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

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1 **Abstract:**

2 *S. costatum* is a common bloom-forming diatom and encounters eutrophication and severe

3 CO_2 limitation during red tides. However, little is known regarding the role of phosphorus in

4 modulating inorganic carbon acquisition in *S. costatum*，particularly under CO_2 limitation

5 conditions. We cultured *S. costatum* under five phosphate levels (0.05, 0.25, 1, 4, 10 μmol L^{-1})

6 and then treated it with two CO_2 conditions (2.8 and 12.6 μmol L^{-1}) for two hours. The lower

7 CO_2 reduced net photosynthetic rate at lower phosphate levels (< 4 μmol L^{-1}) but did not

8 affect it at higher phosphate levels (4 and 10 μmol L^{-1}). In contrast, the lower CO_2 induced

9 higher dark respiration rate at lower phosphate levels (0.05 and 0.25 μmol L^{-1}) and did not

10 affect it at higher phosphate levels (> 1 μmol L^{-1}). The lower CO_2 did not change rETR at

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

12 1 μmol L^{-1}). Photosynthetic CO_2 affinity ($K_{0.5}$) decreased with phosphate levels. The lower

13 CO_2 did not affect $K_{0.5}$ at 0.05 μmol L^{-1} phosphate but reduced it at the other phosphate levels.

14 Activity of extracellular carbonic anhydrase was dramatically induced by the lower CO_2 at

15 phosphate replete conditions (> 0.25 μmol L^{-1}) and the same pattern also occurred for redox

16 activity of plasma membrane. Direct HCO_3^- use was induced when phosphate concentration is

17 more than 1 μmol L^{-1}. This study indicates the essential role of P in regulating inorganic

18 carbon acquisition and CO_2 concentrating mechanisms (CCMs) in *S. costatum* and sheds light

19 on how bloom-forming algae cope with carbon limitation during the development of red tides.

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

21 photosynthesis; redox activity; respiration

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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 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 compelling factor that triggers algal blooms albeit the

occurrence of diatom blooms may be modulated by other environmental factors, such as

temperature, light intensity, salinity etc. (Smetacek & Zingone, 2013; Jeong *et al.*, 2015).

When inorganic nitrogen and phosphorus are replete, diatoms could out-compete

chrysophytes, raphidophytes and dinoflagellates (Berg *et al.*, 1997; Jeong *et al.*, 2015; Barton

et al., 2016) and domainate 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) 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),

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

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

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

46   mechanisms, for instance, active transport of HCO_3^-, the passive influx of CO_2, multiple

47   carbon anhydrase, assumed C4-type pathway, etc. (Hopkinson & Morel, 2011; Hopkinson *et*

48   *al.*, 2016). *S. costatum* is a worldwide diatom species that can be found from equatorial to

49   polar waters. It usually dominates large-scale algal blooms in eutrophic seawaters (Wang,

50   2002; Li *et al.*, 2011). When blooms occur, seawater pH increases and CO_2 decreases. For

51   instance, pH level in the surface waters of the eutrophic Mariager Fjord, Denmark, could be

52   up to 9.75 during algal blooms (Hansen, 2002). Consequently, *S. costatum* experiences very

53   severe CO_2 limitation when blooms occur. To deal with it, *S. costatum* has developed multiple

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

55   Nimer *et al.* (1998) documented that extracellular carbonic anhydrase activity in *S. costatum*

56   was only induced when CO_2 concentration was less than 5 μmol L^{-1} while Rost *et al.* (2003)

57   reported that activity of extracellular CA could be detected even when $_pCO_2$ is 1800 μatm.

58   Chen and Gao (2004) showed that in *S. costatum* had little capacity in direct HCO_3^- utilization.

59   On the other hand, Rost *et al.* (2003) demonstrated that this species could take up CO_2 and

60   HCO_3^- simultaneously.

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

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

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

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

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

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

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

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

69   phosphorus acquisition, utilization and storage (Lin *et al.*, 2016 and the references therein).

70   Some studies show the relationship between phosphorus availability and inorganic carbon

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

72   studies regarding the inorganic carbon acquisition in *S. costatum* focus on its response to

73   variation of CO_2 availability. The role of phosphorus in *S. costatum'*s CCMs remains

74   unknown. Based on the connection between phosphorus and carbon metabolism in diatoms

75   (Brembu *et al.*, 2017), we hypothesize that phosphorus enrichment could enhance the capacity

76   of inorganic carbon utilization and hence maintain high rates of photosynthesis and growth in

77   *S. costatum* under CO_2 limitation conditions. In the present study, we investigated the

78   inorganic acquisition pathways, photosynthetic CO_2 affinity, carbonic anhydrase activity,

79   redox activity of plasma membrane, and photosynthetic rate under five levels of phosphate

80   and two levels of CO_2 conditions to test this hypothesis. Our study would provide helpful

81   insights into how bloom-forming diatoms overcome CO_2 limitation to maintain a quick

82   growth rate during red tides.

83   **2. Materials and Methods**

84   *2.1. Culture conditions*

85   *S. costatum* (Grev.) Cleve from Jinan University, China, was cultured in f/2 artificial

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

87   amounts of NaH_2PO_4 $2H_2O$. The cultures were carried out semi-continuously at 20^oC for

88   seven days. The light irradiance was set 200 μmol m^{-2} s^{-1}, with a light and dark period of 12:

89   12. The cultures were aerated with ambient air (0.3 L min^{-1}) to maintain the pH around 8.2.

90   The cells during exponential phase were collected and rinsed twice with DIC-free seawater

91   that was made according to Xu *et al.* (2017). Afterwards, cells were resuspended in fresh

92   media with two levels of pH (8.2 and 8.7, respectively corresponding to ambient CO_2 (12.6

93   μmol L^{-1}, AC) and low CO_2 (2.8 μmol L^{-1}, LC) under corresponding phosphate levels for two

94   hours before the following measurements, with a cell density of 1.0×10^6 mL^{-1}. This transfer

95   aimed to investigate the effects of phosphate on DIC acquisition under a CO_2 limitation

96   condition. The pH of 8.7 was chosen considering that it is commonly used as a CO_2 limitation

97   condition (Nimer *et al.*, 1998; Chen & Gao, 2004) and also occurs during algal bloom

98   (Hansen, 2002). Two hours should be enough to activate CCMs in *S. costatum* (Nimer *et al.*,

99   1998). All experiments were conducted in triplicates.

100   *2.2.Chlorophyll fluorescence measurement*

101   Chlorophyll fluorescence was measured with a pulse modulation fluorometer

102   (PAM-2100, Walz, Germany). The measuring light and actinic light were 0.01 and 200 μmol

103   photons m^{-2} s^{-1}, respectively. The saturating pulse was set 4,000 μmol photons m^{-2} s^{-1} (0.8 s).

104   Relative electron transport (rETR, μmol e^{-} m^{-2} s^{-1}) = (F$_M$' − F$_t$) / F$_M$' × 0.5 × PFD, where F$_M$'

105   is the maximal fluorescence levels from algae after in light, Ft is the fluorescence at an

106   excitation level and PFD is the actinic light density.

107   *2.3.Estimation of photosynthetic oxygen evolution and respiration*

108   The net photosynthetic rate and respiration rate of *S. costatum* were measured using a

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

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

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

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

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

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

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

116   within ten minutes was defined as dark respiration rate. Net photosynthetic rate and dark

117   respiration rate were presented as $\mu mol\, O_2\, (10^9\, cells)^{-1}\, h^{-1}$.

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

119   0.5, 1, 2, and 4 mM) were made by adding different amounts of $NaHCO_3$ to the Tris buffered

120   DIC-free seawater. The algal samples were washed twice with DIC-free seawater before

121   transferring to the various DIC solutions. Photosynthetic rates at different DIC levels were

122   measured under saturating irradiance of 400 μmol photons $m^{-2}\, s^{-1}$ and growth temperature.

123   The algal samples were allowed to equilibrate for 2–3 min at each DIC level during which

124   period a linear change in oxygen concentration was obtained and recorded. The parameter,

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

126   of Ci-saturated maximum rate of photosynthetic O_2 evolution), was calculated from the

127   Michaelis-Menten kinetics equation (Caemmerer and Farquhar 1981): $V = V_{max} \times [S] / (K_{0.5} +$

128   [S]), where V is the real-time photosynthetic rate, V_{max} is maximum photosynthetic rate and [S]

129   is the DIC concentration. $K_{0.5}$ for CO_2 was calculated via CO2SYS (Pierrot et al., 2006),

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

131   and the KSO_4^- dissociation constant from Dickson (1990). Total alkalinity and pH were the

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132   two input parameters. Seawater pH was measured with a pH meter (pH 700, Eutech

133   Instruments, Singapore) and total alkalinity was measured by titrations.

134   *2.4. Measurement of photosynthetic pigment*

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

136   on a Whatman GF/F filter, extracted in 5 mL of 90% acetone for 12 h at 4°C, and centrifuged

137   (3, 000 *g*, 5 min). The optical density of the supernatant was scanned from 200 to 700 nm

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

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

140    $0.40 \times OD_{630}$, Chl *c* $=24.36 \times OD_{630} - 3.73 \times OD_{664}$.

141   *2.5. Measurement of extracellular carbonic anhydrase activity*

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

143   2009). Cells were harvested by centrifugation at 4, 000 *g* for five minutes at 20°C, washed

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

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

146   decrease from 8.2 to 7.2 at 4°C was recorded. Extracellular carbonic anhydrase (CA_{ext})

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

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

149   pH change in the absence or presence of the samples, respectively.

150   *2.6. Measurement of redox activity in the plasma membrane*

151   The redox activity of plasma membrane was assayed by incubating the cells with 500

152   μmol ferricyanide [$K_3Fe(CN)_6$] that cannot penetrate intact cells and has been used as an

153   external electron acceptor (Nimer *et al.*, 1998; Wu & Gao, 2009). Stock solutions of

154   $K_3Fe(CN)_6$ were freshly prepared before use. Five mL of samples were taken after two hours

155   of incubation and centrifuged at 4000 g for 10 min (20^oC). The absorbance of supernatant at

156   420 nm was measured immediately to assess the rate of exofacial ferricyanide reduction

157   (Nimer et al., 1998).

158   *2.7. pH drift experiment*

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

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

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

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

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

164   *2.8. Statistical analysis*

165   Results were expressed as means of replicates ± standard deviation and data were

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

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

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

169   on differences net photosynthetic rate, dark respiration rate, ratio of net photosynthetic rate to

170   dark respiration rate, rETR, Chl *a*, $K_{0.5}$, CA_{ext}, reduction rate of ferricyanide, and pH

171   compensation point. Least Significant Difference (LSD) was conducted for *post hoc*

172   investigation. Repeated measures ANOVAs were conducted to analyze the effects of DIC on

173   net photosynthetic rate and the effect of incubation time on media pH in a closed system.

174   Bonferroni was conducted for *post hoc* investigation. The threshold value for determining

175   statistical significance was $P < 0.05$.

176 **3. Results**

177 *3.1. Effects of CO_2 and phosphate on photosynthetic and respiratory performances*

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

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

180 on net photosynthetic rate ($F_{(4, 20)} = 3.662$, $P = 0.021$, Fig. 1a), with each factor having a main

181 effect ($F_{(1, 20)} = 11.286$, $P = 0.003$ for CO_2, $F_{(4, 20)} = 157.925$, $P < 0.001$ for phosphate). *Post*

182 *hoc* LSD comparison ($P = 0.05$) showed that LC reduced net photosynthetic rate when the

183 phosphate levels was below 4 μmol L^{-1} but did not affect it at the higher phosphate levels.

184 Under AC, net photosynthetic rate increased with phosphate level and reached the plateau at 1

185 μmol L^{-1} phosphate. Under LC, net photosynthetic rate also increased with phosphate level

186 but did not hit the peak until 4 μmol L^{-1} phosphate. Phosphate had a main effect on dark

187 respiration rate ($F_{(4, 20)} = 169.050$, $P < 0.001$, Fig. 1b), and it interacted with CO_2 ($F_{(4, 20)} =$

188 3.226, $P = 0.034$). Specifically, LC increased dark respiration rate at 0.05 and 0.25 μmol L^{-1}

189 phosphate levels, but did not affect it when phosphate level was above 1 μmol L^{-1} (LSD, $P <$

190 0.05). Regardless of CO_2 level, respiration rate increased with phosphate availability and

191 stopped at 1 μmol L^{-1}.

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

193 phosphate had a main effect ($F_{(1, 20)} = 32.443$, $P < 0.001$ for CO_2, $F_{(4, 20)} = 7.081$, $P = 0.001$ for

194 phosphate), and they interplayed on the ratio of respiration to photosynthesis ($F_{(4, 20)} = 8.299$,

195 $P < 0.001$). LC increased the ratio when phosphate was lower than 4 μmol L^{-1} but did not

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

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198   Both CO_2 and phosphate affected rETR ($F_{(1, 20)} = 28.717$, $P < 0.001$ for CO_2, $F_{(4, 20)} =$

199   127.860, $P < 0.001$ for phosphate) and they also showed an interactive effect ($F_{(4, 20)} = 3.296$,

200      $P = 0.031$, Fig. 3). For instance, *post hoc* LSD comparison showed that LC did not affect

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

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

203   (0.05–4 µmol L^{-1}) but the highest phosphate concentration did not result in a further increase

204   in rETR (LSD, $P = 0.05$).

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

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

207   synthesis of Chl *a* ($F_{(1, 20)} = 32.963$, $P < 0.001$ for CO_2, $F_{(4, 20)} = 92.045$ $P < 0.001$ for

208   phosphate) and they had an interactive effect ($F_{(4, 20)} = 3.871$, $P = 0.017$). *Post hoc* LSD

209   comparison ($P = 0.05$) showed that LC did not affect Chl *a* at 0.05 or 0.25 µmol L^{-1} phosphate

210   but stimulated Chl *a* synthesis at higher phosphate levels (1–10 µmol L^{-1}). Irrespective of CO_2

211   treatment, Chl *a* content increased with phosphate level and reached the plateau at 4 µmol L^{-1}

212   phosphate.

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

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

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

216   interplayed on $K_{0.5}$ ($F_{(4, 20)} = 3.821$, $P = 0.018$) and each had a main effect ($F_{(1, 20)} = 96.182$, P

217   < 0.001 for CO_2, $F_{(4, 20)} = 40.497$, $P < 0.001$ for phosphate). LC did not affect $K_{0.5}$ at the

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

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

220   further decrease in $K_{0.5}$ compared to 0.05 μmol L^{-1} phosphate. The pattern with phosphate

221   under LC was the same as the AC.

222   *3.3. The effects of CO$_2$ and phosphate on inorganic carbon acquisition*

223   To investigate the potential mechanisms that cells overcame CO$_2$ limitation during algal

224   blooms, the activity of CA$_{ext}$, a CCM related enzyme, was estimated under various CO$_2$ and

225   phosphate conditions (Fig. 7a). Both CO$_2$ ($F_{(1, 20)}$ = 569.585, P < 0.001) and phosphate ($F_{(4, 20)}$

226   = 176.392, P < 0.001) had a main effect and they interacted ($F_{(4, 20)}$ = 87.380, P < 0.001) on

227   CA$_{ext}$ activity. *Post hoc* LSD comparison (P = 0.05) showed that LC induced more CA$_{ext}$

228   activity under all phosphate conditions except for 0.05 μmol L^{-1} levels, compared to AC.

229   Under AC, CA$_{ext}$ activity increased with phosphate level and stopped increasing at 1 μmol L^{-1}

230   phosphate. Under LC, CA$_{ext}$ activity also increased with phosphate level but reached the peak

231   at 4 μmol L^{-1} phosphate. The redox activity of plasma membrane was also assayed to

232   investigate the factors that modulate CA$_{ext}$ activity (Fig. 7b). The pattern of redox activity of

233   plasma membrane under various CO$_2$ and phosphate conditions was the same as that of CA$_{ext}$

234   activity. That is, CO$_2$ and phosphate had an interactive effect ($F_{(4, 20)}$ = 137.050, P < 0.001) on

235   redox activity of plasma membrane, each having a main effect ($F_{(1, 20)}$ = 937.963, P < 0.001

236   for CO$_2$; $F_{(4, 20)}$ = 276.362, P < 0.001 for phosphate).

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

238   grown under various CO$_2$ and phosphate levels, changes of media pH in a closed system were

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

240   time ($F_{(10, 100)}$ = 7604.563, P < 0.001). Specifically speaking, there was a steep increase in pH

241   during the first three hours, afterwards the increase became slower and it reached a plateau in

242 six hours (Bonferroni, $P < 0.05$). Phosphate had an interactive effect with incubation time

243 ($F_{(10, 100)} = 47.469$, $P < 0.001$). For instance, there was no significant difference in media pH

244 among phosphate levels during first two hours of incubation but then divergence occurred and

245 they stopped at different points. Two-way ANOVA analysis showed that CO_2 treatment did

246 not affect pH compensation point ($F_{(1, 20)} = 0.056$, $P = 0.816$) but phosphate had a main effect

247 ($F_{(4, 20)} = 226.196$, $P < 0.001$). Under each CO_2 treatment, pH compensation point increased

248 with phosphate level, with lowest of 9.03 ± 0.03 at 0.05 μmol L^{-1} and highest of 9.36 ± 0.04 at

249 10 μmol L^{-1} phosphate.

250 **4. Discussion**

251 *4.1. Photosynthetic performances under various CO_2 and phosphate conditions*

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

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

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

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

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

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

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

259 ~36 μmol L^{-1}, which is replete for physiological activities in *S. costatum*. *S. costatum* grown

260 at higher phosphate levels (4 and 10 μmol L^{-1}) also showed comparative photosynthetic rates

261 between the lower and higher CO_2 treatments. Our finding combined with the previous

262 studies indicates phosphorus plays an important role in dealing with low CO_2 availability for

263 photosynthesis in *S. costatum*.

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

265   (0.05 and 0.25 μmol L^{-1}) and stimulated it at higher phosphate levels (1−10 μmol L^{-1}). This

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

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

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

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

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

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

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

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

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

275   2017). The increased photosynthetic pigment in $S.$ $costatum$ could partially explain the

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

277   *4.2. Ratio of respiration to photosynthesis*

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

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

280    $costatum$ grown at the lower P conditions but did not affect it under the higher P conditions,

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

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

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

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

285   energy used for inorganic carbon acquisition could be from the increased rETR. Therefore,

286 additional dark respiration was not triggered, avoiding carbon loss. Most studies regarding the

287 effect of CO_2 on ratio of respiration to photosynthesis focus on higher plants (Gifford, 1995;

288 Ziska & Bunce, 1998; Cheng *et al.*, 2010; Smith & Dukes, 2013), little is known on

289 phytoplankton. Our study suggests that CO_2 limitation may lead to carbon loss in

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

291 phytoplankton and thus their capacity in carbon sequestration.

292 *4.3. Inorganic carbon acquisition under CO_2 limitation and phosphate enrichment*

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

294 2012; Wu *et al.*, 2012; Raven *et al.*, 2017; Xu *et al.*, 2017). In the present study, the lower

295 CO_2 did increase inorganic carbon affinity when P level was higher than 0.25 μmol L^{-1} but did

296 not affect it when P was 0.05 μmol L^{-1}, indicating the important role of P in regulating cells'

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

298 above 0.25 μmol L^{-1} but did not increase it at 0.05 μmol L^{-1} of P, which could explain the

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

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

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

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

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

304 activity of plasma membrane could induce eextracellular CA activity via protonation

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

306 same as that of redox activity of plasma membrane shows a compelling correlation between

307 CA and redox activity of plasma membrane. The stimulating effect of P on redox activity of

308 plasma membrane may be due to its effect on rETR. The increased rETR could generate

309 excess reducing equivalents, particularly under CO_2 limited conditions. These excess reducing

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

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

312 1999) and triggering CA activity.

313 *4.4. Direct HCO_3^- utilization due to phosphate enrichment*

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

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

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

317 instance, the marine diatom *Phaeodactylum tricornutum*, with strong capacity for direct

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

333   *4.5. CCMs and red tides*

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

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

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

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

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

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

340   *al.*, 2011). However, potential mechanisms are poorly understood. Our study demonstrates *S.*

341   *costatum* has multiple CCMs to cope with CO_2 limitation and the operation of CCMs is

342   regulated by P availability. The CCMs of *S. costatum* is hampered under P limiting conditions

343   and only function when P is replete. Therefore, P enrichment would be critical for *S. costatum*

344   to overcome carbon limitation during algal bloom and to dominate red tides.

345   **5. Conclusions**

346   The present study investigated the role of P in regulating inorganic carbon acquisition and

347   CO_2 concentrating mechanisms in diatoms for the first time. The intensive photosynthesis and

348   quick grow during algal blooms usually result in noticeable increase of pH and decrease of

349   CO_2. Our study demonstrates that P enrichment could induce activity of extracellular carbonic

350   anhydrase and direct utilization of HCO_3^- in *S. costatum* to help overcome the CO_2 limitation,

351   as well as increasing photosynthetic pigment content and rETR to provide required energy.

352　This study provides important insight into the connection of phosphorus and carbon

353　acquisition in diatoms and the mechanisms that *S. costatum* dominates algal blooms.

354　**Author contribution**

355　　JX and GG designed the experiments, and GG, JY, JF and XZ carried them out. GG

356　prepared the manuscript with contributions from all co-authors.

357　**Acknowledgements**

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513

514 **Figure legends**

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

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

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

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

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

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

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

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

523 < 0.05) among phosphate concentrations (capital for AC, lower case for LC). Horizontal lines

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

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

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

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

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

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

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

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

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

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

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

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

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

537   indicate the standard deviations (n = 3).

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

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

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

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

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

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

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

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

546   < 0.05) among phosphate concentrations (capital for AC, lower case for LC). Horizontal lines

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

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

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

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

551

552   **Fig. 1**

553
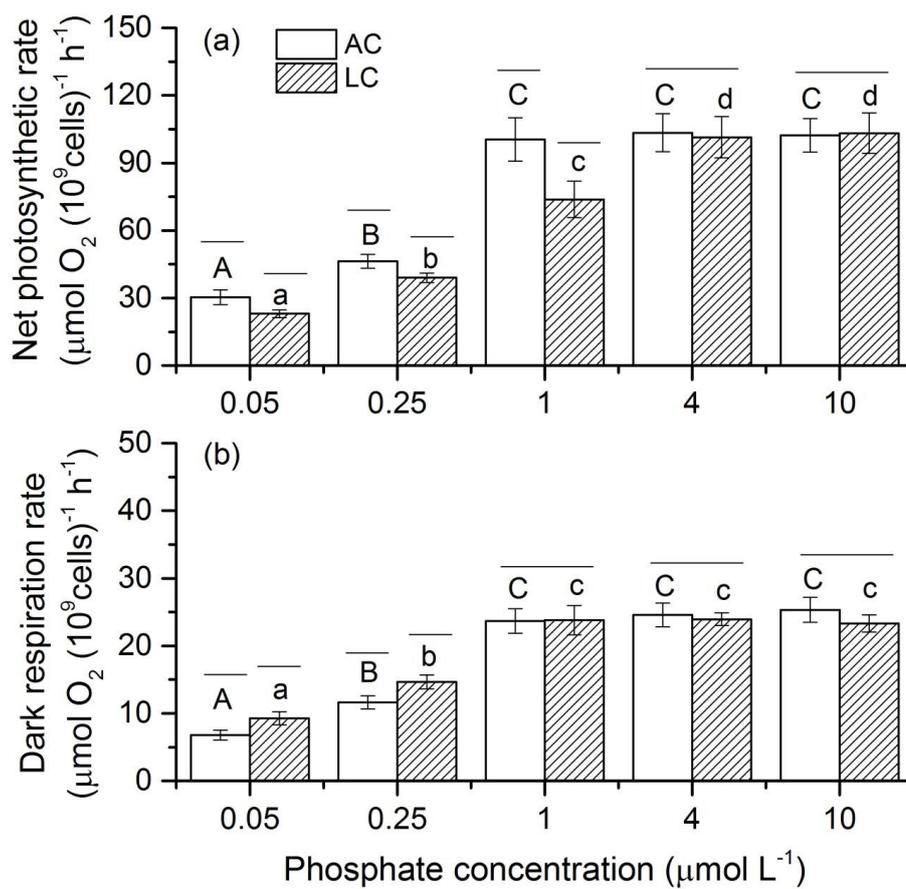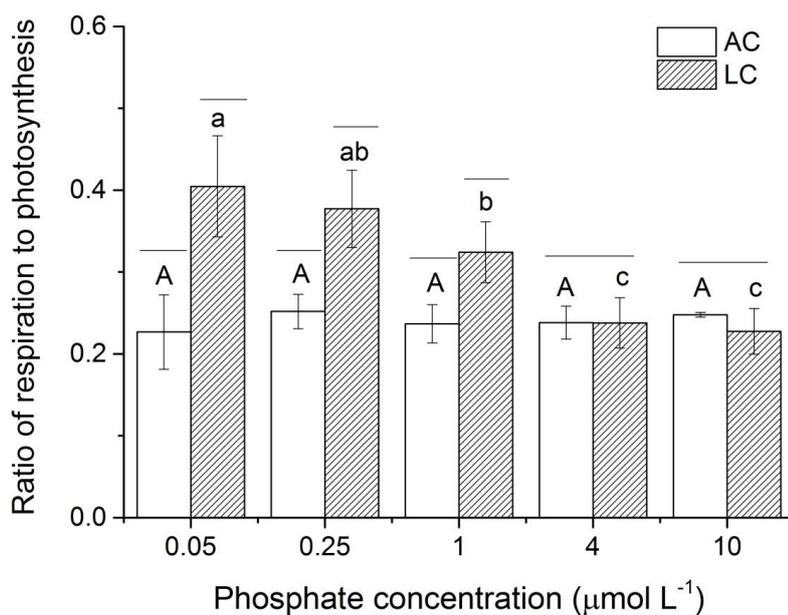
Fig. 2

555

556   **Fig. 3**

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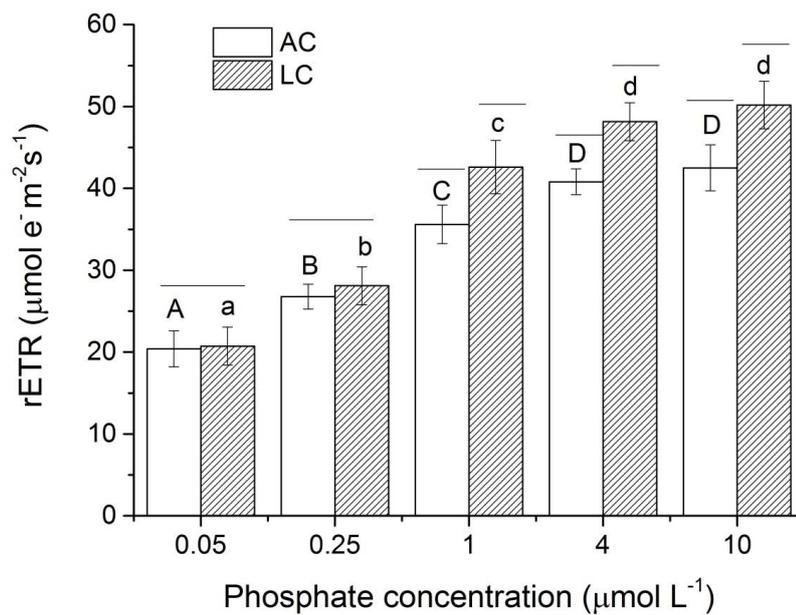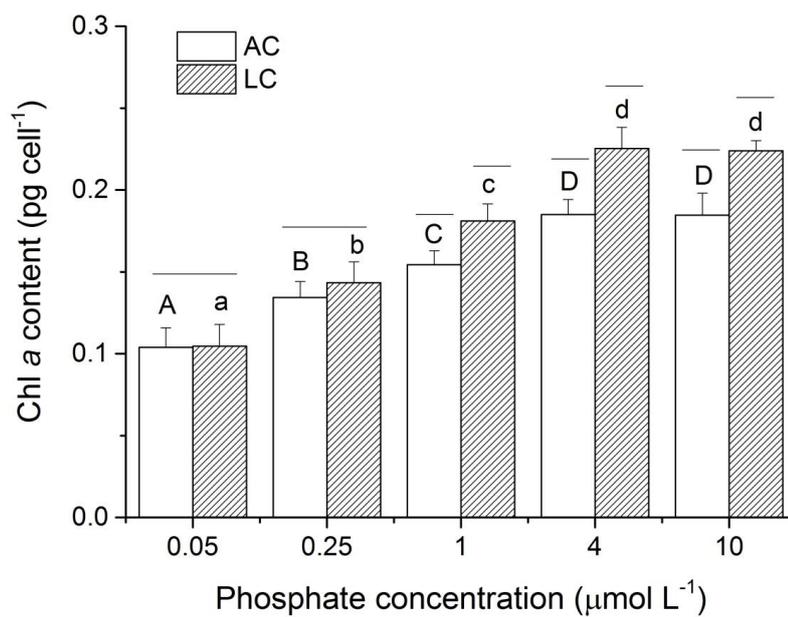
Fig. 4

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560   **Fig. 5**

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562   **Fig. 6**

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Fig. 7

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Fig. 8

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