Food Chemistry 271 (2019) 623–629

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Nitrogen availability modulates the effects of ocean acidification on biomass yield and food quality of a marine crop *Pyropia yezoensis*

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ARTICLE INFO

Keywords:
Amino acid
Carbon dioxide
Flavor
Growth
Nutrient
Phycobiliprotein

ABSTRACT

Pyropia yezoensis is an important marine crop in the world. We cultured it under two levels of partial pressure of carbon dioxide (pCO_2) (408 (LC), 998 (HC) μatm) and nitrate (30 (LN) and 500 (HN) μmol L^{-1}) to investigate the effect of ocean acidification on its growth and food quality under changing nitrogen supply. HC decreased growth rate of *P. yezoensis* under LN but did not affect it under HN. Phycoerythrin and phycocyanin were enhanced by HC, particularly at HN, which contributed to the darker color. HC stimulated the synthesis of sweat amino acids regardless of nitrate condition and umami amino acid only under LN. HN increased the content of umami amino acids regardless of pCO_2 condition and sweet amino acids only under HC. Our findings indicate that future ocean acidification may reduce biomass yield of *P. yezoensis* but increase its color and flavor, which was regulated by nitrate availability.

1. Introduction

Marine macroalgae, in spite of being restricted to a narrow zone of the oceans, contribute to around 10% of the total marine primary productivity, and thus plays a key role in the marine biological CO_2 pump and in sustaining natural and sea-farming ecosystems (Seckbach, Einav, & Israel, 2010). In addition, they are also economically and culturally important for providing food, medicine, cosmetics, biofuel, etc. (Gao, Clare, Rose, & Caldwell, 2018). The red macroalgae genus *Pyropia*, has been an important marine crop in East and Southeast Asia for thousands of years (Bito, Fei, & Watanabe, 2017). Up to 1806 thousand tons of *Pyropia* (fresh weight) were cultivated and harvested in 2014 and the total value is about US$1.3 billion (FAO, 2015). Among the genus *Pyropia*, *Pyropia yezoensis*, previously known as *Porphyra yezoensis* (Sutherland et al., 2011), is the world's most lucrative marine crop due to its high nutrition value and delicious flavor (Bito et al., 2017). *P. yezoensis* is often used in Asian cuisine, e.g., sushi and very popular in East Asian countries. Therefore, *P. yezoensis* is extensively cultivated in China, Japan, and Korea (Zhang et al., 2014). The large-scale cultivation of macroalgae not only increases the supply of human food but also improve the coastal environment by mitigating eutrophication and thus the occurrence of harmful algal blooms (Yang et al., 2015).

The atmospheric CO_2 is continuously rising due to human activity and has reached 408 ppm in February 2018, which means an increase of 46% since the industrial revolution. The uptake of anthropogenic CO_2 by the oceans is leading to substantial variation in seawater carbonate chemistry: the concentrations of CO_2, H^+ and HCO_3^- are increasing, whereas CO_3^{2-} concentration is declining. This ongoing process is called ocean acidification. The trend of ocean acidification will continue and pH in surface seawater of the ocean is predicted to decrease by 0.3–0.4 units by the end of this century based on Representative Concentration Pathway (RCP) 8.5 (Gattuso et al., 2015). The variation of seawater carbonate chemistry can impose significant influences on marine organisms. The previous studies show that ocean acidification could reduce calcification rate of calcifying organisms due to declined CO_3^{2-} concentration and thus threaten their survival (Albright et al., 2018). In terms of non-calcifying macroalgae, contrasting effects were reported. The positive effects of ocean acidification on growth of the green macroalga *U. rigida* (Gao et al., 2018), the brown macroalga *Sargassum muticum* (Xu, Gao, Xu, & Wu, 2017) were shown. Meanwhile, the neutral effects of elevated CO_2 on *U. rigida* were reported because thallus was inorganic carbon saturated under the ambient CO_2 level (Rautenberger et al., 2015). Furthermore, a reduced growth rate under high CO_2 concentrations was observed in *P. linearis* (Mercado, Javier, Gordillo, Niell, & Figueroa, 1999), which was attributed to the promoted dark respiration rate under low pH consuming photosynthate accumulated during light period. The differential effects of ocean

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https://doi.org/10.1016/j.foodchem.2018.07.090
Received 31 March 2018; Received in revised form 28 June 2018; Accepted 15 July 2018
Available online 17 July 2018
0308-8146/

acidulation on non-cacifying macroalgae might be due to interspecies or ecotype differences in response to increased CO_2 and decreased pH.

Apart from growth, rising CO_2 can affect food nutrition. It has been extensively reported that elevated CO_2 decreased the concentrations of zinc, iron, and protein in grains of wheat, barley, and rice (Feng et al., 2015; Myers et al., 2014). Meanwhile, few studies have been conducted to investigate the effect of ocean acidification on food nutrition of macroaglae. The recent study demonstrated that ocean acidification increased protein and lipid content in *U. rigida* (Gao, Clare, Rose, & Caldwell, 2017). It seems that macroalgae might respond differently to elevated CO_2 compared to terrestrial plants. But it is apparent that more studies on other macroalgae are needed to conduct to test this hypothesis. In terms of *Pyropia* species, color and flavor are very important characters in determining algal food quality and thus market potential. Nori sheets, with a darker color and more delicious flavor, usually have a higher value in the market. The color of *P. yezoensis* is mainly determined by photosynthetic pigments, such as phycoerythrin, phycocyanin and chlorophyll (Niwa & Harada, 2013). As for flavor, free amino acids (FAA) are known to be the most important substances that contribute to taste in nori sheets (Nishimura & Kato, 1988). The high levels of aspartic and glutamic acids are responsible for the umami taste of macroalgae while glycine, proline, alanine and serine contribute to sweet taste (Nishimura & Kato, 1988). However, little is known in regard to the effect of ocean acidification on color and flavor in *P. yezoensis*.

The nutrient level in different coastal waters demonstrates dramatic variation. The nitrate level could be 6 μmol L^{-1} in the coastal waters in North Sea (Gao et al., 2017). On the other hand, nitrogen level (NO_2 + NO_3) in the Boston could be 69 μmol L^{-1} (Colvard & Helmuth, 2017) and it could be 600 μmol L^{-1} or even higher in the pond where macroalgae were cultivated (Nelson et al., 2001). Growth of macroalgae usually increases with the availability of nitrate. This has been demonstrated in the green macroalga *U. rigida* (Gao et al., 2017), the brown macroalgae *S. muticum* (Xu et al., 2017) and the red macroalga *P. haitanensis* (Chen, Zou, & Ma, 2016). Nitrogen enrichment can also affect biochemical composition of macroalgae, for instance, stimulating the synthesis of protein in *U. rigida* (Gao et al., 2017). In addition, a growing number of literature has shown that the physiological sensitivity of macroaglae to changes in the environment could be affected by nitrogen availability in seawater (Gouvêa et al., 2017; Gao et al., 2018). For instance, nutrient enrichment can offset the negative effect of ocean acidification on growth of *U. linza* (Gao et al., 2018). On the other hand, high nitrate and high temperature synergistically decreased growth rate in *Laurencia catarinensis* (Gouvêa et al., 2017).

To date, little is known regarding the combined effect of ocean acidification and nitrogen availability on growth and food quality of *P. yezoensis*. Based on the previous study, we hypothesized that ocean acidification and nutrient would interact on growth and food quality (color and flavor) of *P. yezoensis*. In this study, we cultured *P. yezoensis* under two levels of pCO_2 and nitrate and investigated the combined effect of ocean acidification and nutrient on physiological performance, color and amino acid in *P. yezoensis*. Our study can provide helpful insight into how the future ocean environment affects productivity and food quality of marine crops.

2. Materials and methods

2.1. Sample preparation and culture conditions

Porphyra yezoensis was collected from the coastal water of Lianyungang (119.3°E, 34.5°N), Jiangsu province, China in March 2016. The young thalli (~2 cm in length) were selected, transported to the lab in a cooling box (4–6 °C) within one hour. Epiphytes and small grazers were removed from the thalli that were then rinsed with filtered (0.7 μm) and autoclaved seawater to remove sediments. The thalli were grown in 1-L balloon flasks containing 900 mL of media under two levels of pCO_2 (408, 998 μatm) and nitrate (31, 500 μmol L^{-1}). The lower levels of pCO_2 and nitrate were the ambient levels. The higher CO_2 represent the level by 2100 based on RCP 8.5 (Gattuso et al., 2015) and the higher nitrate was set as a nitrate-replete condition. The phosphate level for all cultures was 30 μmol L^{-1}. The nitrate (500 μmol L^{-1}) and phosphate (30 μmol L^{-1}) levels are based on von Stosch's enrichment (VSE) medium that is commonly used for *Pyropia* culture and neither nitrate nor phosphate limiting occurred at such a nutrient level during the culture based on a preliminary experiment. The higher nitrate and phosphate levels were achieved by adding certain amount of $NaNO_3$ and $Na_2HPO_4{\cdot}12H_2O$. The CO_2 levels were maintained by aerating ambient air, and CO_2 enriched air from a CO_2 plant incubator (HP1000 G-D, Wuhan Ruihua Instrument & Equipment Ltd, China) with the variation of CO_2 less than 5%. The thalli were cultured in an intelligent illumination incubator (Jiangnan GXZ-300C, Ningbo, China) at 10 °C with a 10 h: 14 h (light/dark) photoperiod of 112 μmol photons m^{-2} s^{-1} photosynthetically active radiation (PAR). The temperature, light period and density were close to the conditions at the sampling site. The transmission of light in seawater at the sampling site is very low due to the high content of sands from the rivers. The mean daily light density at 2 m depth (floodtime) where *P. yezoensis* grows is 115 μmol photons m^{-2} s^{-1} on March 1st 2017 when the samples were collected. Light density was measured by a diving radiometer (ELDONET, Real Time Computers Inc., Germany). The cultures were conducted for 11 days and in triplicate. The seawater was renewed every two days. The following parameters were measured at the end of the culture period.

2.2. Measurement of carbonate parameters

The pH_{NBS} was measured by a pH meter (pH 700, Eutech Instruments, Singapore) that was equipped with an Orion® 8102BN Ross combination electrode (Thermo Electron Co., USA) and calibrated with standard National Bureau of Standards (NBS) buffers (pH = 4.01, 7.00, and 10.01 at 25.0 °C; Thermo Fisher Scientific Inc., USA). Total alkalinity (TA) was determined at 25.0 °C by Gran acidimetric titration on a 25-ml sample with a TA analyzer (AS-ALK1, Apollo SciTech, USA), using the precision pH meter and an Orion® 8102BN Ross electrode for detection. To ensure the accuracy of TA, the TA analyser was regularly calibrated with certified reference materials from Andrew G. Dickson's laboratory (Scripps Institute of Oceanography, U.S.A.) at a precision of ± 2 μmol kg^{-1}. Other carbonate system parameters, which were not directly measured, were calculated via CO2SYS. All carbonate parameters in the seawater under various pCO_2 and nitrate conditions were presented (Tables S1 and S2).

2.3. Measurement of growth

The growth rate of *P. yezoensis* was determined by measuring thallus area. Thalli were taken from the culture flasks and placed on a smooth glass plate. Surface water of the thalli was removed off with tissue paper and the thalli along with a ruler were then photographed by a Canon camera (EOS 70D, China). The area of thallus was calculated by a software of Adobe Photoshop CS2 based on the pixel ratio of the thallus to a standard area. The relative growth rate (RGR) was estimated as follows: RGR (% d^{-1}) = $(\ln A_{t2} - \ln A_{t1})/t \times 100$, where A_{t1} and A_{t2} are the initial and final thallus area after t days culture, respectively.

2.4. Assessment of photosynthesis and respiration

The net photosynthetic rate of thalli was measured with a Clark-type oxygen electrode (YSI model 5300A, USA). Approximately 0.02 g of fresh weight (FW) algae were harvested from the culture flask and incubated in growth media for 1 h to minimize the cutting damage before being transferred to the oxygen electrode cuvette that contained

2 mL sterilized media and the media were stirred during the measurement. The light and temperature conditions were set the same as those in the growth incubators. The increase rate of the oxygen content in the medium within five minutes was defined as the net photosynthetic rate and the decrease rate of the oxygen content in the medium in darkness within ten minutes was defined as the respiration rate. Before the measurements, the samples were allowed to acclimate to the conditions in the cuvette 5 min for photosynthetic rate and 10 min for respiration rate.

The net photosynthetic rate (NPR) and the respiration rate were normalized to μmol O_2 g^{-1} FW h^{-1}.

2.5. Determination of photosynthetic pigments

To determine the content of light-harvesting proteins in red algae, phycoerythrin and phycocyanin, about 0.2 g FW per sample were extracted in 0.1 M phosphate buffer (pH 6.8) at 4 °C after being homogenized with mortar and pestle. The extraction solution was centrifuged (10,000 *g*, 20 min), and then the supernatant was spectrophotometrically scanned (UV-1800; Shimadzu, Japan) to determine the contents of phycoerythrin and phycocyanin (Beer & Eshel, 1985). Phycoerythrin (mg g^{-1} FW) = [(A564 − A592) − (A455 − A592) × 0.2] × 0.12; phycocyanin (mg g^{-1} FW) = [(A564 − A592) − (A455 − A592) × 0.51] × 0.15.

Approximately 0.02 g FW of thalli were extracted with 5 mL of absolute methanol at 4 °C for 24 h in darkness to measure chlorophyll content. The sample was not grinded before extraction as it has demonstrated that the duration of 24 h is enough to extract the pigments completely by comparing the ungrinded data to grinded data in a preliminary experiment. The extraction solution was centrifuged (5000*g*, 5 min) and the optical density of the supernatant was scanned from 400 to 700 nm with an ultraviolet spectrophotometer (UV-1800, Shimadzu, Japan). The concentration of Chl *a* (mg g^{-1} FW) was calculated based on the optical density (OD) at 630 and 664 nm: Chl $a = 15.65 \times OD_{666} - 7.53 \times OD_{653}$ (Wellburn, 1994).

2.6. Determination of free amino acids

The dried thalli of *P. yezoensis* (0.5 g) were fully ground in 10 mL of 0.1 M phosphate buffer (pH 6.8) using a mortar. The mixture was centrifuged at 5000 *g* for 5 min. An equal volume of 5% sulfosalicylic acid was then added to the supernatant. After 1 h incubation at 25 °C, the mixture was centrifuged again at 5000 *g* for 5 min, and the supernatant was taken for a derivatization treatment. Twenty μL of supernatant were diluted with 180 μL of the reaction buffer (0.15 M sodium hydrogen carbonate, pH 8.6). After thorough mixing with a vortex-mixer, 200 μL of 12.4 mM dabsyl chloride reagent (in acetone) were added and the vials were mixed again. The reaction solution was incubated at 70 °C in a water bath, for 15 min. The reaction was stopped by placing the vials in an ice bath for 5 min. Four hundred μL of the dilution buffer [mixture of 50 mL acetonitrile, 25 mL ethanol, 25 mL sodium dihydrogenphosphate 9 mM, dimethylformamide 4% and triethylamine 0.15% (pH 6.55 with phosphoric acid)] were added, followed by thorough mixing and centrifugation (5000 *g*, 5 min). The supernatants were used for further analysis.

Dabsyl derivatives of free amino acids were determined using a high-performance liquid chromatography (Agilent 1100 Series, USA). They were separated with a reversed-phase ODS HYPERSIL column (250 mm × 4.6 mm; 5 mm particle size) at 40 °C. The solvent system consisted of sodium dihydrogenphosphate 9 mM, dimethylformamide 4%, and triethylamine 0.15% (pH 6.55 with phosphoric acid) (A) and 80% acetonitrile (B). Elution was performed at a flow rate of 1 mL min^{-1}, starting with 20% B and installing a gradient to obtain 20% B at 7 min, 35% B at 35 min, 50% B at 45 min, and 100% B at 66 min. Detection was conducted with a UV–Vis detector set at 436 nm. Free amino acids were quantified by the absorbance recorded in the chromatograms relative to external standards (Aladdin, China). The injection volume for both samples and standards was 20 μL. Content of amino acid was expressed as mg g^{-1} dry weight (DW).

2.7. Statistical 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 multivariate ANOVA was conducted to assess the effects of pCO_2 and nitrate on carbonate parameters. Two-way univariate analysis of variance (ANOVA) was conducted to analyze the effects of pCO_2 and nitrate on relative growth rate, net photosynthesis rate, dark respiration rate, chlorophyll *a*, phycoerythrin, phycocyanin. Two-way multivariate ANOVA was conducted to assess the effects of pCO_2 and nitrate on free amino acids. Two-way univariate analysis of variance (ANOVA) was conducted to analyze the effects of pCO_2 and nitrate on umami fatty acids, and sweet amino acids of *P. yezoensis*. Least Significant Difference (LSD) was conducted for post hoc investigation. A confidence interval of 95% was set for all tests.

3. Results and discussion

3.1. Effect of pCO_2 and nitrate on growth and color

The relative growth rates (RGR) in *P. yezoensis* cultured at different pCO_2 and nitrate conditions varied from 18 to 31% d^{-1} (Fig. 1). Both pCO_2 ($F = 5.619$, $df = 1, 8$, $P = 0.045$) and nitrate ($F = 78.697$, $df = 1, 8$, $P < 0.001$) influenced RGR in *P. yezoensis* and these two factors had an interactive effect ($F = 9.596$, $df = 1, 8$, $P = 0.015$). Specifically, *post hoc* LSD comparison ($P = 0.05$) showed that the higher pCO_2 decreased RGR by 26% at the lower nitrate but did not affect it at the higher nitrate. In contrast, the higher nitrate increased RGR by 27% at the lower pCO_2 and by 76% at the higher pCO_2.

The net photosynthetic rates of *P. yezoensis* cultured at different pCO_2 and nitrate conditions had a large variation (63–273 μmol O_2 g^{-1} FW h^{-1}, Fig. 2A). Both pCO_2 ($F = 7.407$, $df = 1, 8$, $P = 0.026$) and nitrate ($F = 957.393$, $df = 1, 8$, $P < 0.001$) had a significant effect on net photosynthetic rate. *Post hoc* LSD comparison ($P = 0.05$) showed that the higher pCO_2 decreased net photosynthetic rate by 26% at the lower nitrate and the decrease at the higher nitrate was not statistically significant. The higher nitrate increased net

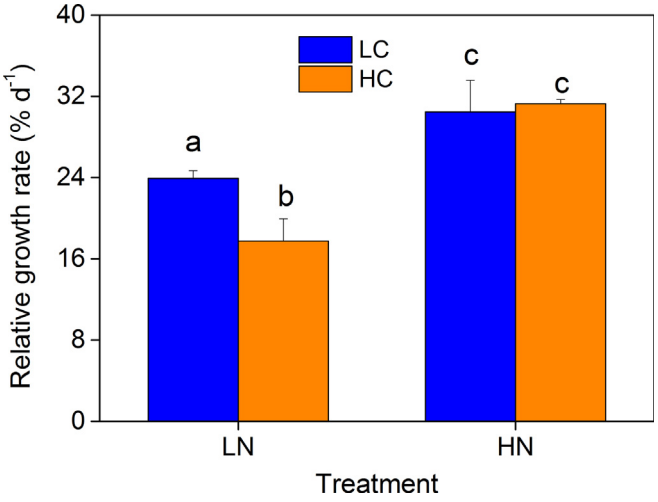

Fig. 1. Relative growth rate (RGR) in *P. yezoensis* cultured at different pCO_2 and nitrate conditions. LC, 408 μatm; HC, 998 μatm; LN, 31 μmol L^{-1}; HN, 500 μmol L^{-1}. The error bars indicate the standard deviations (n = 3). Different letters represent the significant difference ($P < 0.05$) among the treatments.

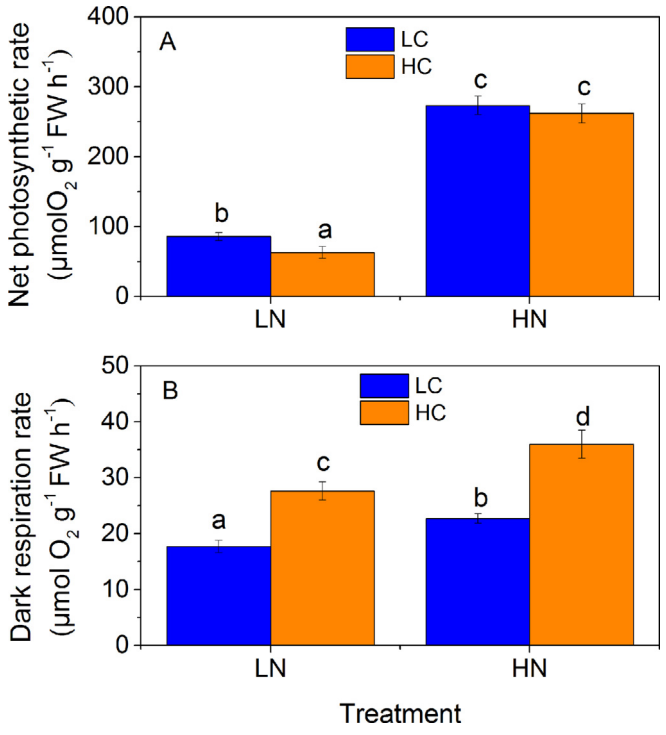

Fig. 2. Net photosynthetic rate (A) and dark respiration rate (B) in *P. yezoensis* cultured at different pCO_2 and nitrate conditions. LC, 408 μatm; HC, 998 μatm; LN, 31 μmol L^{-1}; HN, 500 μmol L^{-1}. The error bars indicate the standard deviations (n = 3). Different letters represent the significant difference ($P < 0.05$) among the treatments.

photosynthetic rate by 219% at the lower pCO_2 and by 316% at the higher pCO_2 (LSD, $P < 0.05$). Contrast to the net photosynthetic rate, dark respiration rate under various conditions had a relatively small variation (18–36 μmol O_2 g^{-1} FW h^{-1}, Fig. 2B). The higher pCO_2 increased dark respiration rate for both the lower and the higher nitrate treatments (F = 48.824, df = 1, 8, $P < 0.001$). The higher nitrate also had a stimulating effect on the dark respiration rate (F = 147.188, df = 1, 8, $P < 0.001$).

As important light-harvesting antennas, the contents of phycoerythrin and phycocyanin in *P. yezoensis* cultured at different pCO_2 and nitrate conditions were measured (Fig. 3). Both pCO_2 (F = 25.473, df = 1, 8, $P = 0.001$) and nitrate (F = 239.929, df = 1, 8, $P < 0.001$) affected the synthesis of phycoerythrin (Fig. 3A). The higher pCO_2 increased it by 125% at the lower nitrate and by 39% at the higher nitrate (LSD, $P < 0.05$). The higher nitrate increased it by 496% at the lower pCO_2 and by 268% at the higher pCO_2 (LSD, $P < 0.05$). In terms of phycocyanin (Fig. 3B), pCO_2 and nitrate had an interactive effect on it (F = 10.189, df = 1, 8, $P = 0.013$). *Post hoc* LSD comparison ($P = 0.05$) showed that the higher pCO_2 did not change phycocyanin at the lower nitrate but increased it by 56% at the higher nitrate. The higher nitrate dramatically increased phycocyanin content regardless of CO_2 condition (LSD, $P < 0.05$). Different contents of phycoerythrin and phycocyanin in *P. yezoensis* grown under pCO_2 and nitrate conditions led to the change of thalli color (Fig. 4). The thalli cultured at the higher nitrate had a much deeper color compared to the lower nitrate. Under the lower nitrate, thalli grown under the higher pCO_2 also had a deeper color compared to the lower pCO_2.

The contents of Chl *a* in thalli grown under various culture conditions ranged from 0.36 to 1.74 mg g^{-1} FW (Fig. 3C). pCO_2 interacted with nitrate to alter Chl *a* content (F = 6.959, df = 1, 8, $P = 0.030$). *Post hoc* LSD comparison ($P = 0.05$) showed that the higher pCO_2 did not affect Chl *a* content at the lower nitrate but decreased it by 18% at the higher nitrate. The higher nitrate increased Chl *a* content regardless

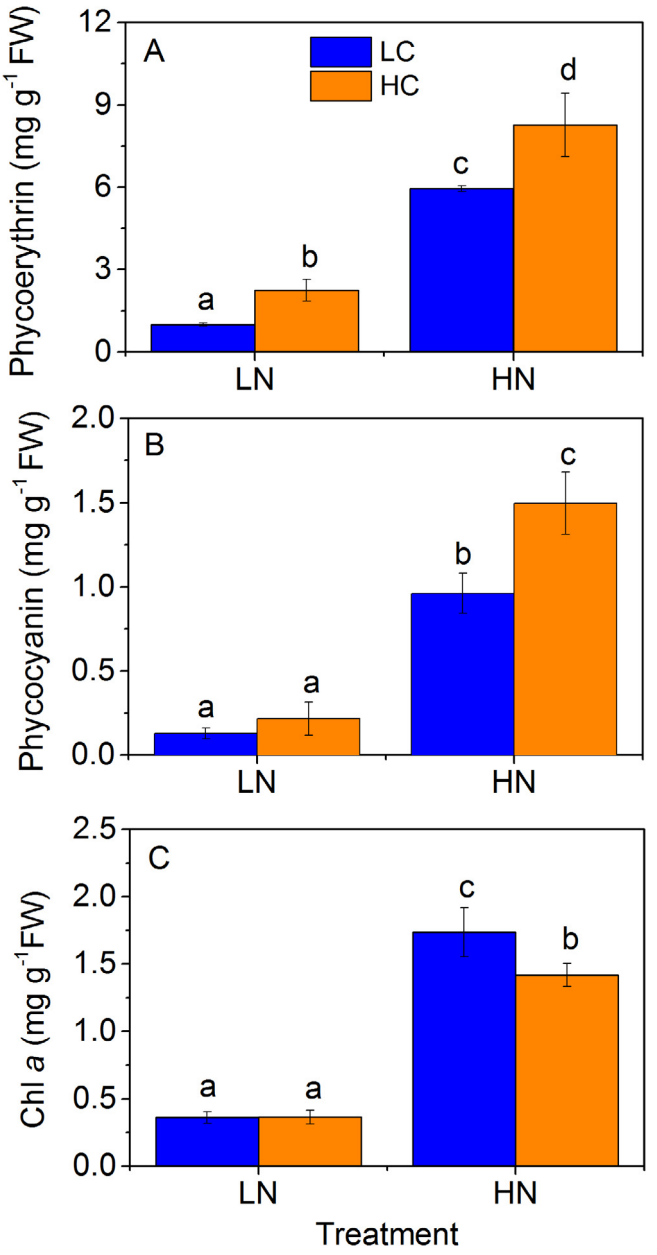

Fig. 3. Phycoerythrin (A), phycocyanin (B) and Chl *a* (C) in *P. yezoensis* cultured at different pCO_2 and nitrate conditions. LC, 408 μatm; HC, 998 μatm; LN, 31 μmol L^{-1}; HN, 500 μmol L^{-1}. The error bars indicate the standard deviations (n = 3). Different letters represent the significant difference ($P < 0.05$) among the treatments.

of pCO_2 level (LSD, $P < 0.05$).

Our previous study demonstrates that *P. yezoensis* has a powerful CO_2 concentrating mechanisms (CCMs) to cope with CO_2 limitation in seawater via carbonic anhydrase and active HCO_3^- uptake (Li, Xu, & He, 2016). However, Gao et al. (1991) showed that higher pCO_2 still enhanced the growth of *P. yezoensis*. This indicates that elevated pCO_2 may stimulate other assimilation processes that support growth. As found in *S. muticum*, elevated pCO_2 did enhance N assimilation and protein synthesis (Xu et al., 2017). However, the elevated pCO_2 did not affect growth of *P. yezoensis* at the higher nitrate and it even reduced it at the lower nitrate in the present study. Apart from the differences in ecotypes, the divergence in the effect of pCO_2 on growth rate between our and Gao, et al. (1991) studies may be caused by different culture conditions used in the two studies. The higher temperature (15 °C) and light density (300 μmol photons m^{-2} s^{-1}) were used in the previous

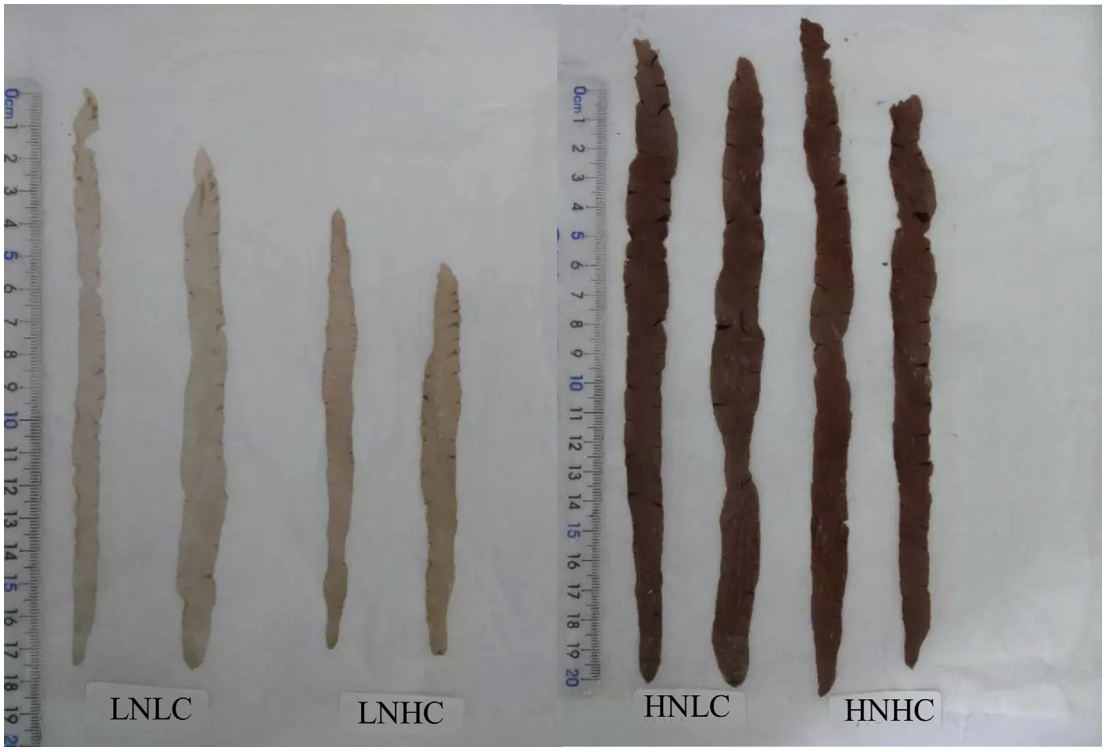

Fig. 4. The color change in *P. yezoensis* cultured at different pCO_2 and nitrate conditions. LC, 408 μatm; HC, 998 μatm; LN, 31 μmol L^{-1}; HN, 500 μmol L^{-1}.

study compared to the present study (10 °C and 112 μmol photons m^{-2} s^{-1}). The lower temperature, particularly the lower light density in the present study may induce more synthesis of photosynthetic pigments, consuming more energy that supports growth. In addition, the negative effect of the higher pCO_2 on growth of *P. haitanensis* in the present study may contribute to the decreased pH. Elevated pCO_2 decreases pH at the surface of cell, which could alter both extracellular and intracellular acid-base balance (Flynn et al., 2012). The disturbed homeostasis could affect intracellular metabolic activities, including photosynthesis and growth (Xu et al., 2017). A reduced growth rate caused by decreased pH was also found in *P. haitanensis* (Xu et al., 2017).

On the other hand, the elevated pCO_2 did not affect growth or photosynthesis of *P. yezoensis* grown at the higher nitrate in the present study. The higher nitrate availability may offset or alleviate the negative effect of pCO_2 via supplying sufficient nitrogen to synthesis functional proteins and enzymes. This hypothesis is supported by the finding that more phycoerythrin and phycocyanin were synthesis under the higher N condition. The higher pCO_2 also induced the synthesis of phycoerythrin and phycocyanin, particularly under the N sufficient condition. Phycoerythrin and phycocyanin are important light-harvesting antennas in cyanobacteria and red algae. However, the increased content of phycoerythrin and phycocyanin under the higher pCO_2 condition did not stimulate net photosynthesis rate in this study. We thus presume that the additional synthesis of phycoerythrin and phycocyanin may act as other roles rather than light-harvesting antennas for photosynthesis. Phycobiliproteins have been proved to have antioxidative activity and be able to scavenge reactive oxygen species (ROS) (Cao, Wang, Wang, & Xu, 2016). Therefore, phycobiliproteins could be induced under stressful environment, such as high temperature, solar UVR and desiccation (Contrerasporcia, Thomas, Flores, & Correa, 2011; Figueroa et al., 1997; Zhang, Yan, & Huang, 2011). Intracellular pH homeostasis plays a critical role in the maintenance of normal cell function. Decreased pH is proposed to increase cellular ROS concentration, disturb intracellular pH homeostasis and inhibit cell proliferation (Lagadicgossmann, Huc, & Lecureur, 2004). The additional synthesis of phycoerythrin and phycocyanin may act as antioxidants to protect cells from the harm caused by the decreased pH. The variation of pH also induced synthesis of PE and PC in *P. haitanensis* (Jiang, Zou, Lou, & Ye, 2017). The additional synthesis of PE and PC under the elevated pCO_2 condition may depress the photosynthesis and carbon assimilation, which is also supported by the enhanced respiration rate under the elevated pCO_2 condition. Contrary to phycoerythrin and phycocyanin, elevated pCO_2 did not stimulate Chl *a* content at the lower nitrate and even reduced it at the higher nitrate, which further supports that additional synthesis of phycoerythrin and phycocyanin did not act as light-harvesting antennas because phycoerythrin, phycocyanin and Chl *a* need to work together for light harvest and transfer.

The enhanced phycoerythrin and phycocyanin resulted in darker color of thalli, which can contribute to a higher market value of *P. yezoensis*. Phycobiliprotein pigment molecules spontaneously fluoresce in vitro and in vivo and these molecules, in particular phycoerythrin, thus have wide applications in biotechnological fields where they are used in fluorescent immunoassays, fluorescent immunohistochemistry assays, biomolecule (proteins, antibody, and nucleic acid) labelling, and fluorescent microscopy (Harnedy & Fitzgerald, 2011). In addition, phycobiliproteins are currently being used as natural colorants for food (i.e., dairy products and chewing gum) and cosmetics (i.e., eyeliners and lipsticks) (Sekar & Chandramohan, 2008). The present study demonstrates that ocean acidification and nitrate enrichment may synergistically enhance the market value of *P. yezoensis* via stimulating the synthesis of its phycobiliproteins.

3.2. Effect of pCO_2 and nitrate on amino acid and flavor

In terms of the profile of free amino acids (Fig. 5 and Table S3), the higher pCO_2 increased the contents of most amino acids except serine, threonine, tyrosine, valine and thus increased the contents of total AA and EAA (Fig. 5 and Table S4). The higher nitrate increased the contents of aspartic acid, glutamic acid, arginine, alanine, tyrosine, cysteine, valine, phenylalanine, total AA and EAA (Fig. 5 and Table S4). In addition, pCO_2 and nitrate interacted on arginine, phenylalanine and

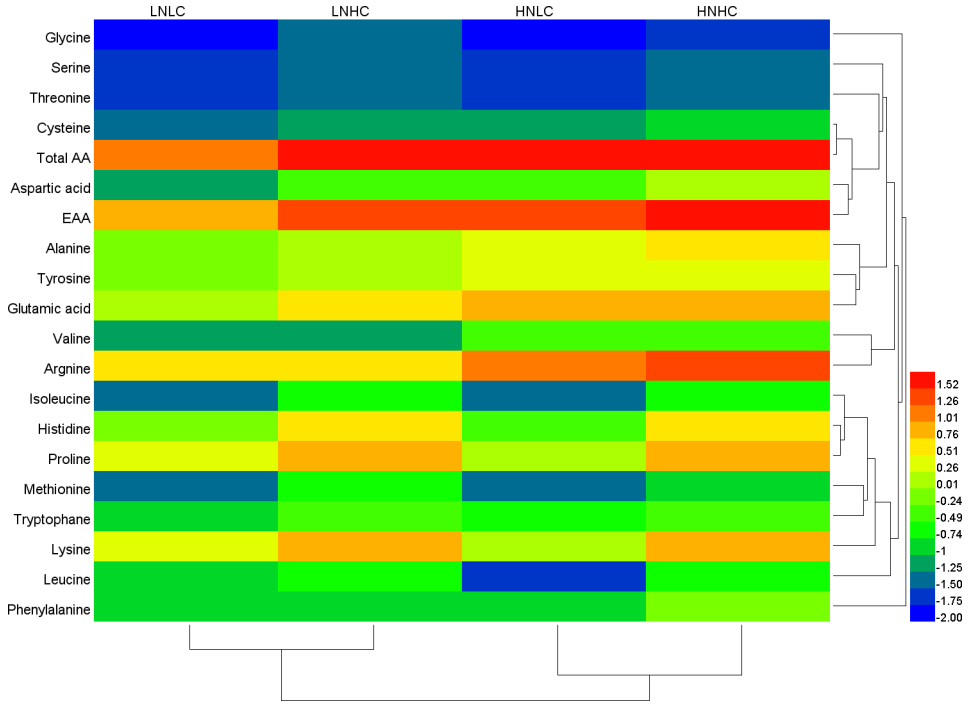

Fig. 5. Heatmap of amino acid composition in *P. yezoensis* cultured at different pCO_2 and nitrate conditions. LC, 408 μatm; HC, 998 μatm; LN, 31 μmol L^{-1}; HN, 500 μmol L^{-1}. To make the map contrasting, the data were taken the logarithm. The original data were presented in Table S3.

EAA (Fig. 5 and Table S4). The higher pCO_2 did not increase arginine or phenylalanine under the lower nitrate but did so at the higher nitrate. The higher pCO_2 and the higher nitrate increased EAA by 166% and 157% respectively while the combination of them resulted in a synergistic increase of 412%.

Umami amino acids (aspartic acid + glutamic acid) and sweet amino acids (glycine + proline + alanine and serine) were calculated to investigate the effect of ocean acidification and nitrate enrichment on flavor of *P. yezoensis* (Fig. 6). Both pCO_2 ($F = 11.870$, df = 1, 8, $P = 0.009$) and nitrate ($F = 68.880$, df = 1, 8, $P < 0.001$) significantly affected umami amino acids. *Post hoc* LSD comparison showed that the higher pCO_2 increased umami amino acids by 423% at the lower nitrate but did not affect it at the higher nitrate. The higher nitrate increased umami amino acids by 302% at the lower pCO_2 and by 102% at the higher pCO_2. In terms of sweet amino acids, both pCO_2 ($F = 77.565$, df = 1, 8, $P < 0.001$) and nitrate ($F = 12.527$, df = 1, 8, $P = 0.008$) also have a significant effect. The higher pCO_2 increased them by 813% at the lower nitrate and by 1098% at the higher nitrate. The higher nitrate did not affect it at the lower pCO_2 but increased it by 40% at the higher pCO_2. The higher pCO_2 alone increased umami and sweet amino acids by 18 and 73 mM, respectively. These changes are enough to lead to the variation of flavor that could be sensed by humans as taste receptors of humans can sense the change of 1–100 mM in amino acids (Nelson et al., 2002).

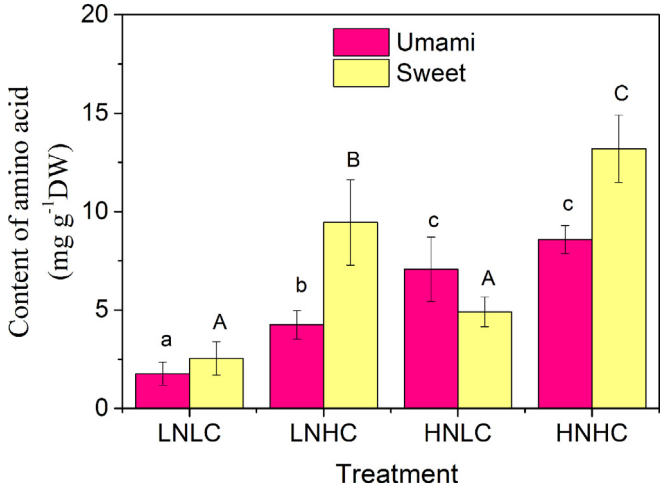

Fig. 6. Content of umami and sweet amino acids in *P. yezoensis* cultured at different pCO_2 and nitrate conditions. LC, 408 μatm; HC, 998 μatm; LN, 31 μmol L^{-1}; HN, 500 μmol L^{-1}. The error bars indicate the standard deviations (n = 3). Different letters represent the significant difference ($P < 0.05$) among the treatments (the lowercase for umami amino acids and the capital for sweet amino acids).

Carbon dioxide plays a critical role in synthesizing amino acids because the carbon skeleton of amino acids derives from CO_2 assimilation. Furthermore, photosynthesis that depends on CO_2 availability can provide reducing equivalents and ATP for amino acid synthesis, including the reduction of nitrate and nitrite, and fixation of NH_4^+. Accordingly, high CO_2 could in theory promote amino acid synthesis. Our results confirm this hypothesis; the higher pCO_2 enhanced the content of most amino acids in *P. yezoensis*. Particularly, the higher pCO_2 increased the content of umami and sweet amino acids, indicating that ocean acidification may improve nutrition and flavor of *P. yezoensis* in spite of its negative effect on growth. On the other hand, the negative effect of the higher pCO_2 on the content of amino acids in *Ulva* species was also documented (Gao, Clare, Chatzidimitriou, Rose, & Caldwell, 2018), and the reasons remain unclear.

Nitrogen is a constitutive element of amino acid. The pathway of amino acid synthesis in plant cells is that nitrate is reduced to nitrite in the cytosol and nitrite is then reduced further to NH^{4+} in the chloroplasts, from which amino acids are finally synthesized (Heldt & Piechulla, 2011). Based on this, the increase in nitrogen level usually stimulates synthesis of amino acid. For instance, the total amino acid content increased by 9.3% in *P. yezoensis* when the nitrate level increase from 4.6 to 100 μmol L^{-1} (Li et al., 2016). Furthermore, it has been reported that the total amino acid content of the green macroalga *U. ohnoi* increased linearly with intracellular nitrogen content ($r = 0.987$) (Angell, Mata, Nys, & Paul, 2014). In our study, the higher nitrate

increased the content of most amino acids and thus the total amino acids. Particularly, the higher nitrate increased umami and sweet amino acids, indicating a positive effect of nitrate enrichment on nutrition and flavor of *P. yezoensis*.

For most amino acid in *P. yezoensis*, the combination of the higher pCO_2 and nitrate resulted in a further increase compared to the single effect of pCO_2 or nitrate. Meanwhile the higher pCO_2 did not affect the content of some amino acids (arginine or phenylalanine) at the lower nitrate but increased them at the higher nitrate. These results suggest the interaction of pCO_2 and nitrate on synthesis of some amino acids in *P. yezoensis*.

4. Conclusion

The oceans are getting more and more acid due to continuous absorption of anthropogenic CO_2. The present study demonstrates the negative effect of ocean acidification on growth of *P. yezoensis* and its positive effect on color and flavor for the first time. It seems that nitrate enrichment could offset the negative of ocean acidification and magnify its positive effect on amino acid synthesis. This study provides important information in regard to the effects of climate change on biomass yield and food quality of the important marine crop *P. yezoensis*. Further investigations into other marine crops are needed to have a more comprehensive view of impacts of clime change on macroalgae cultivation and nutrition.

Acknowledgement

This work was supported by the China Postdoctoral Science Foundation (2018T110463 & 2017M620270), the Science and Technology Bureau of Lianyungang (SH1606), the Jiangsu Planned Projects for Postdoctoral Research Funds (1701003A), the Science Foundation of Huaihai Institute of Technology (Z2016007), and the Priority Academic Program Development of Jiangsu Higher Education Institutions of China.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.foodchem.2018.07.090.

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