	96.182	<0.001
Phosphate	4	127.860	<0.001	4	92.045	<0.001	4	40.497	<0.001
CO_2*phosphate	4	3.296	0.031	4	3.871	0.017	4	3.821	0.018
Error	20			20			20		

565 Table 3 Two-way analysis of variance for the effects of CO_2 and phosphate on CA_{ext} activity,

566 redox activity of plasma membrane and pH compensation point of *S. costatum.*

567 CO_2*phosphate means the interactive effect of CO_2 and phosphate, df means degree of

568 freedom, F means the value of F statistic, and Sig. means p-value.

569

Source	CA_{ext} activity			redox activity of plasma membrane			pH compensation point		
	df	F	Sig.	df	F	Sig.	df	F	Sig.
CO_2	1	569.585	<0.001	1	937.963	<0.001	1	0.056	0.816
Phosphate	4	176.392	<0.001	4	276.362	<0.001	4	226.196	<0.001
CO_2*phosphate	4	87.380	<0.001	4	137.050	<0.001	4	0.040	0.997
Error	20			20			20		

570 Table 4 Repeated measures analysis of variance for the effects of CO_2 and phosphate on pH

571 change during 10 hours of incubation. Time*CO_2 means the interactive effect of incubation

572 time and CO_2, Time*phosphate means the interactive effect of incubation time and phosphate,

573 Time*CO_2*phosphate means the interactive effect of incubation time, CO_2 and phosphate,

574 df means degree of freedom, F means the value of F statistic, and Sig. means p-value.

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Time	40.766	10	4.077	8737.941	<0.001
Time*CO_2	0.003	10	<0.001	0.569	0.838
Time*phosphate	0.886	40	0.022	47.496	<0.001
Time*CO_2*phosphate	0.002	40	<0.001	0.112	1.000
Error	0.093	200	<0.001		

Figure legends

575

576 **Fig. 1.** Net photosynthetic rate (a) and dark respiration rate (b) in *S. costatum* grown at

577 various phosphate concentrations after ambient (AC) and low CO_2 (LC) treatments. The error

578 bars indicate the standard deviations (n = 3). Different letters represent the significant

579 difference ($P < 0.05$) among phosphate concentrations (capital for AC, lower case for LC).

580 Horizontal lines represent significant difference ($P < 0.05$) between CO_2 treatments.

581 **Fig. 2.** Ratio of respiration rate to net photosynthetic rate in *S. costatum* grown at various

582 phosphate concentrations after ambient (AC) and low CO_2 (LC) treatments. The error bars

583 indicate the standard deviations (n = 3). Different letters represent the significant difference (P

584 < 0.05) among phosphate concentrations (capital for AC, lower case for LC). Horizontal lines

585 represent significant difference ($P < 0.05$) between CO_2 treatments.

586 **Fig. 3.** Relative electron transport rate (rETR) in *S. costatum* grown at various phosphate

587 concentrations after ambient (AC) and low CO_2 (LC) treatments. The error bars indicate the

588 standard deviations (n = 3). Different letters represent the significant difference ($P < 0.05$)

589 among phosphate concentrations (Capital for AC lower case for LC). Horizontal lines

590 represent significant difference ($P < 0.05$) between CO_2 treatments.

591 **Fig. 4.** Photosynthetic Chl *a* content in *S. costatum* grown at various phosphate concentrations

592 after ambient (AC) and low CO_2 (LC) treatments. The error bars indicate the standard

593 deviations (n = 3). Different letters represent the significant difference ($P < 0.05$) among

594 phosphate concentrations (capital for AC, lower case for LC). Horizontal lines represent

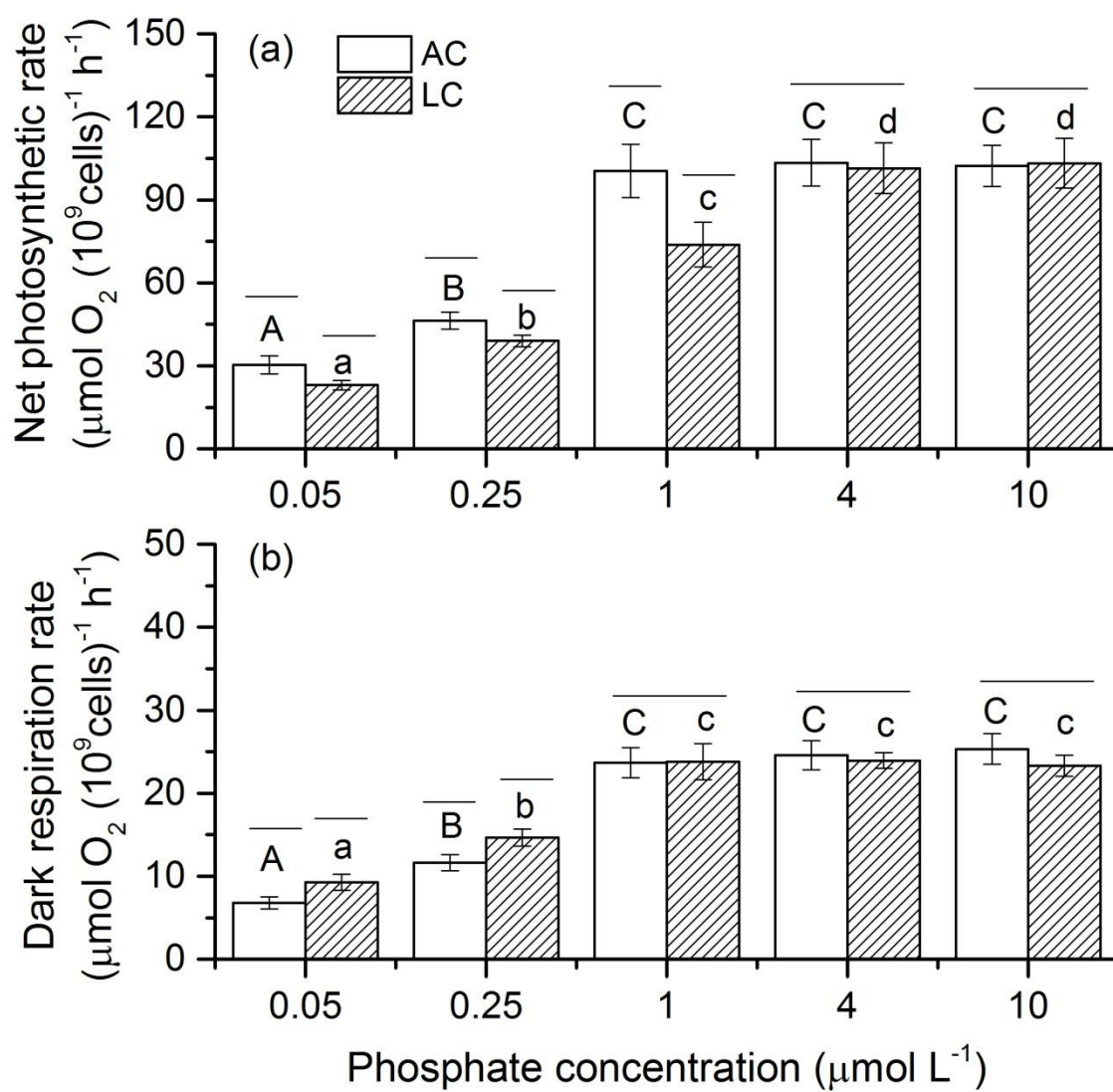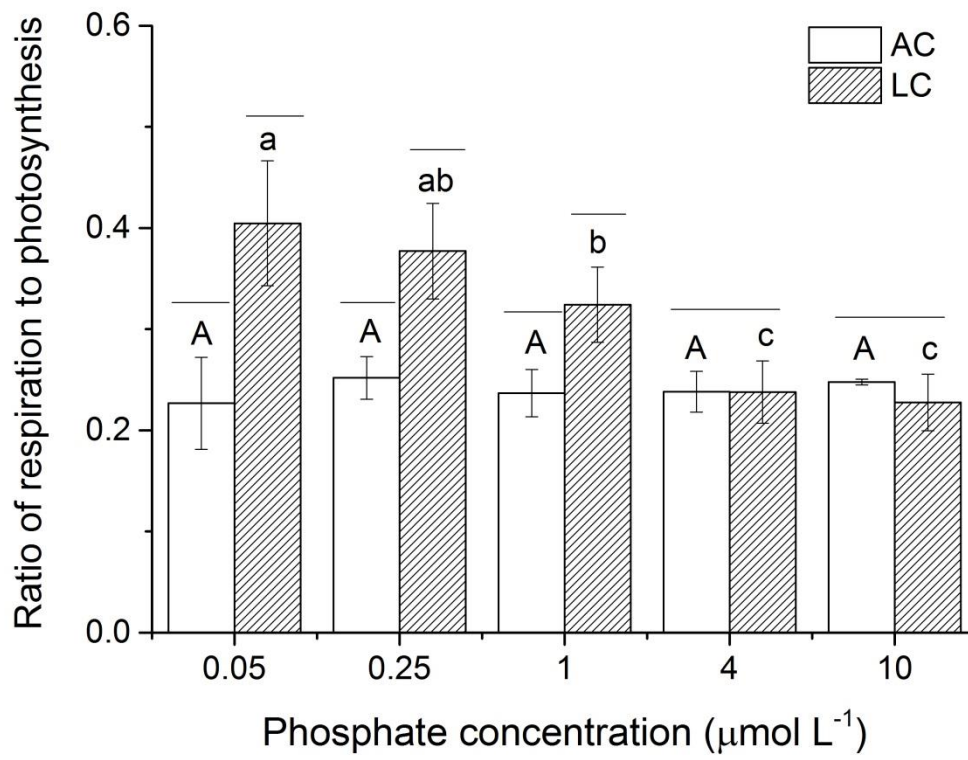595 significant difference ($P < 0.05$) between CO_2 treatments.

596 **Fig. 5.** Net photosynthetic rate as a function of DIC for *S. costatum* grown at various

597   phosphate concentrations after ambient (a) and low CO_2 (b) treatments. The error bars

598   indicate the standard deviations (n = 3).

599   **Fig. 6.** Half saturation constant ($K_{0.5}$) for CO_2 in *S. costatum* grown at at various phosphate

600   concentrations after ambient (AC) and low CO_2 (LC) treatments. The error bars indicate the
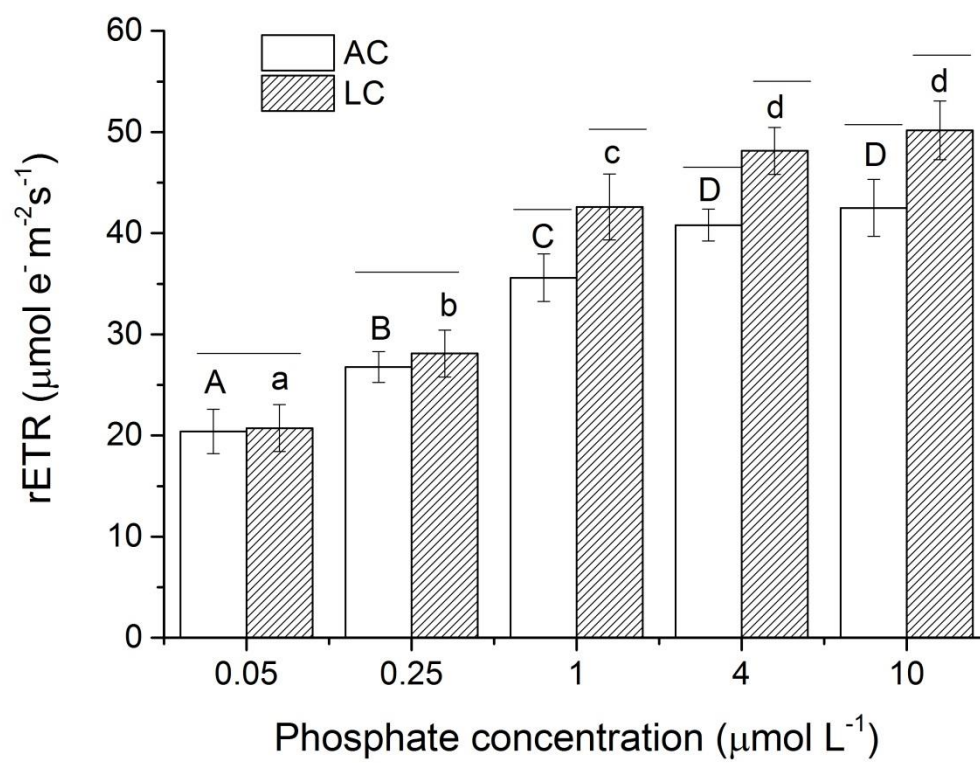
601   standard deviations (n = 3). Different letters represent the significant difference ($P < 0.05$)

602   among phosphate concentrations (capital for AC, lower case for LC). Horizontal lines

603   represent significant difference ($P < 0.05$) between CO_2 treatments.

604   **Fig. 7.** CA_{ext} activity (a) and reduction rate of ferricyanide (b) in *S. costatum* grown at various

605   phosphate concentrations after ambient (AC) and low CO_2 (LC) treatments. The error bars

606   indicate the standard deviations (n = 3). Different letters represent the significant difference (P

607   < 0.05) among phosphate concentrations (capital for AC, lower case for LC). Horizontal lines

608   represent significant difference ($P < 0.05$) between CO_2 treatments.

609   **Fig. 8.** Changes of pH in a closed system caused by photosynthesis of *S. costatum* grown at

610   various phosphate concentrations after ambient (AC) and low CO_2 (LC) treatments. The error

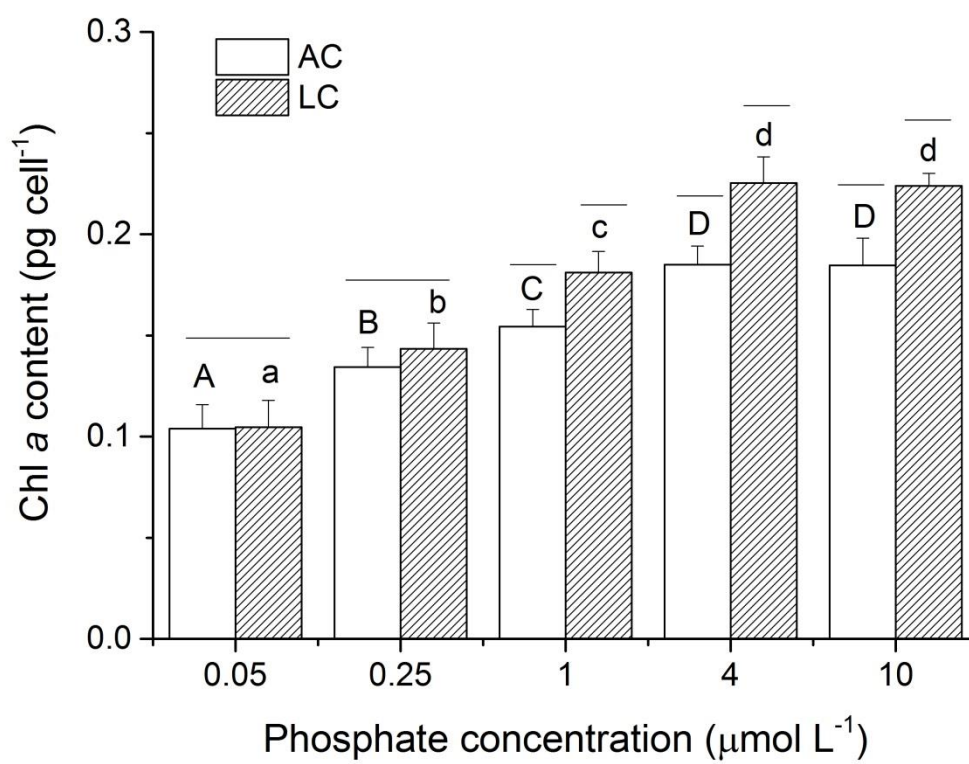611   bars indicate the standard deviations (n = 3).

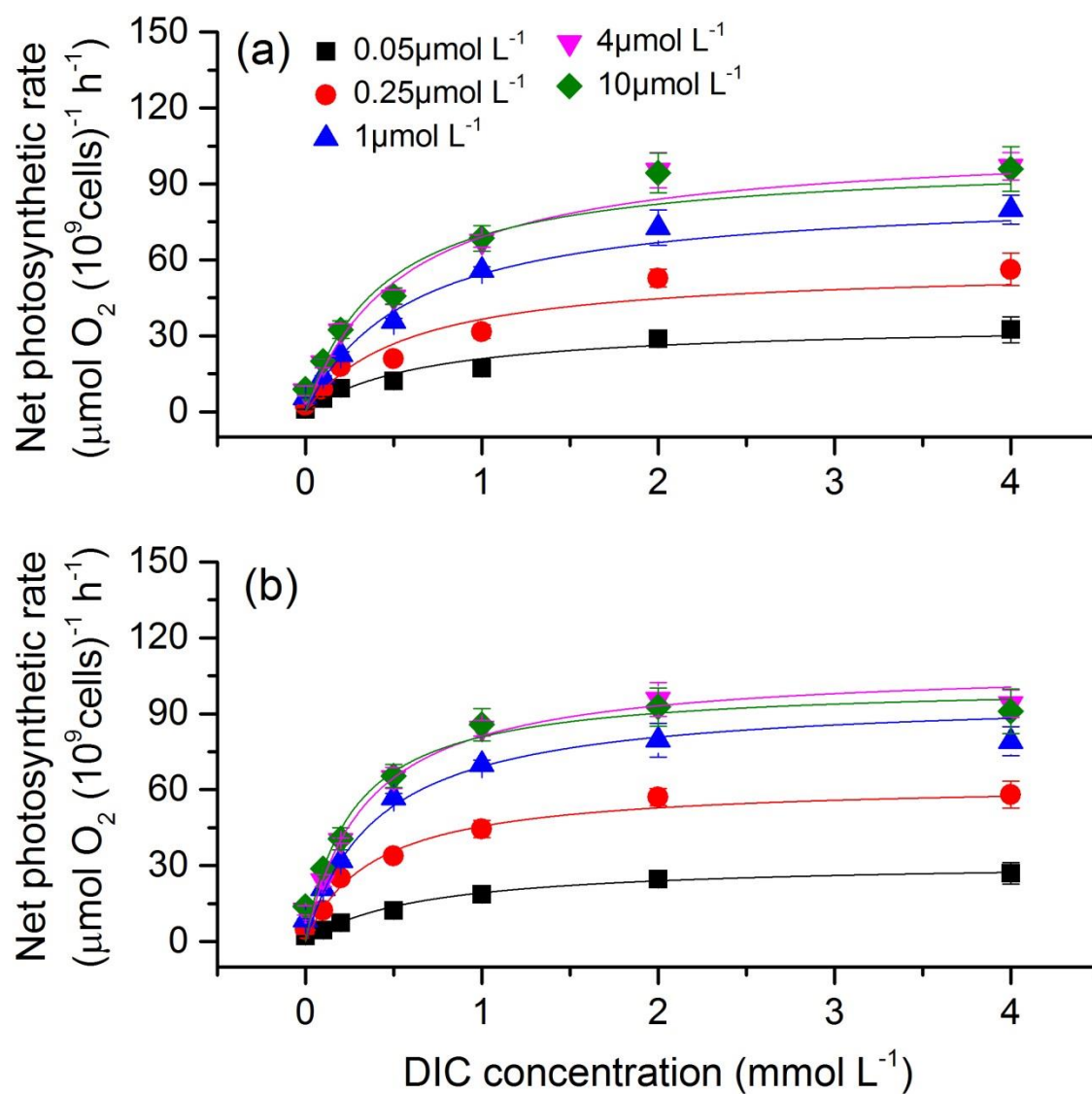Fig. 1

612

613

Fig. 2

616

617   **Fig. 3**

Fig. 4

618

619

Fig. 5

Fig. 6

622

623

Fig. 7

624

625

Fig. 8

626

627

628

629