Interactive comment on “Physiological response of a golden tide alga (Sargassum muticum) to the interaction of ocean acidification and phosphorus enrichment” by Zhiguang Xu et al.

Anonymous Referee #1

Received and published: 25 October 2016

The present manuscript provides interesting and useful information on the influence of future ocean acidification and eutrophication on a golden tide alga, Sargasssum muticum. The authors suggested that future ocean acidification and eutrophication may promote the growth of S. muticum and thus occurrence of gold tide events however, ocean acidification and eutrophication may not boost the gold tides events synergistically. The authors discussed their results reasonably within a physiological and ecological context. The experiments were reasonably performed and described. The data analysis was satisfactory and the results were clearly presented. The conclusions were sufficiently justified. The figures and tables were all adequate and essential. Therefore, in my opinion, this manuscript is suited for publication in BIOGEOSCIENCES.

Response: We really appreciate these comments.

Anonymous Referee #2

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This is an interesting paper describing the combined effects of elevated CO2 (and hence ocean acidification) and elevated P levels on growth and physiology of Sargassum muticum. The work is well designed and executed and the data presented and discussed thoroughly, although English expression is a little strange in places.

Response: We sincerely thank the anonymous referee for these comments. Thanks to Dr. Douglas A. Campbell, English expression has been improved.

I do though draw the authors attention to a couple of points:

Line 239: It is stated that projected ocean acidification increased pCO2 by 138.29% (LP) and 134.08% (HP) but surely it is the changes in pCO2 that cause OA?

Response: We totally agree with the reviewer. The text has been corrected to "elevated pCO_2 decreased pH by 0.31 unit at both LP and HP, CO_3^{2-} by 45% (LP) and 45% (HP), but increased DIC by 10% (LP) and 9% (HP), HCO_3^- by 14% (LP) and 14% (HP), and CO_2 by 139% (LP) and 134% (HP)." at lines 238-241.

Line 348-9: Here it is stated that "The evidence above indicates that the CO2 in seawater should be carbon limited for marine macroalgae". This is based on the high k0.5 CO2 for Rubisco and the diffusive resistance to CO2 on seawater - that the k0.5 CO2 values for intact thalli are very much lower than those for Rubisco is prima facie evidence that an active CCM is present. More could be made of this and the fact that it appears CCM activity is not down regulated by the high CO2 conditions. The explanation on lines 359-61 that this is "mainly because of increased CO2 availability for Rubisco and depressed photorespiration at the elevated ratio of CO2 to O2" would not apply to P vs DIC curves.

Response: We do agree that most algae have an active CCM, contributing to much lower $K_{0.5}$ values for intact thalli in comparison with those for Rubisco. Meanwhile, we think the CCM was down regulated by increased pCO_2 in the present study based on the increased $K_{0.5}$ that is deemed as a signal of down regulation of CCMs (Giordano et al., 2005, Gao and Campbell, 2014). The lines 359-61 was not used to explain the P vs DIC curves but the decrease of photosynthetic affinity for DIC did not lead to reduced photosynthesis in *S. muticum*. We have clarified it to "But this decrease of photosynthetic affinity for DIC at the higher pCO_2 did not lead to reduced photosynthesis in *S. muticum* compared to that at the lower pCO_2 in the present study, mainly because of increased CO_2 availability for Rubisco and depressed photorespiration at the elevated ratio of CO_2 to O_2, which has been confirmed in red seaweed *Lomentaria articulate* (Kübler et al., 1999)." at lines 358-362.

Gao, K. and Campbell, D. A.: Photophysiological responses of marine diatoms to elevated CO_2 and decreased pH: a review, Funct. Plant Biol., 41, 449-459, 2014.

Giordano, M., Beardall, J. and Raven, J. A.: CO_2 concentrating mechanisms in algae: mechanisms, environmental modulation, and evolution. Annu. Rev. Plant Biol., 56: 99-131, 2005.

Kübler, J. E., Johnston, A. M., and Raven, J. A.: The effects of reduced and elevated CO_2 and O_2 on the seaweed *Lomentaria articulata*, Plant Cell & Environment, 22, 1303-1310, 1999.

The authors suggest in several places (e.g. lines 388-91) that the HC conditions may have down-regulated CCMs in S. muticum, but there is no evidence for this in their

data
(Fig 3, Table 2).

Response: In a review (Gao and Campbell, 2014), it states: “Downregulation of CCMs can include decreased CO_2 affinity resulting in an increased requirement for pCO_2 to support photosynthesis, inhibition of carbonic anhydrase activity, depressed HCO_3^- transport, and downregulation of PEPCase and PEPCKase (Reinfelder et al. 2000; Giordano et al. 2005; Roberts et al. 2007a, 2007b; Raven 2010; Reinfelder 2011).” Giordano et al. (2005) also thought that high CO_2 could down regulate the CCM by suppressing expression of a high-affinity DIC state. Therefore, we think the increased $K_{0.5}$ could be considered as a hint for the down regulated CCM. In our study, the higher pCO_2 increased $K_{0.5}$ (Table 2) although the increase at the higher P level was not statistically significant.

Gao, K. and Campbell, D. A.: Photophysiological responses of marine diatoms to elevated CO_2 and decreased pH: a review, Funct. Plant Biol., 41, 449-459, 2014.

Giordano, M., Beardall, J. and Raven, J. A.: CO_2 concentrating mechanisms in algae: mechanisms, environmental modulation, and evolution. Annu. Rev. Plant Biol., 56: 99-131, 2005.

D. Campbell (Referee)
dcampbell@mta.ca
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The authors grew an invasive Sargassum species under an ecophysiologically reasonable matrix of pCO2 and [phosphate]. They analyzed the growth rate, photosynthetic rates, nitrate uptake and reduction rates and composition of the algae. They show interactive effects of pCO2 and [phosphate].

The study is well designed and potentially interesting. The current discussion spends words on entirely speculative interpretations that might well be true, but which are not directly supported by the data presented. On the other hand, intriguing ratios and discrepancies in the presented results are not discussed. For example, how can algal nitrate uptake rates exceed measured nitrate reduction rates? Does the tissue store NO3- differentially depending upon conditions? Are there variable rates of denitrification in the media?

Response: We appreciate these comments. We believe our manuscript has been improved by answering the reviewer’s queries. Please see the following response for details.

What happens to the environmental effects upon photosynthesis if it is normalized to chlorophyll rather than fresh weight?

Response: The reviewer raised a valuable point. We have normalized photosynthesis rate to chl *a*. The net photosynthetic rates under different treatments were 135.4 ± 27.0 (LCLP), 142.2 ± 6.5 (LCHP), 161.1 ± 4.4 (HCLP), and 193.0 ± 7.6 (HCHP) μmol O_2 mg^{-1} chl *a* h^{-1} respectively. The higher pCO$_2$ increased the net photosynthetic rate by 35% at HP and the higher P increased it by 20% at HC.

I offer some suggestions below for the authors. best regards, Doug Campbell

Abstract: ’the development of golden tides...’ (not ’evolvement’)

Response: Corrected.

39.31% etc. over precision. It is not possible to report such values to 1 part per 10,000 but that is what is implied by 39.31%

Response: It has been changed to 39%.

Introduction: ’...it originates from Japan..." (not ’it origins...’)

Response: Corrected.

Materials & Methods line 155: units for total alkalinity?

Response: We presume the reviewer meant the unit for salinity here. The unit for salinity has been developing. The Practical Salinity Scale (PSS) was defined in 1978 and later promulgated by the UNESCO/ICES/SCOR/IAPSO Joint Panel on Oceanographic Tables and Standards in Sidney, BC, Canada, 1-5 September 1980. Because it makes no sense to say the salinity is, for example, 35 PSS, the term Practical Salinity Unit (PSU) was introduced. However, the use of PSU is discouraged because salinity is by definition a dimensionless parameter. For now, most oceanographers follow the recommendation of the Scientific Committee for Oceanic Research (SCOR) that salinity be represented by a unitless number, as it's a unitless ratio and its measurement is now based on conductivity instead of the long time gone determination of evaporated mass.

Line 195: Decrease in NO3- in the media could result from microbial denitrification? A cross check would be whether nitrate reductase activity matched 1;1 with decrease in NO3-2 in the media?

Response: The reviewer raised a point worthy of discussion. We agree that nitrate reductase activity should match 1:1 with decrease in NO_3^- in the media, in theory. However, the undoupling between them is not uncommon and could be found in both microalgae (Collos 1982; Blasco et al., 1984) and macroalgae (Gordillo et al., 2001; Zou, 2005). One possible cause that leads to the NO_3^- uptake from the media exceeding NO_3^- reductase activity in the present study may be the intercellular NO_3^- storage (Collos 1982; Viaroli et al., 1996). It has been reported that the NO_3^- reductase activity (NRA) peak was 11-fold less than the NO_3^- uptake rate in *Ulva* sp. , suggesting that the reduction of NO_3^- reductase to nitrite NO_2^- by nitrate reductase was the rate-limiting step in NO_3^- assimilation (Lartigue and Sherman, 2005). Another reason might be the underestimation of NRA as the NO_2^- release may be limited not only by NRA, but also by the diffusion rates of NO_3^- into the cells and NO_2^- out of the cells in the assay used in the present study (Lartigue and Sherman, 2002). As for the microbial denitrification, we presume there is less possibility that the additional decrease of NO_3^- was caused by it. As far as we know, denitrification only takes place in anoxic environments while our cultures were aerated by ambient or CO_2 enriched air. Apparently, we do not have evidence to support these specific interpretations. To minimize the content of speculation, we would like to add one sentence to the text “It is worth noting that the nitrate uptake rates were commonly higher than the corresponding reduction rates of NO_3^- to nitrite NO_2^- by nitrate reductase in the present study, which might be due to the intercellular nitrate storage (Collos, 1982; Lartigue and Sherman, 2005) and the underestimation of RNA measured by the in situ assay (Lartigue and Sherman, 2002).” at lines 416-420.

Blasco, D., MacIsaac, J. J., Packard, T. T, and Dugdale, R. C.: Relationship between nitrate reductase and nitrate uptake in phytoplankton in the Peru upwelling region, Limnol. Oceanogr., 29, 275-286, 1984.

Collos Y.: Transient situations in nitrate assimilation by marine diatoms. III. Short-term uncoupling of nitrate uptake and reduction, J. Exp. Mar. Bio. Ecol., 62, 285-295, 1982.

Gordillo, F. J. L., Niell, F. X., and Figueroa, F. L.: Non-photosynthetic enhancement of growth by high CO_2 level in the nitrophilic seaweed *Ulva rigida* C. Agardh (Chlorophyta), Planta, 213, 64-70, 2001.

Lartigue, J., and Sherman, T. D.: Field assays for measuring nitrate reductase activity in *Enteromorpha* sp. (Chlorophyceae), *Ulva* sp. (Chlorophyceae), and *Gelidium* sp. (Rhodophyceae), J. Phycol., 38, 971-982, 2002.

Lartigue, J., and Sherman, T. D.: Response of *Enteromorpha* sp. (Chlorophyceae) to a nitrate pulse: nitrate uptake, inorganic nitrogen storage and nitrate reductase activity, Mar. Ecol. Prog. Ser., 292,147-157, 2005.

Viaroli, P., Naldi, M., Bondavalli, C. and Bencivelli, S. Growth of the seaweed *Ulva rigida* C. Agardh in relation to biomass densities, internal nutrient pools and external nutrient supply in the Sacca di Goro lagoon (Northern Italy), Hydrobiologia, 329, 93–103, 1996.

Zou, D.: Effects of elevated atmospheric CO_2 on growth, photosynthesis and nitrogen metabolism in the economic brown seaweed, *Hizikia fusiforme* (Sargassaceae, Phaeophyta), Aquaculture, 250, 726-735, 2005.

Fig. 3: There is an inhibition response in HCLP that is not apparent in other treatments.

Response: It appears that the last two points are lower than the two points before them but there are no statistical differences between these four points.

Fig. 4: Would a renormalization of photosynthetic rates (fig. 2) to chlorophyll content (fig. 4) eliminate some of the differences among treatments? I think maybe yes. Then some of the photosynthetic data can be explained by nutrient effects on content of photosynthetic units.

Response: The reviewer raised a valuable point. We have normalized photosynthesis rate to chl *a*. The net photosynthetic rates under different treatments were 135.4 ± 27.0 (LCLP), 142.2 ± 6.5 (LCHP), 161.1 ± 4.4 (HCLP) and 193.0 ± 7.6 (HCHP) μmol O_2 mg^{-1} chl *a* h^{-1} respectively. The higher pCO_2 increased the net photosynthetic rate by 35% at HP and the higher P increased it by 20% at HC. Compared to the results normalized to fresh weight, it does eliminate the differences at LC or LP. We would say this renormalization could partially explain the effects of pCO_2 and P on photosynthetic rate. Meanwhile, to the best of our knowledge, the photosynthesis rate of macroalgae in most studies is normalized to fresh weight/dry weight. We hope we can keep the current results to compare our study with others'.

Fig. 5, Fig 6 There is a discrepancy. NO3- uptake from the media cannot exceed NO3- reductase rates, unless the tissue is storing NO3-.

Response: Yes. We think it is mainly because of the intercellular nitrate storage as explained in the above response.

Fig 2 vs. Fig 8 dark respiration = _1/2 of photosynthetic rates?

Response: We realize that this ratio may be a little higher, particularly compared to microalgae. However, it might not be surprising for macroalgae. For instance, the ratio of respiration to photosynthesis varies between 0.14 and 0.54 in *Gracilaria lemaneiformis* (Zou and Gao, 2013), around 0.2–0.7 in *Hizikia fusiform* (Zou et al., 2011) and it could even be close to 1 in *Gracilaria tikvahiae* (Lapointe and Tenore, 1984), depending on different culture conditions.

Lapointe, B. E., Tenore, K. R.: Dawes C J. Interactions between light and temperature on the physiological ecology of *Gracilaria tikvahiae* (Gigartinales: Rhodophyta). Mar. Biol., 80, 161-170, 1984.

Zou, D., Gao, K.: Thermal acclimation of respiration and photosynthesis in the marine macroalga *Gracilaria lemaneiformis* (Gracilariales, Rhodophyta), J. Phycol., 49, 61-68, 2013.

Zou D, Gao K, Luo H: Short - and long - term effects of elevated CO_2 on photosynthesis and respiration in the marine macroalga *Hizikia fusiformis* (Sargassaceae, Phaeophyta) grown at low and high n supplies, J. Phycol., 47, 87-97, 2011.

Results Lines 237-241 Over precision in reporting of results to 1 part in 10,000. This is a problem throughout.

Response: It has been revised to 1 part in 100 throughout the text.

Discussion Lines 428 to 440 are entirely speculative. They might be true, but there is no evidence supporting these specific interpretations, in this paper.

Response: We agree with the reviewer. The length of speculation needs to be reduced, although it can supply a direction for future research. It has been shortened to seven lines and it reads now “The increased soluble protein and decreased NRA at the condition of higher $p\mathrm{CO}_2$ and higher P suggest some H^+ transport-related protein, such as plasma membrane H^+-ATPase, might be synthesized to counteract the acid–base perturbation caused by increased $p\mathrm{CO}_2$ and H^+. The additional production of H^+ transport-related protein like plasma membrane H^+-ATPase could competitively decrease the synthesis of nitrate reductase. This hypothesis needs further experimental evidence to stand even though it could explain the results in the present study.” at lines 431-440.

Physiological response of a golden tide alga (*Sargassum muticum*) to the interaction of ocean acidification and phosphorus enrichment

Zhiguang Xu[a], Guang Gao[b,c*], Juntian Xu[b], Hongyan Wu[d]

[a]Marine Biology Institute of Shandong Province, Qingdao 266104 , China
[b]Marine Resources Development Institute of Jiangsu, Huaihai Institute of Technology, Lianyungang, 222005, China
[c]School of Marine Science and Technology, Ridley Building, Newcastle University, Newcastle upon Tyne, NE1 7RU, England, UK
[c]Hubei University of Technology, Wuhan, 430068, China

*Corresponding author, g.gao@ncl.ac.uk, Phone/Fax: +44(0)1912085048

Abstract

The development~~evolvement~~ of golden tides would be influenced by global change factors, such as ocean acidification and eutrophication, but the related studies are very scarce. In this study, we cultured a golden tide alga, *Sargasssum muticum*, at two levels of $p\mathrm{CO}_2$ (400, 1000 µatm) and phosphate (0.5 µM, 40 µM) conditions to investigate the interactive effects of elevated $p\mathrm{CO}_2$ and phosphate on physiological properties of the thalli. The higher $p\mathrm{CO}_2$ level and phosphate (P) level alone increased the relative growth rate by 4~~0.82~~1% and 4~~7.78~~8%, net photosynthetic rate by 46~~.34~~% and 55~~.16~~%, soluble carbohydrates by 3~~2.78~~3% and 6~~1.83~~2% respectively whilst the combination of these two levels did not promote growth or soluble carbohydrates further. The higher levels of $p\mathrm{CO}_2$ and P alone also enhanced the nitrate uptake rate by 68~~.27~~% and 3~~5.89~~6%, nitrate reductase activity by 89~~.08~~% and 39~~.31~~%, and soluble protein by 19~~.05~~% and 15~~.13~~% respectively. The nitrate uptake rate and soluble protein was further enhanced although the nitrate reductase activity was reduced when the higher levels of $p\mathrm{CO}_2$ and P worked together. The higher $p\mathrm{CO}_2$ level and higher P level alone did not affect the dark respiration rate of thalli but they together increased it by 32~~.30~~% compared to the condition of the lower $p\mathrm{CO}_2$ and lower P. The mute effect of the higher level of $p\mathrm{CO}_2$ and higher P on growth, soluble carbohydrates, combined with the promoting effect of it on soluble protein and dark respiration, suggests more energy was drawn from carbon assimilation to nitrogen assimilation at the condition of higher $p\mathrm{CO}_2$ and higher P, probably to act against the higher $p\mathrm{CO}_2$ caused acid-base perturbation via synthesizing H^+ transport-related protein. Our results indicate ocean acidification and eutrophication may not boost the gold tides events synergistically although each of them alone has a promoting effect.

Key words: carbohydrates, growth, photosynthesis, protein, respiration, *Sargassum muticum*

1. Introduction

Sargassum C. Agardh (1820) is the most species-rich genus in the Phaeophyta and has a global distribution (Mattio and Payri, 2011). The species of this genus constitutes an important part of the marine flora and is considered as a valuable and unique habitat for a number of highly adapted marine animal species (Laffoley et al., 2011). Some species of *Sargassum* are economically important, being used as animal fodder, manure in agriculture, as well as alginates production (Ashok-Kumar et al., 2012; Fenoradosoa et al., 2010; González-López et al., 2012). On the other hand, *Sargassum* is an aggressive genus and it can rapidly spread and invade new areas (Sfriso and Facca, 2013). The invasion of *Sargassum* would accordingly compete with indigenous species for nutrients and light and lead to the alteration of macroalgal community structure (Rueness, 1989; Stæhr et al., 2000). For instance, the increased abundance of *S. muticum* in Limfjorden (Denmark) between 1990 and 1997 led to decreased cover of several indigenous species belonging to the genera of *Codium*, *Fucus*, and *Laminaria*, and thus reduced species richness and diversity of the macroalgal community (Stæhr et al., 2000). Recently, the species of *Sargassum* inundate the coasts along Gulf of Mexico, West African, Caribbean, and Brazil in unprecedented biomass, termed as golden tides (Schell et al., 2015; Smetacek and Zingone, 2013). Apart from the negative effect on aesthetics and tourism, the occurrence of golden tides could kill the fish within the algal mass, mainly due to hypoxia or anoxia in the waters caused by decomposition of *Sargassum* thalli (Cruzrivera et al., 2015). In addition, the dense *Sargassum* accumulation could clog fishing nets and impede the passage of boats, leading to food shortages for local people who live on artisanal fisheries (Smetacek and Zingone, 2013). The occurrence of golden tides has been linked to higher nutrient levels in the seawaters (Lapointe, 1995; Smetacek and Zingone, 2013). The distribution pattern and biomass of *Sargassum* spp. are environment (temperature, light, nutrients, etc.)-dependent (Ang, 2006; Sfriso and Facca, 2013).

Due to burning fossil fuels and changes to land use, the atmospheric concentrations of carbon dioxide have increased to the level of 401.72 ppm in July 2016 (http://www.esrl.noaa.gov/gmd/ccgg/trends/global.html), which is unprecedentedly high in at least the last 800,000 years (IPCC, 2013). When CO_2 dissolves in seawater it forms carbonic acid and as more CO_2 is taken up by the ocean's surface, the pH decreases, moving towards a less alkaline and therefore more acidic state, termed ocean acidification. The mean surface ocean pH has already decreased by 0.1 units since the beginning of the industrial era, corresponding to a 26% increase in hydrogen ion concentration (IPCC, 2013). By 2100, concentrations of CO_2 (aq) and HCO_3^- are predicted to increase by 192% and 14%, respectively, and CO_3^{2-} to decrease by 56%, with a concomitant decline in pH to 7.65 (Raven et al., 2005). Increased CO_2 could exert positive, neutral, or negative on physiological properties of macroalgae (Ji et al., 2016; Wu et al., 2008). In terms of *Sargassum* species, increased CO_2 (800 ppm) enhanced photosynthetic rate (based on CO_2 uptake) in *S. muticum* (Longphuirt et al., 2014). On the other side, the same level of increased CO_2 (750 ppm) did not affect growth, Rubisco's maximal activity, affinity for CO_2 or quantity in *S. vulgare* (Alvaro and Mazal, 2002). Furthermore, increased CO_2 (750 ppm) significantly decreased net photosynthetic rate and light saturation point of *S. henslowianum* (Chen and Zou, 2014).

Apart from ocean acidification, eutrophication is another environmental challenge. Eutrophication can occur naturally in lakes via transferring nutrients from the sediment to water by living or decomposing macrophytes, resuspension, diffusion, and bioturbation (Carpenter, 1981). However, anthropogenic activities have accelerated the rate and extent of eutrophication (Carpenter et al., 1998). Inevitable urbanization of a growing human population, increased use of coastal areas, and rising fertilizer use for agricultural intensification has led to accelerated nutrient inputs from land-water to coastal waters (Smith et al., 1999). These changes in nutrient availability result in eutrophication, an increasing threat for coastal ecosystems (Bricker et al., 2008). One consequence of eutrophication is that it can lead to algal bloom, such as green tides and golden tides (Smetacek and Zingone, 2013). There are relatively intensive studies regarding the effect of nutrients on physiological properties of *Sargassum* species (Hwang et al., 2004; Incera et al., 2009; Lapointe, 1995; Liu and Tan, 2014; Nakahara, 1990). Enrichment of nutrients usually can enhance the growth and photosynthetic parameters of *Sargassum*. For instance, the growth rate of *S. baccularia* almost doubled when nutrients increased from 3 μM ammonium plus 0.3 μM phosphate to 5 μM ammonium plus 0.5 μM phosphate

(Schaffelke and Klumpp, 1998) and the photosynthetic rates of *S. fluitans* and *S. natans* were also two-fold higher with 0.2 mM PO_3^- enrichment compared to the control (Lapointe, 1986). Furthermore, some studies have demonstrated that macroalgae experience more phosphorus limit instead of nitrogen limit (Lapointe, 1986; Lapointe et al., 1987, 1992; Littler et al., 1991). For instance, nitrogen enrichment did not affect growth rates of *S. fluitans* or *S. natans* whilst phosphorus enrichment increased them from 0.03–0.04 (control) to 0.05–0.08 doublings d^{-1} (Lapointe, 1986).

Neither ocean acidification nor eutrophication is proceeding in isolation; rather they occur simultaneously, particularly in coastal areas. The interactive effects of two factors may be completely different, or be of greater magnitude, compared to effects of any single stressor. To the best of our knowledge, no studies have been reported in regard to the interactive effects of ocean acidification and eutrophication on *Sargassum*. In this study, we chose the species *S. muticum* to investigate its responses to interaction of ocean acidification and eutrophication. *S. muticum* is an invasive macroalga and commonly habitats on rocky shores (Karlsson and Loo, 1999). It originates~~origins~~ from Japan and was introduced to the northern Pacific coast of the United States in the early 20th century (Scagel, 1956), and was also introduced to Europe along with the imported Japanese oyster in the late 1960s (Jones and Farnham., 1973). Nowadays, its distribution is worldwide due to the introduction and the subsequent rapid expansion (Cheang et al., 2010). Our study would supply insight into how ocean acidification and eutrophication affect the physiological properties of *S. muticum* and thus the evolvement of golden tides.

2. Materials and methods

2.1. Sample collection and experiment design

S. muticum was collected from lower intertidal rocks on the coast of Lidao, Rongcheng, China (37 °15′N, 122 °35′E). The samples were transported to the laboratory in an insulated polystyrene cooler (4–6 ℃) within 3 hours. Healthy thalli were selected and rinsed with sterile seawater to remove sediments, epiphytes and small grazers. The thalli were maintained in an intelligent illumination incubator

(MGC-250P, Yiheng Technical Co. Ltd., Shanghai, China) for 24 hours before the experiment. The temperature in the incubator was set as 20°C with a 12h: 12h (light/dark) photoperiod of 150 μmol photons m^{-2} s^{-1} photosynthetically active radiation (PAR). After the maintenance, a two-way factorial experiment was set up to investigate the interactive effects of $p\mathrm{CO_2}$ and phosphate on *S. muticum.* The thalli were placed in 3 L flasks with 2 L sterile seawater (one thallus per flask) and cultured at fully crossed two $p\mathrm{CO_2}$ (400 μatm, LC; 1000 μatm, HC) and two phosphate (0.5 μM, LP; 40 μM, HP) levels with continuous aeration for 13 days. Phosphorus was selected as a nutrient variable since some findings have displayed that phosphorus, rather than nitrogen, is the primary limiting nutrient for macroalgae (Lapointe, 1986; Lapointe et al., 1987, 1992; Littler et al., 1991). The 400 μatm $p\mathrm{CO_2}$ and 0.5 μM phosphate are the conditions of natural seawater. The 400 μatm $p\mathrm{CO_2}$ was achieved by bubbling ambient air and 1000 μatm $p\mathrm{CO_2}$ was obtained through a CO_2 plant chamber (HP1000 G-D, Wuhan Ruihua Instrument & Equipment Ltd, China) with the variation of CO_2 less than 5%. The higher P level (40 μM) was achieved by adding NaH_2PO_4 to natural seawater and the nitrate concentration was set as 200 μM for all treatments to avoid N limit. The media were refreshed every day.

2.2. Carbonate chemistry parameters

The seawater pH was recorded with a pH meter (pH 700, Eutech Instruments, Singapore) and total alkalinity (TA) was measured by titrations. The salinity of seawater was 29. Other carbonate system parameters, which were not directly measured, were calculated via CO2SYS (Pierrot et al., 2006), using the equilibrium constants of K_1 and K_2 for carbonic acid dissociation (Roy et al., 1993).

2.3. Measurement of growth

The growth of *S. muticum* was determined by weighing fresh thalli. The thalli of *S. muticum* were blotted gently with tissue paper to remove water on the surface of the thalli before weighing. The relative growth rate (RGR) was estimated as follows: $\mathrm{RGR} = (\ln W_t - \ln W_0) / t \times 100$, where W_0 is the initial fresh weight (FW) and W_t is the weight after t days culture.

2.4. Determination of photosynthesis and respiration

The net photosynthetic rate of thalli was measured by a Clark-type oxygen electrode (Chlorolab-3, Hansatech, Norfolk, UK) at the end of the experiment. Approximately 0.1 g of fresh weight algae harvested from the culture flask was transferred to the oxygen electrode cuvette with 8 ml sterilized media, and the media were stirred during measurement. The irradiance and temperature conditions were set as the same as that in the growth incubators. The increase of oxygen content in seawater within five minutes was defined as net photosynthetic rate and the decrease of oxygen content in seawater in darkness within ten minutes was defined as respiration rate. Net photosynthetic rate (NPR) and respiration rate were presented as μmol O_2 g^{-1} FW h^{-1}.

Photosynthetic rates at different dissolved inorganic carbon (DIC) levels were measured under saturating irradiance of 600 μmol photons m^{-2} s^{-1} at the end of the experiment. The various DIC concentrations (0–13.2 mM) were obtained by adding different amounts of $NaHCO_3$ to the Tris buffered DIC-free seawater. DIC was removed from the natural seawater by reducing pH to approximately 4.0 with the addition of 1.0 M HCl, and then sparging for 2 h with pure N_2 gas (99.999%). Finally, Tris buffer (25mM) was added and the pH was adjusted to 8.1 with freshly prepared 1 M NaOH and 1 M HCl. The parameters, maximum photosynthetic rate (V_{max}) and the half saturation constant ($K_{0.5}$, i.e., the DIC concentration required to give half of Ci-saturated maximum rate of photosynthetic O_2 evolution), were calculated from the Michaelis-Menten kinetics equation (Caemmerer and Farquhar, 1981):

$V = V_{max} \times [S] / (K_{0.5} + [S])$, where [S] is the DIC concentration.

2.5. Assessment of photosynthetic pigments

Approximately 100 mg of fresh weight thalli from each culture condition at the end of the experiment was ground thoroughly in 2 ml 80% acetone and placed in darkness for 12 hours. Then the homogenate was centrifuged for 10 minutes at 5, 000 *g* and the supernatant was used to determine Chl *a* content spectrophotometrically according to the equation of Lichtenthaler (1987).

2.6. Measurement of nitrate uptake rate

The nitrate uptake rate (NUR) of thalli was estimated from the decrease of NO_3^-

concentration in the culture medium over a given time interval (12 hours) during light period using the following equation: NUR = $(N_0 - N_t) \times V / W / 12$, where N_0 is the initial concentration of NO_3^-, N_t is the concentration after 12 hours, V is the volume of the culture medium, and W is the fresh weight of the thalli in culture. NO_3^- concentration in the seawater was measured according to Strickland and Parsons (1972).

2.7. Estimate of nitrate reductase activity

Nitrate reductase activity of thalli was assayed according to modified in situ method of Corzo and Niell (1991). The measurement was conducted during the local noon period (13:00) since the activity of nitrate reductase usually displays circadian periodicity a maximum during the light period and a minimum in darkness (Deng et al., 1991; Velasco and Whitaker, 1989). Approximately 0.3 g (FW) of thalli from each culture condition was incubated for 1 h at 20°C in darkness in the reaction solution (10 mL), which contained 0.1 M phosphate buffer, 0.1% propanol (v/v), 50 mM KNO_3, 0.01 mM glucose, and 0.5 mM EDTA, with a pH of 8.0. The mixture was flushed with pure N_2 gas (99.999%) for 2 minutes to obtain an anaerobic state before the incubation. The concentration of nitrite produced was determined colorimetrically at 540 nm (Zou, 2005). The NR activity was expressed as μmol NO_2^- g^{-1} FW h^{-1}.

2.8. Analysis of biochemical composition

About 0.2 g of FW thalli from each culture condition at the end of the experiment were ground in a mortar with distilled water and soluble carbohydrates were extracted in a water bath of 80°C for 30 min. After being centrifuged for 10 minutes at 5, 000 *g*, supernatant was volumed to 25 ml with distilled water, and soluble carbohydrates content was determined by phenol-sulfuric acid method (Kochert, 1978).

Approximately 0.2 g of FW thalli from each culture condition at the end of the experiment were ground in a mortar with extraction buffer (0.1 mol L^{-1} phosphate buffer, pH 6.8) and then centrifuged for 10 minutes at 5, 000 *g*. Soluble protein was estimated from the supernatant using the Bradford (1976) assay with bovine serum albumin as a standard.

2.9. Data Analysis

Results were expressed as means of replicates ± standard deviation. Data were analyzed using the software SPSS v.21. The data under every treatment conformed to a normal distribution (Shapiro-Wilk, $P > 0.05$) and the variances could be considered equal (Levene's test, $P > 0.05$). Two-way ANOVA was conducted to assess the effects of $p\mathrm{CO}_2$ and P on carbonate parameters, relative growth rate, net photosynthesis rate, V_{max}, $K_{0.5}$, Chl *a*, nitrate uptake rate, nitrate reductase activity, soluble carbohydrates, soluble protein, and dark respiration rate. Tukey HSD was conducted for *post hoc* investigation. A confidence interval of 95% was set for all tests.

3. Results

The effects of ocean acidification and P enrichment on seawater carbonate parameters were detected first (Table 1). Two-way ANOVA analysis ($P = 0.05$) showed that pCO_2 had a main effect on all parameters except TA whilst P did not affect any parameter. *Post hoc* Tukey HSD comparison ($P = 0.05$) showed that ~~projected ocean acidification~~elevated $p\mathrm{CO}_2$ decreased pH by 0.31 unit at both LP and HP, CO_3^{2-} by 45~~.24~~% (LP) and 45~~4.70~~% (HP), but increased ~~$p\mathrm{CO}_2$ by 138.29% (LP) and 134.08% (HP),~~ DIC by ~~9.53~~10% (LP) and 9~~.26~~% (HP), HCO_3^- by 14~~.11~~% (LP) and 1~~3.79~~4% (HP), and CO_2 by 13~~8.88~~9% (LP) and 134~~.20~~% (HP).

The growth of *S. muticum* cultured at different $p\mathrm{CO}_2$ and P conditions was recorded (Fig. 1). $p\mathrm{CO}_2$ and P had an interactive effect on the relative growth rate of *S. muticum* (ANOVA, F = 5.776, df = 1, 8, $P = 0.043$) and each factor had a main effect (ANOVA, F = 19.145, df = 1, 8, $P = 0.002$ for $p\mathrm{CO}_2$; ANOVA, F = 30.592, df = 1, 8, $P = 0.001$ for P). *Post hoc* Tukey HSD comparison ($P = 0.05$) showed that the higher levels of $p\mathrm{CO}_2$ and higher P alone increased the relative growth rate by 4~~0.82~~1% and 4~~7.78~~8% respectively, compared to the relative growth rate (3.~~05~~ 1 ± 0.~~36~~4%) at the condition of lower $p\mathrm{CO}_2$ and lower P. The combination of the higher $p\mathrm{CO}_2$ and higher P levels did not enhance the relative growth rate as much as the sum of the higher $p\mathrm{CO}_2$ alone plus the higher P alone, with an increase of 59.66%. Although the higher P level increased the relative growth rate at the condition of lower $p\mathrm{CO}_2$, it did not affect the relative growth rate at the condition of higher $p\mathrm{CO}_2$.

In terms of the net photosynthetic rate (Fig. 2), both $p\mathrm{CO}_2$ (ANOVA, F = 26.556, df = 1, 8, $P = 0.001$) and P had main effects (ANOVA, F = 38.963, df = 1, 8, $P < 0.001$) on it. *Post hoc* Tukey HSD comparison ($P = 0.05$) showed the higher $p\mathrm{CO}_2$ level increased the net photosynthetic rate by 46~~.34~~% and 2~~3.96~~4% at the conditions of lower P and higher P respectively. The higher P level increased the net photosynthetic rate by 55~~.16~~% and 31~~.43~~% at the conditions of lower $p\mathrm{CO}_2$ and higher $p\mathrm{CO}_2$ respectively. The difference in the net photosynthetic rate between LCHP and HCLP was statistically insignificant.

The carbon-saturating maximum photosynthetic rate (V_{max}) and the half saturation constant ($K_{0.5}$), obtained from the photosynthesis versus DIC curves (Fig. 3), are shown in Table 2. The $p\mathrm{CO}_2$ and P had an interactive effect on V_{max} of *S. muticum* (ANOVA, F = 10.095, df = 1, 8, $P = 0.013$) and each factor had a main effect (ANOVA, F = 31.402, df = 1, 8, $P = 0.001$ for $p\mathrm{CO}_2$; ANOVA, F = 105.116, df = 1, 8, $P < 0.001$ for P). *Post hoc* Tukey HSD comparison ($P = 0.05$) showed the higher $p\mathrm{CO}_2$ level increased the V_{max} by 42~~.44~~% at the condition of lower P while the increase at the condition of higher P was statistically insignificant. The higher P level increased the V_{max} at the conditions of both lower $p\mathrm{CO}_2$ (6~~4.90~~5%) and higher $p\mathrm{CO}_2$ (24~~.01~~%), with the larger promoting effect at the condition of lower $p\mathrm{CO}_2$.

$p\mathrm{CO}_2$ and P interacted on the $K_{0.5}$ of *S. muticum* (ANOVA, F = 5.928, df = 1, 8, $P = 0.041$) and each factor had a main effect (ANOVA, F = 14.713, df = 1, 8, $P = 0.005$ for $p\mathrm{CO}_2$; ANOVA, F = 20.857, df = 1, 8, $P = 0.002$ for P). *Post hoc* Tukey HSD comparison ($P = 0.05$) showed the higher $p\mathrm{CO}_2$ level increased the $K_{0.5}$ by 9~~7.85~~8% at the condition of lower P but did not affect it at the condition of higher P. In contrast, the higher P level decreased the $K_{0.5}$ by 55~~.22~~% at the condition of higher $p\mathrm{CO}_2$ and the negative effect of the higher P level at the condition of lower $p\mathrm{CO}_2$ was insignificant.

The contents of photosynthetic pigment Chl *a* under various treatments were also estimated (Fig. 4). $p\mathrm{CO}_2$ and P had an interactive effect on the Chl *a* content (ANOVA, F = 8.184, df = 1, 8, $P = 0.021$), P had a main effect (ANOVA, F = 22.828, df = 1, 8, $P = 0.001$), while $p\mathrm{CO}_2$ did not affect it (ANOVA, F = 0.676, df = 1, 8, $P =$

0.435). *Post hoc* Tukey HSD comparison ($P = 0.05$) showed the higher P level increased the Chl *a* content from 0.17 ± 0.00 to 0.25 ± 0.02 mg g^{-1} FW at the condition of lower $p\text{CO}_2$ whereas the difference in the Chl *a* content between HCLP (0.21 ± 0.02 mg g^{-1} FW) and HCHP (0.23 ± 0.02 mg g^{-1} FW) was not statistically significant.

To assess the effects of ocean acidification and P enrichment on the nitrogen assimilation in *S. muticum*, nitrate uptake rate under various $p\text{CO}_2$ and P treatments was investigated first (Fig. 5). Both $p\text{CO}_2$ (ANOVA, F = 139.916, df = 1, 8, $P < 0.001$) and P (ANOVA, F = 43.923, df = 1, 8, $P < 0.001$) had main effects on the nitrate uptake rate of *S. muticum*. The nitrate uptake rates at the conditions of lower $p\text{CO}_2$ were 0.18 ±0.01(LP) and 0.25 ±0.03 μmol NO_3^- g^{-1} FW h^{-1} (HP) respectively. *Post hoc* Tukey HSD comparison ($P = 0.05$) showed the higher $p\text{CO}_2$ level increased the nitrate uptake rate to 0.31 ±0.02 μmol NO_3^- g^{-1} FW h^{-1} at the condition of lower P and to 0.39 ±0.01 μmol NO_3^- g^{-1} FW h^{-1} at the condition of higher P, compared to those at the conditions of lower $p\text{CO}_2$. The higher P level also increased the nitrate uptake rate by 3~~5.89~~6% at the condition of lower $p\text{CO}_2$ and by 2~~7.71~~8% at the condition of higher $p\text{CO}_2$, compared to those at the conditions of lower P.

Apart from nitrate uptake, the nitrate reductase activity (NRA) of *S. muticum* under various $p\text{CO}_2$ and P treatments was also detected (Fig. 6). $p\text{CO}_2$ and P interacted on NRA of *S. muticum* (ANOVA, F = 28.435, df = 1, 8, $P = 0.001$) and $p\text{CO}_2$ had a main effect (ANOVA, F = 59.038, df = 1, 8, $P < 0.001$). The NRA at the conditions of lower $p\text{CO}_2$ were 0.10 ± 0.01 (LP) and 0.14 ± 0.02 μmol NO_2^- g^{-1} FW h^{-1} (HP) respectively, and the higher $p\text{CO}_2$ level increased it to 0.19 ± 0.00 μmol NO_2^- g^{-1} FW h^{-1} at the condition of lower P and to 0.15 ± 0.02 μmol NO_2^- g^{-1} FW h^{-1} at the condition of higher P. The higher P level increased the NRA by 39~~.31~~% at the condition of lower $p\text{CO}_2$, however, it decreased NRA by 1~~7.81~~8% at the condition of higher $p\text{CO}_2$.

The soluble carbohydrates (Fig. 7a) and protein (Fig. 7b) were estimated to understand the effects of ocean acidification and P enrichment on the products of carbon and nitrogen assimilation in *S. muticum*. $p\text{CO}_2$ and P had an interactive effect on the soluble carbohydrates (ANOVA, F = 18.294, df = 1, 8, $P = 0.003$) and P had a main effect (ANOVA, F = 23.129, df = 1, 8, $P = 0.001$). The higher P level increased the soluble carbohydrates from 25.40 ± 1.66 to 41.10 ± 1.74 mg g^{-1} FW at the condition of lower $p\mathrm{CO}_2$ but did not alter it at the condition of higher $p\mathrm{CO}_2$. The higher $p\mathrm{CO}_2$ level increased the soluble carbohydrates to 33.72 ± 3.31 mg g^{-1} FW at the condition of lower P while the decrease of soluble carbohydrates caused by the higher $p\mathrm{CO}_2$ level was not statistically significant at the condition of higher P.

Both $p\mathrm{CO}_2$ (ANOVA, F = 106.663, df = 1, 8, $P < 0.001$) and P (ANOVA, F = 75.003, df = 1, 8, $P < 0.001$) had main effects on the soluble protein of *S. muticum* and the interactive effect of the two factors was not detected (ANOVA, F = 4.961, df = 1, 8, $P = 0.057$). The soluble protein contents at the conditions of lower $p\mathrm{CO}_2$ were 8.49 ± 0.49 (LP) and 9.77 ± 0.14 mg g^{-1} FW (HP) respectively. The higher $p\mathrm{CO}_2$ level increased it to 10.11 ± 0.16 mg g^{-1} FW at the condition of lower P and to 12.28 ± 0.44 mg g^{-1} FW at the condition of higher P. The higher P level also increased the soluble protein contents by 15~~.13~~% at the condition of lower $p\mathrm{CO}_2$ and by 21~~.51~~% at condition of higher $p\mathrm{CO}_2$.

Finally, the effects of ocean acidification and P enrichment on the dark respiration rate of *S. muticum* were investigated (Fig. 8). $p\mathrm{CO}_2$ and P had an interactive effect on the dark respiration rate (ANOVA, F = 19.584, df = 1, 8, $P = 0.002$) and each factor had a main effect (ANOVA, F = 6.428, df = 1, 8, $P = 0.035$ for $p\mathrm{CO}_2$; ANOVA, F = 6.754, df = 1, 8, $P = 0.032$ for P). The higher $p\mathrm{CO}_2$ level increased the dark respiration rate from 14.21 ± 1.94 to 21.24 ± 1.28 μmol O_2 g^{-1} FW h^{-1} at the condition of higher P but did not affect it at the condition of lower P. Likewise, The higher P level increased the respiration rate from 14.15 ± 0.65 to 21.24 ± 1.28 μmol O_2 g^{-1} FW h^{-1} at the condition of higher $p\mathrm{CO}_2$ but did not change it at the condition of lower $p\mathrm{CO}_2$.

4. Discussion

4.1. Effects of $p\mathrm{CO}_2$ *and P on carbon assimilation*

The higher $p\mathrm{CO}_2$ level increased the net photosynthetic rate in *S. muticum* at the condition of lower P in the present study. Although the dissolved inorganic carbon in seawater is around 2 mM, the dominant form is HCO_3^-, with CO_2 typically accounting for less than 1% (Dickson, 2010). In addition, CO_2 in seawater diffuses ~8, 000 times slower than in air (Gao and Campbell, 2014). Furthermore, the marine macroalgae have high $K_{0.5}$ values (40–70 µM CO_2) for Rubisco, the carbon assimilating enzyme (Ji et al., 2016). The evidence above indicates that the CO_2 in seawater should be carbon limited for marine macroalgae. The promoting effect of elevated CO_2 on photosynthesis was also reported in other macroalgae species, such as green algae *Ulva linza* (Gao et al., 1999), red algae *Pyropia haitanensis* (Zou and Gao, 2002), and brown algae *Petalonia binghamiae* (Gao and Kunshan, 2010). Meanwhile, the higher pCO_2 level increased $K_{0.5}$ of *S. muticum* at the condition of lower P in the present study, which indicates the plant grown at the condition of higher pCO_2 reduced its photosynthetic affinity for DIC. This phenomenon is commonly found in both microalgae and macroalgae (Gao and Campbell, 2014; Ji et al., 2016; Wu et al., 2008) and is considered as a sign of down-regulated CCMs at high CO_2 conditions (Gao and Campbell, 2014). But this decrease of photosynthetic affinity for DIC did not lead to reduced photosynthesis in *S. muticum* compared to that at the lower pCO_2 in the present study, mainly because of increased CO_2 availability for Rubisco and depressed photorespiration at the elevated ratio of CO_2 to O_2, which has been confirmed in red seaweed *Lomentaria articulate* (Kübler et al., 1999).

The higher P level also increased the net photosynthetic rate of *S. muticum* in the present study, which can be partially explained by the decreased $K_{0.5}$ at the condition of higher P. The decreased $K_{0.5}$ is an indication of increased photosynthetic carbon-use capability. Phosphorus is a key macronutrient component for organisms and high levels of P availability is not only essential for chloroplast DNA and RNA synthesis (Vered and Shlomit, 2008), but is required for various chloroplast functions, referring to phosphorylation of photosynthetic proteins , synthesis of phospholipids and generation of ATP (Zer and Ohad, 2003). Therefore, High P levels could speed up the transport of Ci from media to the site of Rubisco by supplying necessary energy. In addition, P enrichment can increase both activity and amount of Rubisco (Lauer et al., 1989). Meanwhile, phosphorus, with low concentration in seawater, is generally considered to be limiting for marine primary producer (Elser et al., 2007; Howarth, 1988; Müller and Mitrovic, 2015). Therefore, adding extra phosphorus to natural seawater can stimulate photosynthesis of algae. For instance, the midday (12:00) photosynthetic rates increased from 1.3 to 2.3 mg C g^{-1} DW h^{-1} for *S. natans*, from 0.9 to 2.1 mg C g^{-1} DW h^{-1} for *S. fluitans* when 0.2 mM P was added (Lapointe, 1986). In the present study, the addition of 40 μmol P also resulted in nearly two-fold increase of the net photosynthetic rate and V_{max}, which suggests the significant importance of P in photosynthesis of this alga. In addition, the higher P level promoted the synthesis of Chl *a* at the condition of lower pCO_2, which may also contribute to the increased net photosynthetic rate in *S. muticum* at the condition of higher P. Although P is not the component constituting Chl *a*, higher P supply may stimulate the content of Chl *a* synthesis-related enzymes and thus the production of Chl *a*. The positive effect of P on Chl *a* was also reported in *S. thunbergii* (Nakahara, 1990). On the other hand, the higher P level did not increase the Chl *a* content at the condition of higher pCO_2 in the present study. The possible reason is that there is more ATP available at the condition of higher pCO_2 due to the down-regulation of CCMs and thus there is no need to synthesize more Chl *a* to capture more light for cells as excessive energy can lead to the harm to photosynthesis and growth of algae (Gao et al., 2012; Xu and Gao, 2012).

4.2. Effect of pCO_2 *and P on nitrogen assimilation*

The higher pCO_2 level noticeably enhanced the nitrate uptake rate in *S. muticum* regardless of P concentration in the present study. This could be attributed to the increased nitrate reductase activity (NRA) at the condition of higher pCO_2. The enhanced NRA at the conditions of high CO_2 was also reported in *U. rigida* (Gordillo et al., 2001), *Hizikia fusiforme* (Zou, 2005), *P. haitanensis* (Liu and Zou, 2015), *Corallina officinalis* (Hofmann et al., 2013), as well as the higher plants *Plantago major* (Fonseca et al., 1997), tomato (Yelle et al., 1987), etc. Taken together, these findings indicate that the response of NRA in plants to elevated CO_2 may be homogeneous.

The higher P level also enhanced the nitrate uptake in *S. muticum* regardless of pCO_2 level, which can be partially due to the increased NRA at the condition of higher P. This is very evident at the condition of lower pCO_2. However, the higher P level decreased the NRA at the condition of higher pCO_2, which did not lead to reduced nitrate uptake. This indicates there should be other mechanisms to account for the promoting effect of the higher P level on the nitrate uptake. One possible mechanism is that the higher P level can increase the availability of ATP that is required for the active uptake of nitrate across the plasma membrane. The phenomenon that ATP concentration increases with P level has been found in higher plants (Olivera et al., 2004; Rychter et al., 2006). Apart from *S. muticum*, the positive effect of higher P level on nitrate uptake was also reported in red macroalgae *Gracilaria lemaneiformis* (Xu et al., 2010) and higher plant *Phaseolus vulgaris* (Gniazdowska and Rychter, 2000). The increased nitrate uptake, NRA and soluble protein at the condition of higher P in the present study suggest that high P availability promoted nitrogen assimilation in *S. muticum*. It is worth noting that the nitrate uptake rates were commonly higher than the corresponding reduction rates of NO_3^- to nitrite NO_2^- by nitrate reductase in the present study, which might be due to the intercellular nitrate storage (Collos, 1982; Lartigue and Sherman, 2005) and the underestimation of RNA measured by the in situ assay (Lartigue and Sherman, 2002).

The higher P level increased the nitrate uptake rate and soluble protein at the conditions of both lower pCO_2 and higher pCO_2 but it only increased the NRA in *S. muticum* at the condition of lower pCO_2 in the present study. Surprisingly, it decreased the NRA at the condition of higher pCO_2. The reason for that may be not onefold but must be related to interaction of pCO_2 and P. High pCO_2, on one hand, could enhance photosynthetic carbon fixation and thus growth by supplying sufficient CO_2. On the other hand, it also results in the decrease of pH and increase of seawater acidity, which can disturb the acid-base balance on cell surface of algae (Flynn et al., 2012). Algae may accordingly allocate additional energy to act against the acid–base perturbation in some way. This hypothesis is supported by increased respiration at the condition of higher pCO_2 and higher P in the present study. The increased soluble protein and decreased NRA at the condition of higher pCO_2 and higher P suggest some H^+ transport-related protein, such as plasma membrane H^+-ATPase, might be synthesized to counteract the acid–base perturbation caused by increased pCO_2 and H^+. ~~The plasma membrane H^+-ATPase plays an essential role in maintaining an electrochemical proton gradient across the plasma membrane (Morth et al., 2011; Sondergaard et al., 2004).~~ The additional production of H^+ transport-related protein like plasma membrane H^+-ATPase could competitively decrease the synthesis of nitrate reductase. This hypothesis needs further experimental evidence to stand even though it could explain the results in the present study. ~~Meanwhile, the higher pCO_2 can also deliver the signal to induce the synthesis of H^+ transport-related protein, but low P supply may limit the synthesis. Accordingly, the nitrate reductase activity did not decrease at the condition of higher pCO_2 and lower P.~~

4.3. Connection between carbon and nitrogen assimilation

The increased net photosynthetic rate at the condition of higher pCO_2 and higher P did not result in higher soluble carbohydrates compared to the condition of higher pCO_2 and lower P. The additional ATP produced by photosynthetic electron transport at the condition of higher pCO_2 and higher P may be drawn to nitrogen assimilation as more soluble protein was synthesized at the condition of higher pCO_2 and higher P. The additional energy allocation to protein synthesis, possibly H^+ transport-related protein to maintain the balance of acid-base, hindered the increase of growth, which may be the reason that the higher P increased the net photosynthetic rate but not the growth rate at the condition of higher pCO_2. Although synthesized protein can also contribute to the increase of thalli weight, it is not as energy-effective as carbohydrates (Norici et al., 2011; Raven, 1982). It seems that *S. muticum* tends to maintain a steady state in vivo even if it can sacrifice growth to some extent, considering that regulation of intracellular acid-base balance is crucial for organismal homoeostasis (Flynn et al., 2012; Smith and Raven, 1979). The increased respiration at HC was also demonstrated in *G. lemaneiformis* (Xu et al., 2010) and *U. prolifera* (Xu and Gao, 2012). The respiration at the condition of higher pCO_2 and lower P did not increase compared to at the condition of lower pCO_2 and lower P in the present study, suggesting the action against acid-base perturbation did not commence. The acid-base perturbation at the condition of higher pCO_2 and lower P may lead to the decreased photosynthetic rate compared to that at the condition of lower $p\mathrm{CO}_2$ and lower P.

5. Conclusion

Our study, for the first time, demonstrates the combined effects of elevated $p\mathrm{CO}_2$ and P enrichment on the physiological traits of a golden alga, *S. muticum.* It suggests current ocean environment is both CO_2 and P limited for the photosynthesis and grow of *S. muticum*. Therefore, future ocean acidification and eutrophication may promote the growth of *S. muticum* and thus occurrence of gold tide events. Meanwhile, *S. muticum* tends to maintain homoeostasis taking advantage of phosphate enrichment, at the cost of growth. Accordingly, the combination of ocean acidification and eutrophication may not boost gold tides further compared to ocean acidification or eutrophication alone.

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Table 1. Parameters of the seawater carbonate system at different CO_2 and phosphate conditions. Measurements and estimation of the parameters are described in Materials and Methods. Data are the means ± SD (n = 3). LCLP, the low pCO_2 and low P condition, LCHP, the low pCO_2 and high P condition, HCLP, the high pCO_2 and low P condition, HCHP, the high pCO_2 and P condition, DIC = dissolved inorganic carbon, TA = total alkalinity. Different superscript letters indicate significant differences in one parameter between treatments ($P < 0.05$).

Treatment	pH	pCO_2 (µatm)	HCO_3^- (µmol kg^{-1})	CO_3^{2-} (µmol kg^{-1})	CO_2 (µmol kg^{-1})	DIC (µmol kg^{-1})	TA (µmol kg^{-1})
LCLP	8.07±0.02^{b}	426.9±31.1^{a}	2000.2±51.7^{a}	200.9±5.8^{b}	14.2±1.0^{a}	2215.3±49.7^{a}	2475.2±44.2
LCHP	8.07±0.02^{b}	423.9±21.1^{a}	1987.6±10.9^{a}	199.8±11.4^{b}	14.1±0.7^{a}	2201.5±19.3^{a}	2504.7±33.8
HCLP	7.76±0.02^{a}	1017.2±83.2^{b}	2282.5±27.6^{b}	110.0±10.0^{a}	34.0±2.9^{b}	2426.5±32.5^{b}	2541.5±44.2
HCHP	7.76±0.02^{a}	992.2±44.9^{b}	2261.8±35.9^{b}	110.5±5.9^{a}	33.1±1.5^{b}	2405.4±39.4^{b}	2563.6±44.2

Table 2. The carbon-saturating maximum photosynthetic rate ($V_{\max}$, µmol O_2 g^{-1} FW h^{-1}) and half saturation constant ($K_{0.5}$, mM) for *S. muticum* cultured under different pCO$_2$ and P conditions for 13 days. Different superscript letters indicate significant differences in one parameter between treatments ($P < 0.05$).

	LCLP	LCHP	HCLP	HCHP
V_{max}	57.00 ± 2.88^a	93.99 ± 0.98^c	81.18 ± 5.94^b	100.67 ± 6.81^c
$K_{0.5}$	0.21 ± 0.02^a	0.14 ± 0.05^a	0.42 ± 0.08^b	0.19 ± 0.05^a

Fig. 1. Relative growth rate (RGR) of *S. muticum* grown at different pCO_2 and P conditions for 13 days. Data are the means ± SD (n = 3). LCLP, the low pCO_2 and low P condition, LCHP, the low pCO_2 and high P condition, HCLP, the high pCO_2 and low P condition, HCHP, the high pCO_2 and high P condition. Different letters above error bars indicate significant differences between treatments ($P < 0.05$).

Fig. 2. Net photosynthetic rate (RGR) of *S. muticum* after being grown at different pCO_2 and P conditions for 13 days. Data are the means ± SD (n = 3). LCLP, the low pCO_2 and low P condition, LCHP, the low pCO_2 and high P condition, HCLP, the high pCO_2 and low P condition, HCHP, the high pCO_2 and high P condition. Different letters above error bars indicate significant differences between treatments ($P < 0.05$).

Fig. 3. The photosynthesis versus DIC curves of *S. muticum* after being cultured under pCO_2 and P conditions for 13 days. Data are the means ± SD (n = 3). LCLP, the low pCO_2 and low P condition, LCHP, the low pCO_2 and high P condition, HCLP, the high pCO_2 and low P condition, HCHP, the high pCO_2 and high P condition. DIC = dissolved inorganic carbon.

Fig. 4. Chl *a* content of *S. muticum* after being grown at different pCO_2 and P conditions for 13 days. Data are the means ± SD (n = 3). LCLP, the low pCO_2 and low P condition, LCHP, the low pCO_2 and high P condition, HCLP, the high pCO_2 and low P condition, HCHP, the high pCO_2 and high P condition. Different letters above error bars indicate significant differences between treatments ($P < 0.05$).

Fig. 5. Nitrate uptake rate of *S. muticum* after being grown at different pCO_2 and P conditions for 13 days. Data are the means ± SD (n = 3). LCLP, the low pCO_2 and low P condition, LCHP, the low pCO_2 and high P condition, HCLP, the high pCO_2 and low P condition, HCHP, the high pCO_2 and high P condition. Different letters above error bars indicate significant differences between treatments ($P < 0.05$).

Fig. 6. Nitrate reductase activity (NRA) of *S. muticum* after being grown at different pCO_2 and P conditions for 13 days. Data are the means ± SD (n = 3). LCLP, the low pCO_2 and low P condition, LCHP, the low pCO_2 and high P condition, HCLP, the high pCO_2 and low P condition, HCHP, the high pCO_2 and high P condition. Different letters above error bars indicate significant differences between treatments ($P < 0.05$).

Fig. 7. The contents of soluble carbohydrates (a) and protein (b) of *S. muticum* after being grown at different pCO_2 and P conditions for 13 days. Data are the means ± SD (n = 3). LCLP, the low pCO_2 and low P condition, LCHP, the low pCO_2 and high P condition, HCLP, the high pCO_2 and low P condition, HCHP, the high pCO_2 and high

P condition. Different letters above error bars indicate significant differences between treatments ($P < 0.05$).

Fig. 8. Dark respiration rate of *S. muticum* after being grown at different $p\text{CO}_2$ and P conditions for 13 days. Data are the means ± SD (n = 3). LCLP, the low $p\text{CO}_2$ and low P condition, LCHP, the low $p\text{CO}_2$ and high P condition, HCLP, the high $p\text{CO}_2$ and low P condition, HCHP, the high $p\text{CO}_2$ and high P condition. Different letters above error bars indicate significant differences between treatments ($P < 0.05$).

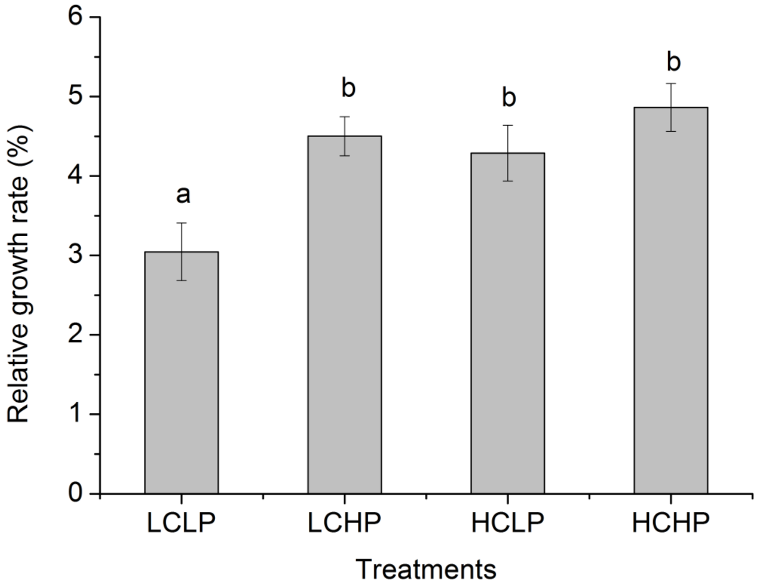
6
5
4
3
2
1
0
Relative growth rate (%)
a
b
b
b
LCLP
LCHP
HCLP
HCHP
Treatments

Fig. 1

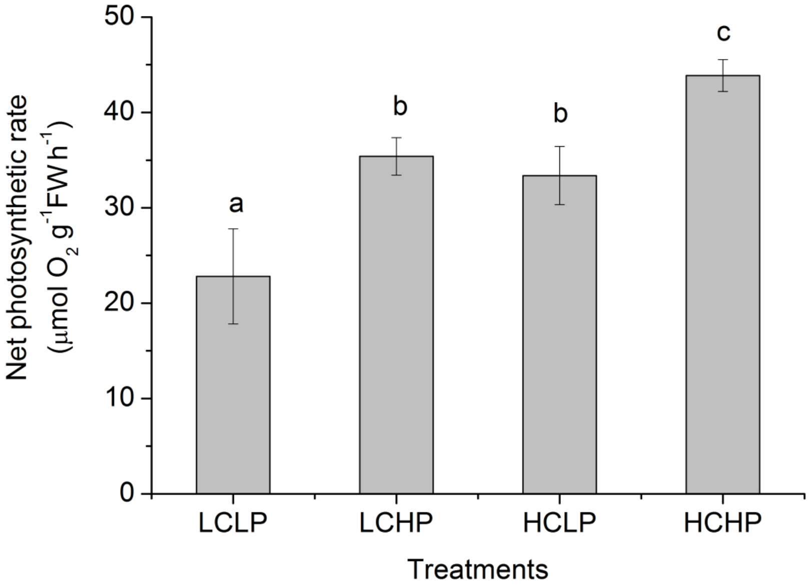
50
40
30
20
10
0
Net photosynthetic rate
(μmol O_2 g^{-1}FWh^{-1})
a
b
b
c
LCLP
LCHP
HCLP
HCHP
Treatments

Fig. 2

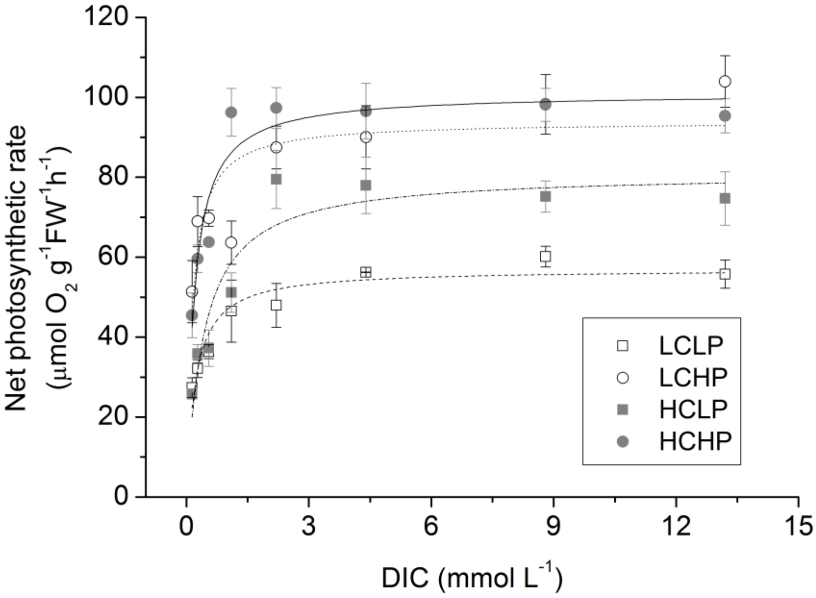
Net photosynthetic rate
(μmol O_2 g^{-1}FW^{-1}h^{-1})
DIC (mmol L^{-1})
0
20
40
60
80
100
120
0
3
6
9
12
15
LCLP
LCHP
HCLP
HCHP

Fig. 3

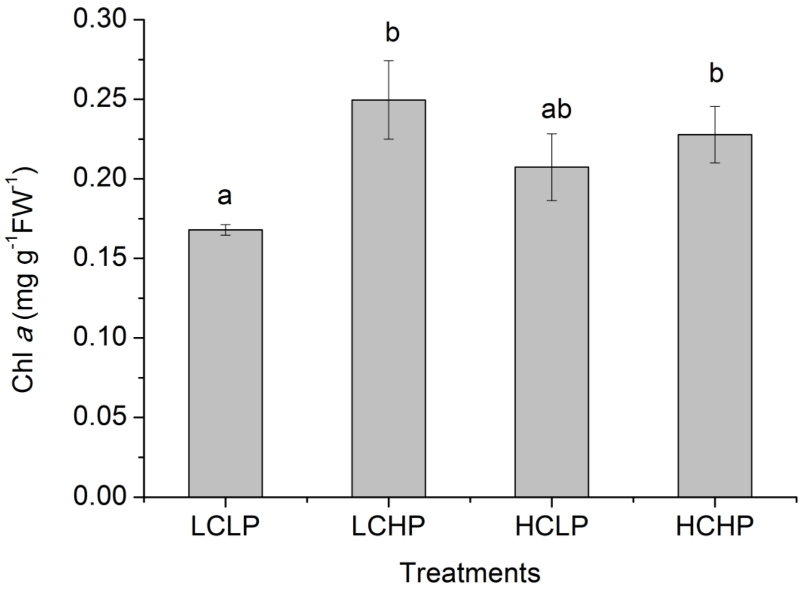
0.30
0.25
0.20
0.15
0.10
0.05
0.00
Chl *a* (mg g^{-1}FW^{-1})
a
b
ab
b
LCLP
LCHP
HCLP
HCHP
Treatments

Fig. 4

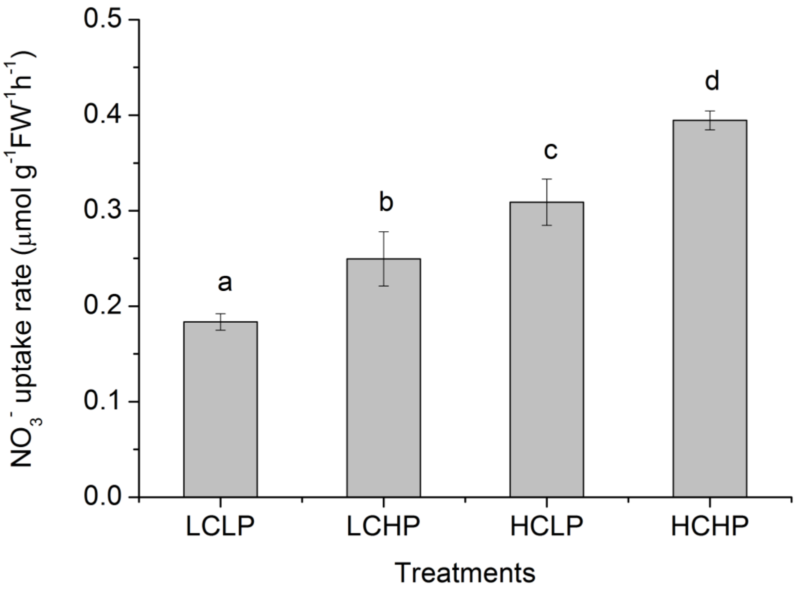
0.5
0.4
0.3
0.2
0.1
0.0
NO3- uptake rate (μmol g-1FW-1h-1)
a
b
c
d
LCLP
LCHP
HCLP
HCHP
Treatments

Fig. 5

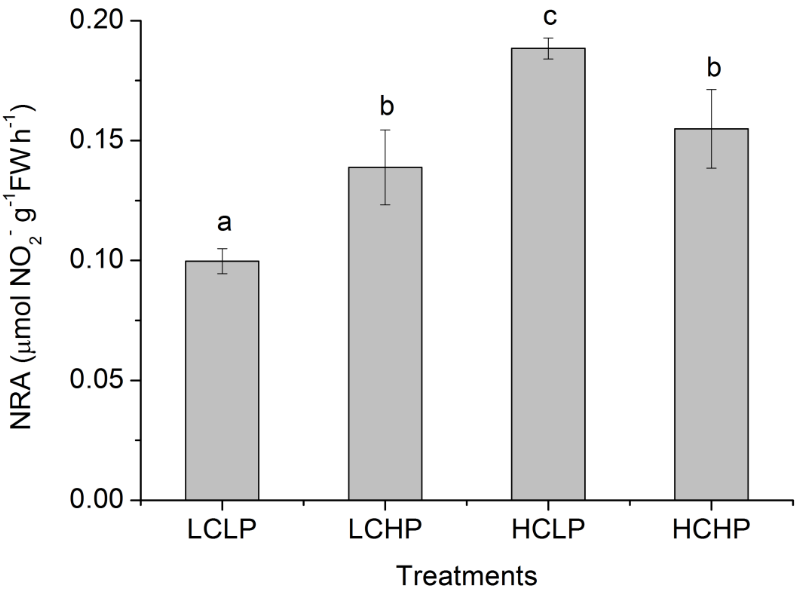
0.20
0.15
0.10
0.05
0.00
NRA (μmol NO2- g-1FW h-1)
a
b
c
b
LCLP
LCHP
HCLP
HCHP
Treatments

Fig. 6

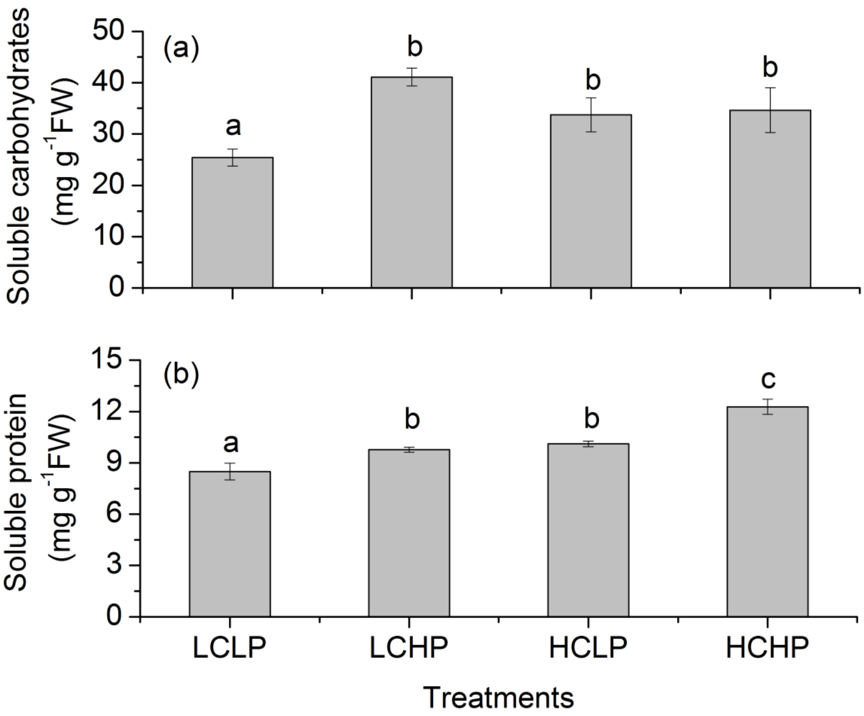
(a)
Soluble carbohydrates
(mg g-1FW)
50
40
30
20
10
0
a
b
b
b
(b)
Soluble protein
(mg g-1FW)
15
12
9
6
3
0
a
b
b
c
LCLP
LCHP
HCLP
HCHP
Treatments

Fig. 7

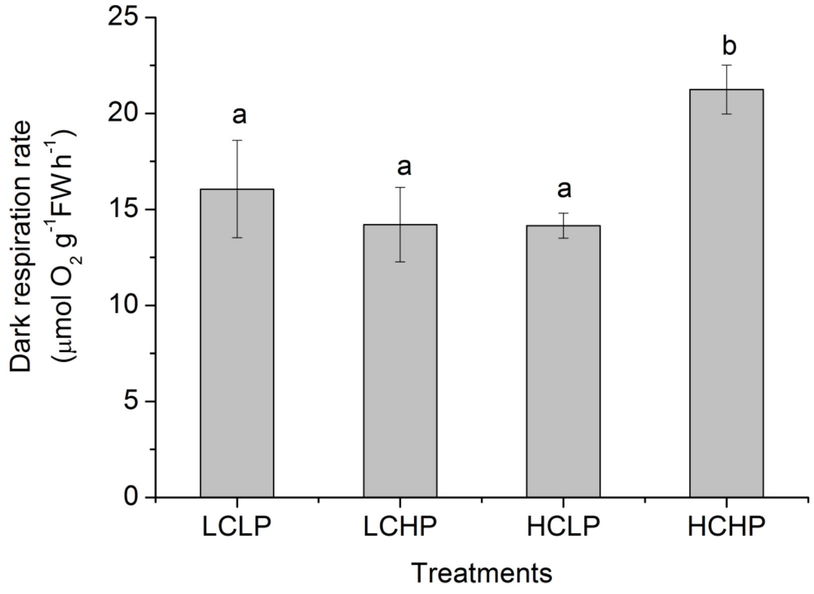
Dark respiration rate
(μmol O2 g-1 FW h-1)
25
20
15
10
5
0
a
a
a
b
LCLP
LCHP
HCLP
HCHP
Treatments

Fig. 8