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Nutrient Enrichment Regulates the Growth and Physiological Responses of *Saccharina japonica* to Ocean Acidification

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Abstract Environmental changes, such as ocean acidification and eutrophication, have created threats to kelp mariculture. In this study, the growth, photosynthesis, respiration and nutrient composition of *Saccharina japonica* were evaluated at different levels of pCO_2 (400 and 800 μL L^{-1}) and nutrients (nutrient-enriched and non-enriched seawater). Elevated pCO_2 decreased the relative growth rate (RGR), net photosynthetic rate and contents of tissue carbon and tissue nitrogen under non-enriched nutrient conditions, but it had no significant effect on these parameters under nutrient-enriched conditions. The dark respiration rate was positively affected by elevated pCO_2 regardless of the nutrient conditions. However, the C:N was unaffected by elevated pCO_2 at both nutrient levels. These results implied that ocean acidification could reduce the production and nutrient contents in the tissues of *S. japonica*, which was associated with nutrient conditions.

Key words eutrophication; growth; nutrient composition; ocean acidification; *Saccharina japonica*

1 Introduction

Because of human activities, the atmospheric CO_2 has continuously risen to a recent level of 400 μL L^{-1} (IPCC, 2014) and is predicted to reach 530–1000 μL L^{-1} by the end of this century (IPCC, 2013). When excessive CO_2 is absorbed by the ocean, the seawater pH decreases. This process is called ocean acidification (OA). OA not only alters the fundamental chemistry of the ocean but also imposes great effects on marine organisms (Johnson *et al*., 2017; Huang *et al*., 2018; Jia *et al*., 2019). Previous studies have shown that OA could influence the growth of many kind of macroalgae (Schmid *et al*., 1996; Mercado *et al*., 1999; Olischlaeger *et al*., 2012), such as *Hizikia fusiforme* (Zou, 2015), *Sargassum muticum* (Xu *et al*., 2017), *Pyropia haitanensis* (Chen *et al*., 2019), *Ulva lactuca* (Chen *et al*., 2019) and *Prionitis cornea* (Kim *et al*., 2016). Additionally, OA could exert various effects on the physiological response of different macroalgae. For instance, in terms of photosynthesis and biochemical compositions, a high CO_2 concentration had a positive effect on *Ecklonia cava* (Oh *et al*., 2015) and *Cystoseira tamariscifoliawas* (Celis-Plá *et al*., 2017), and a negative effect on *Fucus vesiculosus* (Gutow *et al*., 2014) and *P. cornea* (Kim *et al*., 2016).

In addition to OA, eutrophication is another issue in the coastal seawater environment. Due to anthropogenic activities, a large amount of wastewater inputs are generated in coastal areas, which affect the coastal ecosystem (Geertz-Hansen *et al*., 1993; Smith *et al*., 2003). Nutrients are a main factor that influences the growth and physiology of macroalgae. Some previous studies have reported that the photosynthesis and biochemical content of macroalgae are likely to increase under high nutrient availability (Mizuta *et al*., 2001; Agatsuma *et al*., 2014; Endo *et al*., 2017; Kang and Chung, 2018). The maturation of *Saccharina religiosa* gametophytes was enhanced at higher nutrient concentrations (Mizuta, 2001), and an increased synthesis of biochemical compositions under a high nutrient availability was observed in *Saccharina latissima* (Boderskov *et al*., 2016).

Large kelps constitute an important part of the lower intertidal and subtidal zones and provide a nursery ground and habitat for a variety of marine animals (Graham, 2004; Agatsuma *et al*., 2014; Endo *et al*., 2017). As an important population of seaweed, kelps also act as ecosystem engineers and carbon sinks in coastal areas (Gao *et al*., 1999), which shows the great potential for CO_2 bioremediation (Gao and McKinley, 1994). In addition to their important ecological significance, kelps are also widely used as food and raw industrial materials (Endo *et al*., 2017; Gao *et al*., 2017; Xu *et al*., 2019). Given their significant commercial values and ecological effects, many studies have been conducted to investigate the interactive effect of OA and eutrophication on them, and the results suggested that the combination of OA and nutrient enrichment could produce synergism or a neutral effect on the

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growth and physiology of kelp (Russell *et al*., 2009; Falkenberg *et al*., 2013). For instance, the turf algal percent cover multiplied under higher CO_2 and nutrient level conditions (Russell *et al*., 2009). In contrast, the growth and C:N ratio of *Ecklonia radiata* were influenced by nutrients, but CO_2 and the combination of the two factors did not show significant effects on these parameters (Falkenberg *et al*., 2013).

The canopy-forming kelp *Saccharina japonica* is an important commercial alga and is widely cultivated in China, Japan and Korea (Selivanova *et al*., 2007; Liu *et al*., 2009; Hwang *et al*., 2018). Some studies have investigated the responses of their early stage and sporophyte to OA (Xu *et al*., 2015, 2019). The results of these studies showed that the meiospore germination, fecundity and reproductive success were reduced (Xu *et al*., 2015), and the sporophyte growth of *S. japonica* was increased by elevated CO_2 concentration (Xu *et al*., 2019). However, these studies examined the effects of OA individually or the interactions of OA and other environmental factors. Few studies assessed the combined effects of nutrient and OA on the growth and nutrient composition of *S. japonica*.

Therefore, in the current study, we investigated the combined effects of elevated CO_2 and NO_3^- on the growth, photosynthesis, respiration, and nutrient composition of *S. japonica*. According to previous studies, we hypothesized that an elevated CO_2 level would suppress the growth and nutrient composition of this kelp, and a high NO_3^- level would affect its physiological responses to CO_2. The results of this study are expected to provide valuable information on how the future oceanic environment will influence the cultivation and production of *S. japonica* in China and assess the potential effect of this kelp as a carbon sink under the future oceanic conditions.

2 Materials and Methods

2.1 Algal Collection and Maintenance

Adult sporophytes of *S. japonica* (approximately 110 cm in average length, $n=20$) were collected from the cultivated populations in Rongcheng, Shandong, China (36°07′N, 120°19′E) in January 2019. The samples were kept in 7℃ cool seawater and taken to the laboratory within 5 h. Healthy sporophytes were selected and rinsed with sterilized seawater to remove any sediments and epiphytic organisms. More than 60 discs (1.4 cm in diameter) were cut from the meristem using a cork borer for the subsequent experiments. They were stock-cultured in a 7-L plastic tank containing 6 L of filtered seawater, which was acquired from the coastal area of Taipingjiao, Qingdao, with a salinity of approximately 30. The light intensity was maintained at 90 μmol photons $m^{-2} s^{-1}$ with a 12 h:12 h light/dark cycle, and the temperature was controlled at 7℃, which was close to the seawater temperature at the sampling site, for 3 d to recover the cut wounds.

2.2 Culture Experiment and Growth

The culture experiment used a two-way factorial design (2×2 treatments) consisting of two pCO_2 levels (ambient: 400 μL L^{-1}; elevated: 800 μL L^{-1}) and two nutrient levels (non-enriched natural seawater and nutrient-enriched seawater). The cultures were conducted for 6 d, and three replicates were prepared for each treatment. During the experiment, a light/dark photoperiod of 12 h:12 h and a light intensity of 90 μmol photons $m^{-2} s^{-1}$ were held constantly. This experiment used 12 side-arm flasks with each flask containing 500 mL of natural seawater or 50% Provasoli's ES Iodine medium (PESI)-enriched seawater (Tatewaki, 1966). In the 50% PESI-enriched seawater treatment, the culture medium contained either 438 μmol L^{-1} NO_3^- and 28 μmol L^{-1} $H_2PO_4^-$, or 28 μmol L^{-1} NO_3^- and 2 μmol L^{-1} $H_2PO_4^-$ in the natural seawater. The elevated nutrient level was based on studies referring to eutrophication (Wu *et al*., 2015; Ménesguen *et al*., 2018), and nutrient limitation did not occur at the applied nutrient level during the experiment (based on a preliminary experiment). Four discs were introduced into each flask, which was then gently aerated. The culture medium was regularly renewed every 3 d.

For the experimental treatment, two pCO_2 levels were maintained in two CO_2 incubators, respectively, 400 μL L^{-1} (ambient air) and 800 μL L^{-1} (elevated pCO_2). The CO_2 levels were automatically manipulated in two incubators (GXZ-380C-C02, Jiangnan Instruments Factory, Ningbo, China) by regulating the flow of the ambient air and pure CO_2 gas. Autoclaved natural seawater with the current pCO_2 level (approximately 400 μL L^{-1}) was used as the control. A pH meter (Orion STAR A211; Thermo Scientific) was used to measure the pH value of the medium in each flask. The total alkalinity (TA) was measured using the automatic alkalinity titrator by Gran acidimetric titration (848MPT, Titrino) on 50-mL samples filtered through cellulose acetate membranes (0.22 μm). The seawater carbonate chemistry parameters were estimated from the pH and TA values, salinity, nutrients, temperature, the equilibrium constants K1 and K2 for carbonic acid dissociation (Roy *et al*., 1993), and KB for boric acid (Dickson, 1990) using the CO_2SYS software programme (Lewis and Wallace, 1998).

After 6 days of culture, the fresh weights of the discs were calculated after being softly blotted with tissue paper. The relative growth rate (RGR) of each culture was calculated using the following formula (Gao *et al*., 2017):

$$RGR\,(\%\,d^{-1}) = 100\ln(W_t / W_0)/t\,,$$

where W_0 and W_t represent the initial and final fresh weights of the 4 discs, respectively, and t is the number of days.

2.3 Photosynthesis and Respiration Measurements

After the culture experiment, the net photosynthetic rate (P_n) and the dark respiration rate (R_d) of the discs were determined using a FireStingO_2II oxygen meter (FirestingO$_2$, Pyro Science). After measuring the fresh weight, four discs were transferred to the oxygen electrode cu-

vette with 330 mL of medium from the culture flask. Then the medium was magnetically stirred during the measurement to ensure the even diffusion of oxygen. The temperature and light conditions were the same as the mentioned culture experiment above. The value of P_n was obtained by increasing the oxygen content in the medium and the value of R_d was obtained by decreasing the oxygen content. Prior to the measurements, the samples were acclimated to the conditions in the cuvette for 5 min. The oxygen concentration in the medium was recorded every 1 min for 10 min. The P_n and R_d values were normalized to μmol O_2 g^{-1} FW h^{-1}.

2.4 Tissue Carbon, Tissue Nitrogen and C:N

For the detection of the tissue carbon (TC) and tissue nitrogen (TN) contents, the samples were dried at 80℃ until the dry weight was constant. Before the further processing, the dried samples were ground to powder with a mortar and 2–3 mg of the powder was selected. The TC and TN contents were analyzed with an elemental analyzer (Vario EL III, Elementar, Germany). Data were represented in percentage of the dry weight. The C:N ratio was expressed as a molar ratio.

2.5 Statistical Analysis

All data in the present study are reported as the mean± SD (n=3). Prior to the analysis, tests for the normal distribution (Shapiro-Wilk, P>0.05) and homogeneity (Levene's test, P>0.05) of variance were conducted. A two-way analysis of variance (ANOVA) was used to test the effects of the nutrient and pCO_2 levels on the *RGR*, net photosynthetic rate, dark respiration rate, and the contents of TC, TN and C:N. A Tukey's HSD test was conducted to determine the significance level of the factors (P< 0.05). Different letters indicated the significant differences (P<0.05) among the different experimental treatments. All statistical analyses were performed using SPSS 22.0 software.

3 Results

3.1 Seawater Carbonate Chemistry

The effects of pCO_2 and the nutrient level on the seawater carbonate parameters were detected (Table 1). The two-way ANOVA analysis (P=0.05) showed that pCO_2 had a significant effect on all parameters except for TA, while the nutrient level did not influence any parameter. Elevated pCO_2 reduced the pH value by 0.3 at both the enriched and non-enriched nutrient treatments and CO_3^{2-} by 48% (non-enriched) and 46% (enriched), but it enhanced the dissolved inorganic carbon (DIC) by 8% (non-enriched) and 7% (enriched), CO_2 by 147% (non-enriched) and 130% (enriched), and HCO_3^- by 13% (non-enriched) and 12% (enriched).

Table 1 The parameters of seawater carbonate system in different treatments

Nutrient	pCO_2 (μL L^{-1})	pH	DIC (μmol kg^{-1})	TA (μmol kg^{-1})	CO_3^{2-} (μmol kg^{-1})	HCO_3^- (μmol kg^{-1})	CO_2 (μmol kg^{-1})
Non-enriched	400	8.27±0.05^a	1788.95±26.84^a	2037±11.31^a	169.68±16.94^a	1610.50±42.43^a	8.78±1.35^a
	800	7.91±0.06^b	1925.52±22.04^b	2038±6.37^a	87.73±12.21^b	1816.09±30.69^b	21.70±3.57^b
Enriched	400	8.25±0.05^a	1801.47±24.20^a	2041±9.19^a	163.77±15.24^a	1628.42±38.17^a	9.29±1.27^a
	800	7.95±0.04^b	1928.68±11.74^b	2042±4.95^a	88.16±6.44^b	1819.08±16.17^b	21.43±2.00^b

Notes: DIC, dissolved inorganic carbon; TA, total alkalinity. Data are presented as the mean± SD (n=3). Different letters indicate significant differences (P<0.05) among the different experimental treatments. The units for TA and carbonate chemistry parameters are μmol kg^{-1}. The different letters indicate significant differences (P<0.05) between different experimental treatments.

3.2 Growth

The *RGR* values were significantly affected by pCO_2 ($F_{(1,10)}$=14.498, P=0.005) and nutrients ($F_{(1,10)}$=44.779, P<0.001), respectively (Fig.1). A significant interaction between pCO_2 and nutrient was not detected ($F_{(1,10)}$= 2.097, P=0.186). Under the non-enriched condition, the *RGR* value was significantly higher at 400 μL L^{-1} than at 800 μL L^{-1}. However, in the nutrient-enriched condition, the *RGR* values had no significant difference between the two pCO_2 levels. For both pCO_2 levels, the *RGR* values were higher at nutrient-enriched condition than at the non-enriched condition. The *RGR* showed a maximum of 13.164 % d^{-1} at 400 μL L^{-1} and the nutrient-enriched conditions.

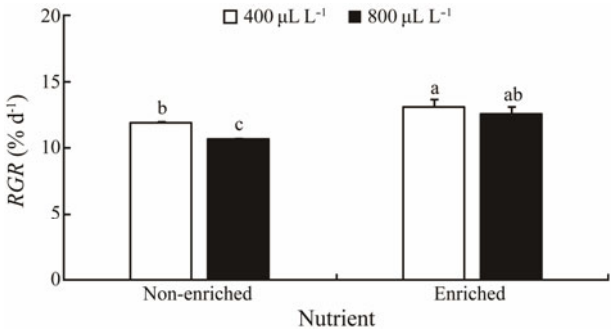

Fig.1 The relative growth rate (*RGR*) of *S. japonica* cultured for 6 d under two pCO_2 levels and two nutrient levels. Data represent the mean± SD (n=3). Different letters indicate significant differences (P<0.05) among different experimental treatments.

3.3 Photosynthesis and Respiration

The P_n values were not significantly affected by pCO_2 ($F_{(1,10)}$=2.353, P=0.164) and nutrient ($F_{(1,10)}$=1.874, P= 0.208), respectively (Fig.2A). However, there was a significant combined effect between pCO_2 and nutrient ($F_{(1,10)}$=15.482, P=0.004). At the non-enriched condition, the P_n values were higher at 400 μL L^{-1} than at 800 μL L^{-1}. However, in the enriched-nutrient treatment, the P_n values did not significantly differ between the two pCO_2 levels. Additionally, at the 400 μL L^{-1}, the P_n values did not show

difference between two nutrient levels. However, at 800 $\mu L\,L^{-1}$, the P_n value in the nutrient-enriched treatment was higher than that in the non-enriched treatment. The P_n values showed a minimum of 0.493 $\mu mol\,O_2\,g^{-1}\,FW\,h^{-1}$ at 800 $\mu L\,L^{-1}$ and the non-enriched conditions.

The R_d values were significantly affected by pCO_2 ($F_{(1,10)}$ =19.645, P=0.002) (Fig.2B). There was no significant effect of nutrient ($F_{(1,10)}$=0.028, P=0.872) on the R_d values. A significant interaction between pCO_2 and nutrient was not detected ($F_{(1,10)}$=1.569, P=0.246). For both nutrient levels, the R_d value at 800 $\mu L\,L^{-1}$ was higher than that at 400 $\mu L\,L^{-1}$. At both pCO_2 conditions, the R_d values did not show a significant difference between two nutrient levels. The R_d showed a maximum of 0.248 $\mu mol\,O_2\,g^{-1}\,FW\,h^{-1}$ at 800 $\mu L\,L^{-1}$ under the enriched-nutrient conditions.

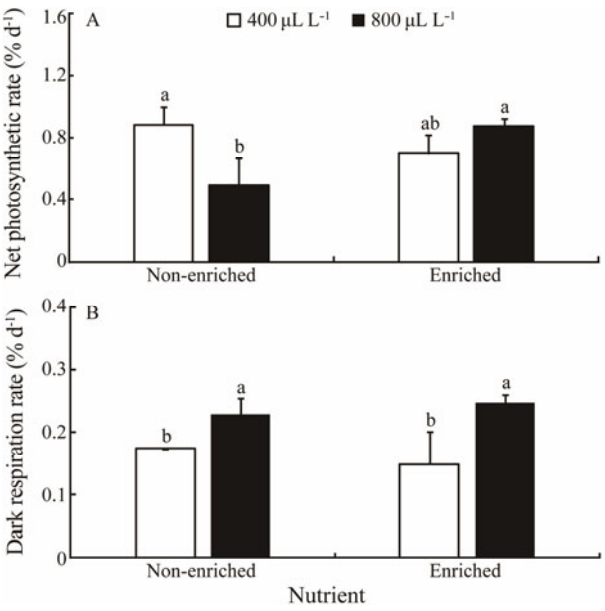

Fig.2 The net photosynthetic rate (A) and dark respiration rate (B) of *S. japonica* cultured for 6 d under two pCO_2 levels and two nutrient levels. Data represent the mean± SD (n=3). Different letters indicate significant differences (P<0.05) among different experimental treatments.

3.4 TC and TN Contents and C:N Ratio

Both the contents of TC ($F_{(1,10)}$=7.884, P=0.023) and TN ($F_{(1,10)}$=26.475, P=0.001) were significantly affected by nutrient. pCO_2 significantly affected the contents of TC ($F_{(1,10)}$=13.824, P=0.006), but it had no significant effect on the contents of TN ($F_{(1,10)}$=3.973, P=0.081) (Figs.3A and B). Additionally, pCO_2 and nutrient had an interactive effect on the contents of TC ($F_{(1,10)}$=31.752, P <0.001) and TN ($F_{(1,10)}$=18.026, P=0.003). In the non-enriched nutrient condition, the contents of both TC and TN contents at 400 $\mu L\,L^{-1}$ were significantly higher than those at 800 $\mu L\,L^{-1}$, but showed no significant difference in the nutrient-enriched condition. Similarly, at 400 $\mu L\,L^{-1}$, the TC and TN contents did not show a significantly difference in either nutrient level. However, at 800 $\mu L\,L^{-1}$, the TC and TN contents were higher at the nutrient-enriched condition than at the non-enriched condition. At 800 $\mu L\,L^{-1}$ and non-enriched nutrient conditions, the TC and TN contents showed a minimum of 26.81% DW and 3.176% DW, respectively.

The C:N ratio was significantly affected by nutrients ($F_{(1,10)}$=7.148, P=0.028) (Fig.3C). The pCO_2 did not have a significant effect on the C:N ratio ($F_{(1,10)}$=0.015, P= 0.905). Moreover, a significant interaction between pCO_2 and nutrients was not detected ($F_{(1,10)}$=0.680, P=0.433). At two pCO_2 levels, the C:N ratio showed no significant difference between the two nutrient levels. Similarly, the C:N ratio showed no significant difference between the two pCO_2 levels regardless of the nutrient level.

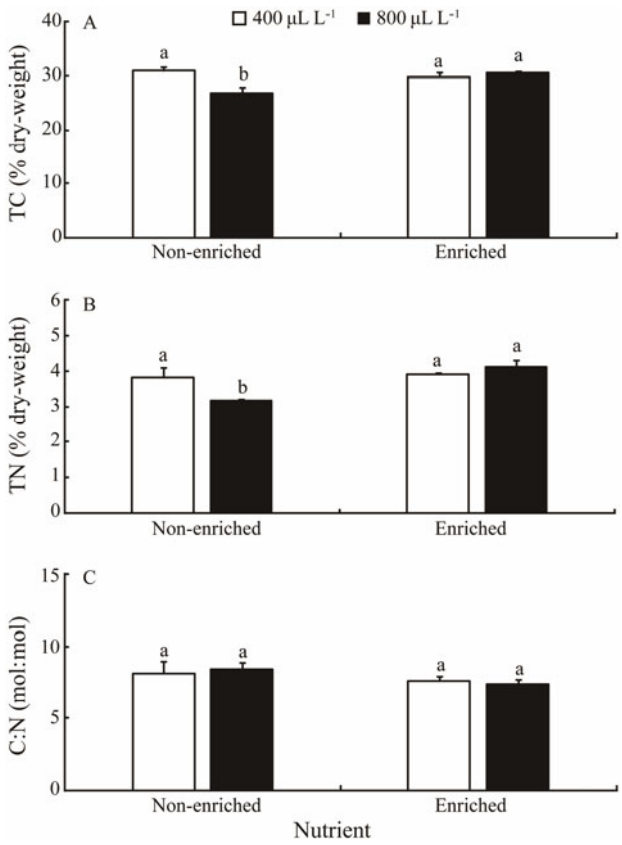

Fig.3 The TC (A) and TN (B) contents and C:N (C) of *S. japonica* cultured for 6 d under two pCO_2 levels and two nutrient levels. Data represent the mean± SD (n=3). Different letters indicate significant differences (P<0.05) among different experimental treatments.

4 Discussion

In the present study, the higher pCO_2 level decreased the growth of *S. japonica* under the non-enriched nutrient condition while growth was not affected by OA under the nutrient-enriched condition. It has been reported that elevated pCO_2 could reduce the growth of kelp (Swanson and Fox, 2007; Gutow *et al*., 2014; Gordillo *et al*., 2015). For instance, the growth of *Alaria esculenta* was inhibited when pCO_2 increased from 380 $\mu L\,L^{-1}$ to 1000 $\mu L\,L^{-1}$ (Gordillo *et al*., 2015). According to previous studies, the negative effect of elevated pCO_2 on growth may occur because decreased pH could disturb the acid-base balance on the cell surface (Flynn *et al*., 2012; Xu *et al*., 2017), which can increase the cellular reactive oxygen species

(ROS) concentration and affect the normal cellular function such as enzyme activities and nutrient uptake and assimilation (García-Sânchez *et al*., 1994; Israel *et al*., 1999). However, in this study, elevated pCO_2 did not affect the growth of *S. japonica* in the nutrient-enriched condition, which indicates that high nutrient availability alleviated the negative effect of the high pCO_2 level. As observed in *Pyropia yezoensis*, high nutrient level could provide nitrogen enough for the synthesis of functional proteins and enzymes to scavenge ROS and prevent the cell from harm derived from the decreased pH (Gao *et al*., 2019). Moreover, because *S. japonica* acted as an ecosystem engineer and formed great canopies with biomass in the lower intertidal and subtidal rocky zones, the benefits in growth rate of this kelp under the high nutrient condition might boost its great potential and mitigate the effect of OA in future oceanic environment.

Like *RGR*, photosynthesis was significantly reduced by elevated pCO_2 in the non-enriched nutrient condition and was not significantly affected by pCO_2 under the nutrient-enriched condition. The negative effect of pCO_2 on photosynthesis was also reported in *Desmarestia aculeata* (Iñiguez *et al*., 2016) and *Ulva prolifera* (Xu and Gao, 2012). According to the previous studies, the carbon concentrating mechanisms (CCMs) of seaweed could be down-regulated by elevated pCO_2 (Rost *et al*., 2003; Wu *et al*., 2010) and thus affect the sustainability of the intracellular CO_2 concentration and availability of CO_2 around Rubisco (Xu and Gao, 2012). In addition, an elevated pCO_2 level could reduce the Rubisco content and activity, which is the primary enzyme of carbon assimilation (García-Sáinchez *et al*., 1994). These findings could explain the lower photosynthesis of the alga grown at the elevated pCO_2 level. However, elevated pCO_2 had no significant effect on photosynthesis under nutrient-enriched conditions, which showed that high nutrient availability modified the response of this alga to CO_2. It had been reported that sufficient nutrient can stimulate the synthesis of related enzymes (Dawes and Koch, 1990; Crawford, 1995), which can promote photosynthesis. Thus, the negative effect of the elevated pCO_2 level on photosynthesis was offset by high nutrient availability. Additionally, in this study, the dark respiration rate for *S. japonica* was significantly enhanced by elevated pCO_2 regardless of the nutrient. Similar results have also been observed in *D. aculeata* (Iñiguez *et al*., 2016) and *P. yezoensis* (Gao *et al*., 2019). It was also showed that the positive effect of elevated pCO_2 on dark respiration may be associated with the changes of proton gradients across the mitochondrial membrane or pH-dependent changes in the functioning of respiratory enzymes (Amthor, 1991; Iñiguez *et al*., 2016). However, increased respiration under the OA condition might play a role in counteracting external pH reduction and provide an additional energy demand for maintaining the internal acid-base stability (Yang and Gao, 2012).

Changes in TC and TN contents were in accordance with the response of the growth rate. The negative effects of elevated pCO_2 on the contents of TC and TN were also reported in *D. aculeata* (Iñiguez *et al*., 2016) and *A. esculenta* (Iñiguez *et al*., 2016). In contrast, the C:N ratio was not affected by elevated pCO_2 and nutrient level while similar results have also been observed in *Hypnea spinell* (Suárez-Álvarez *et al*., 2012), *Ulva pertusa* (Kang and Chung, 2017) and *F. vesiculosus* (Gordon and Carol, 2017). This finding suggests that elevated pCO_2 has the ability to alter the chemical composition in tissue. This may be because the uptake and assimilation of carbon and nitrogen were decreased by elevated pCO_2, which contributes to decreased intracellular inorganic carbon or nitrogen pools (García-Sânchez *et al*., 1994; Israel *et al*., 1999; Spijkerman, 2011; Raven *et al*., 2012; Qu *et al*., 2017). Moreover, a similar result that TC and TN contents were synergistically decreased by OA was reported in *D. aculeata* (Iñiguez *et al*., 2016). According to the previous study (Iñiguez *et al*., 2016), a decrease of TN content could lead to an increase of dissolved organic carbon release and thus maintain the internal C:N ratio.

In summary, the present study demonstrated the negative effects of OA on the growth, photosynthesis, TC and TN contents of *S. japonica* under non-enriched nutrient condition in natural seawater. It also indicated that OA showed a positive effect on the dark respiration rate and a neutral effect on the C:N ratio. Moreover, continuous eutrophication seems to alleviate the negative effects of OA. This study provided important information of the effect of OA on the production and nutrient composition of *S. japonica*. However, to further understand how OA affects the biomass yield and physiology of this kelp, it is necessary to conduct more experiments to study the responses of *S. japonica* to the interactive effects of OA and other variables, such as salinity, temperature and irradiance levels.

5 Conclusions

In this study, the combined effects of OA and eutrophication on the growth and nutrient composition of *S. japonica* were investigated. The results showed that OA had a significant negative effect on growth, photosynthesis, tissue carbon and nitrogen of this kelp. Moreover, nutrient enrichment could alleviate the negative effects of OA. These results indicate that the effect of OA on the production of *S. japonica* would be regulated by nutrient condition. Furthermore, the beneficial effect of high nutrient supply on the growth rate could enhance the ability of this kelp to mitigate the risks from OA and greenhouse in future oceanic conditions.

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