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Elevated CO_2 affects kelp nutrient quality: A case study of *Saccharina japonica* from CO_2 enriched coastal mesocosm systems

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ABSTRACT

Kelps provide critical services for coastal food chains and ecosystem, and they are important food source for some segments of human population. Despite their ecological importance, little is known about long-term impacts of elevated CO_2 (eCO_2) on nutrient metabolites in kelps and the underlying regulation mechanisms. In this study, the kelp *Saccharina japonica* was cultured in CO_2 enriched coastal mesocosm systems for up to 3 months. We found that though eCO_2 significantly increased the growth rate, carbon concentrations and C/N ratio of *S. japonica*, it had no effect on total nitrogen and protein contents at the end of cultivation period. Meanwhile it decreased the lipid, magnesium, sodium, calcium contents and changed the amino acid and fatty acid composition. Combining the genome-wide transcriptomic and metabolic evidence, we obtained a systems-level understanding of metabolic response of *S. japonica* to eCO_2. The unique ornithine-urea cycle (OUC) and aspartate-argininosuccinate shunt (AAS), coupled with TCA cycle balanced the carbon and nitrogen metabolism under eCO_2 by providing carbon skeleton for amino acid synthesis and reduced power for nitrogen assimilation. This research provides a major advance in the understanding of kelp nutrient metabolic mechanism in the context of global climate change, and such CO_2-induced shifts in nutritional value may induce changes in the structure and stability of marine trophic webs and affect the quality of human nutrition resources.

KEY WORDS: elevated CO_2, kelp, marine ecosystem, nutrition, metabolite

Abbreviations: AA, amino acid; AAS, aspartate-argininosuccinate shunt; ALA, linolenic acid; ASL, argininosuccinate lyase; ARG, arginase; ASS, argininosuccinate synthetase; CA, carbonic anhydrase; CLL, cellulose; CPS, Carbamoyl-phosphate synthase; DHAP, Dihydroxyacetonephosphate; EAA, amino acids essential to humans; eCO2, elevated CO2; F6P, Fructose 6-phosphate; FAME, fatty acid methyl esters; FPKM, fragments per kilobase of transcript per million mapped reads; Fum, fumarate hydratase; GAP, glyceraldehyde 3-phosphate; GDH, glutamate dehydrogenase; GOGAT, glutamate synthase; GS, glutamine synthetase; ICDHm, Isocitrate dehydrogenase; LA, linoleic acid; MDH, malate dehydrogenase; N, total

nitrogen; NiR, Nitrite reductase; NR, Nitrate reductase; OAA, oxaloacetate; OTC, ornithine transcarbamoylase; OUC, ornithine-urea cycle; OXG, oxoglutarate; PEP, Phosphoenolpyruvate; PK, pyruvate kinase; Pro, crude protein; PUFA, polyunsaturated fatty acid; Pyr, Pyruvate; RubisCO, Ribulose-1,5-bisphosphate carboxylase/oxygenase; TAA, total amino acids; TCA, tricarboxylic acid cycle; Ure, urease

INTRODUCTION

Marine systems in coastal areas are among the most ecologically and socio-economically important on the planet (Barbier et al. 2011). The effects of global climate change, such as increased atmospheric CO_2 levels and associated ocean acidification, are currently threatening the marine ecosystems at multiple scales with unprecedented ecological and economic consequences (Schröter et al. 2005, Hoegh-Guldberg and Bruno 2010). Marine macroalgae (seaweeds) are foundational species prevalent in shallow coastal ecosystems, where they provide basic ecosystem services providing food and forming substrata for settlement to support highly complex and productive food webs (Byrnes et al. 2011, Radulovich et al. 2015, Krause-Jensen et al. 2016). They are also critical constituents in nutrient cycling processes that influence global cycling of key biogeochemical elements such as carbon (C) and nitrogen (N; Vézina et al. 2008). Moreover, seaweeds are also integral to human health due to their unique and rich bioactive compounds, such as polysaccharides and polyphenols that are not present in terrestrial food (Brown et al. 2014). Though humans lack the ability to digest such as β (1,4) linkages in glucan polysaccharides, microbial co-metabolism could ferment them into a wider variety of compounds that provide nutritional or functional benefits (Wells et al. 2017). In addition, they accumulate much higher levels of essential minerals than terrestrial plants, such as calcium and phosphorus (Bocanegra et al. 2009, Gao et al. 2018). Therefore, determining the effects of increased atmospheric CO_2 on marine macroalgae is critical to better characterize marine ecosystems, biogeochemical cycling, and ocean-based food security under future ocean conditions.

Until now, the effects of CO_2 enrichment on macroalgae have focused mainly on how elevated CO_2 concentrations will affect photosynthetic performance, productivity and biomass (Zou et al. 2010, 2011, Chen et al. 2014, Olischläger et al. 2017). Indeed, nutrient qualitative traits of primary producers are often more important than their quantity in trophic transfer efficiency, food web stability and food safety (Müeller-Navarra et al. 2000, 2004, Gilbert et al. 2014). Shifts in algal nutrient content can propagate upward by altering feeding rates, assimilation efficiencies, and growth rates of grazers, which can drastically impact the structure and function of coastal food webs and downstream fisheries (Loladze et al. 2000, Rosenblatt and Schmitz 2016, Ullah et al.

2018). Understanding how environmental drivers impact algal nutrition is fundamental for anticipating food-web responses to global climate change. It has been shown that ocean acidification and warming could reduce energy flow from the marine primary producers to the higher levels (Loladze et al. 2000). The reduction in energy flow caused by declines in the nutrient quality of primary producers can reduce herbivore populations or even drive their extinction and the collapse of the food web (Loladze et al. 2000, Ullah et al. 2018). For higher plants, though rising atmospheric CO_2 concentrations stimulate the growth of C3 crops by increasing photosynthesis rates, nitrogen and water use efficiency (Kimball 2016), increases in crop quantity appear to come at the expense of crop quality with higher carbohydrate-to-protein and carbohydrate-to-minerals ratios but lower mineral and vitamin in C3 plants (Loladze 2002, Ziska et al. 2016), which will negatively impact the quality of human nutrition and exacerbate 'hidden hunger' (Loladze et al. 2014, Myers et al. 2014, Weyant et al. 2018, Zhu et al. 2018).

Despite the importance of nutrient alteration to climate change, limited attention has been paid on how eCO_2 and ocean acidification affects nutrient quality in macroalgae although carbon and nitrogen metabolism were extensively studied (e.g., Gutow et al. 2014, Olischläger et al. 2014, Ober and Thornber 2017, Chen et al. 2017, 2019). In addition, most studies on the response of seaweeds to climate change are conducted in confined short-term and controlled conditions mainly because long-term cultivation is difficult to perform in the laboratory due to their longer life cycle, seasonality and lack of community interactions (Widdicombe et al. 2010, Kumar et al. 2017, Xu et al. 2017). Based on these studies, it is difficult and questionable to predict the long-term effects in natural environment (Kumar et al. 2017). Moreover, mechanism studies on metabolic response of seaweeds to eCO_2 were scarce. Brown seaweeds, which arose from a secondary endosymbiosis of a red alga, are phylogenetically distant from the green lineages (e.g., land plants and green algae; Ye et al. 2015). They were found to hold several unique metabolism pathways in carbon, nitrogen and energy metabolisms, such as the ornithine-urea cycle (OUC). The OUC, which is similar to that of metazoans but is absent in green lineages, functions as repackaging hub connecting carbon metabolism and nitrogen fixation/remobilization by combining the tricarboxylic acid cycle (TCA cycle) and the glutamine synthetase-glutamate synthase cycle (GS-GOGAT cycle) in

stramenopiles (Allen et al. 2011). However, functions of these metabolic pathways in the adaptation of kelp to future climate change are poorly understood.

To address these issues, we constructed stable coastal mesocosm settings (Xu et al. 2017, 2019), and in this study we used this coastal mesocosm setting to explore three main questions: (a) the effects of increased CO_2 and ocean acidification on nutrient variation in cultivated kelp *Saccharina japonica*; (b) the dynamic nutrient contents variation during the treatment period; and (c) the underlying molecular mechanisms involved in regulating nutrient metabolism at the transcriptional, protein and metabolic levels. Together, the research presented here will be of help to assess the acclimation of kelp to future climate change, as well as the potential impacts on coastal ecosystem and human nutrition.

MATERIAL AND METHODS

Experimental materials and mesocosm experimental design

Saccharina japonica samples were collected from the same mesocosm experiment in Xu et al. (2019). The mesocosms were designed following. Six net cages (three per treatment) were used, applying two pCO_2 levels, ambient pCO_2 (400 ppm, bubbled with air, average pH around 8.1) and elevated pCO_2 (1,000 ppm, bubbled with air/CO_2 premixed gas using a CO_2 Enrichlor, CE-100B; Wuhan Ruihua Instrument & 25 Equipment Ltd; average pH around 7.8; Fig. S1 in the Supporting Information). Each net cage measured 8.0 m in length, 8.0 m in width, and 4 m in height. Polythene nets with mesh size of 1 mm in diameter were used around all sides of the cube net, except for the upside. To achieve the desired seawater chemistry, the air/CO_2 mixed gas was delivered around four sides of net cages and dispersed by air stones to allow for the pCO_2 to be well-mixed throughout the cages and the polythene nets were cleaned every week and replaced every month to maintain the stable pH level. Seawater carbonate chemistry in the ambient (400 ppm) or elevated (1,000 ppm) pCO_2 culture environments were monitored every 2 days, and the carbonate chemistry was calculated using the CO2SYS Package in MS Excel based on pH, temperature, salinity, and TA (Fig. S2 in the Supporting Information).

Young sporophytes of *Saccharina japonica* were collected from semi - enclosed Sungo Bay, located on the northwestern coast of the Yellow Sea, China (37°01′–37°09′ N, 122°24′–122°35′ E). The algal species of similar size was inserted into ropes (2.5 m in length, 2.5 cm in diameter) and hanged about 20 cm below the seawater surface. One hundred and twenty individuals with similar size were grown in each cage for approximately three months, from December 2016 to March 2017. During this period, 9 individuals (three from each cage per CO_2 level) were randomly selected for monitoring growth. To determine the effect of algal size during the culture period, samples were taken at 0, 1, 2 and 3 months. The specific growth rate (SGR) was calculated using the equation: SGR (increase $\cdot$ d^{-1}) = (lnw2-lnw1)/(T2-T1) where w1 = wet weight, blade length or width at time T1, w2 = wet weight, blade length or width at time T2. To obtain enough samples of sufficient weight, each of nine individuals from the same net cage were randomly divided into three groups to determine kelp nutrient contents.

Biochemical composition

The *Saccharina japonica* samples were collected after cultivation for 2 or 3 months. Each replicate represent samples taken from each cages. The samples were washed with deionized water to eliminate salt and residues from the thalli surfaces and then stored at -80ºC. Prior to the analytical procedures, the algae samples were defrosted, cut into small pieces and then dried at 60ºC for 24 h. The thalli weight was measured discontinuously during the course of dehydration until the dry weight achieved constant and then homogenized into a fine powder.

Carbon, nitrogen and nitrate determination.

The total C and N content (% DW) was determined against an acetanilide standard with a Vario EL III automatic elemental analyzer (Elementar Analysensysteme). The nitrate contents were determined using a Plant Nitrate Quantification Assay kit (Comin Biotechnology, China) based on salicylic acid colorimetry methods.

Analysis of protein, amino acid content and amino acid composition.

The organic nitrogen content was quantified in a kjeldahl apparatus. The crude protein content was

calculated multiplying the organic nitrogen content by a conversion factor of 6.25. Amino acid content was determined using an Amino acid (AA) Quantification Assay kit (Comin Biotechnology, China) based on ninhydrin colorimetry methods.

For amino acids profiles analysis, the kelp sample was placed in 20 mL acid hydrolysis tube and 16 mL of 6 N hydrochloride acid was then added. The vials were closed under nitrogen, placed in a heating block at 110°C for 24 h. The tubes were cooled and their contents were vacuum-filtered through Whatman no. 41 paper and diluted to 25 mL with ultrapure water in a volumetric flask. Then 1 mL of amino acid standard solution or sample hydrolysate was placed in a tube and dried in a vacuum oven at 85℃. Ultrapure water was then added to the residue and the resulting solution was vacuum-dried at ambient temperature. The contents were diluted with 10 mL 0.02M hydrochloride acid was added. Then 500 μL of the resulting solution was used for derivatization for 1 h by adding 250 μL 0.1 M phenyl isothiocyanate and 250 μL 1 M Triethylamine acetonitrile. Added 2 mL n-hexane to the resulting derivatization products, vortexed and then the bottom liquid was membrane-filtered (Millipore 0.45 lm) for HPLC. Amino acids profiles were evaluated on an Agilent 1260 HPLC system. The mobile phase was a gradient prepared from two solutions, A and B. Solution A was 0.1 M sodium acetate buffer containing 3% (v/v) acetonitrile (pH adjusted to 6.5 with glacial acetic acid). Solution B was 80:20 (v/v) acetonitrile - water. The elution gradient (min:A%) was: 0:100, 14:85, 29:66, 30:0, 37:0, 38:100, 45:100. The flow rate was 1.0 mL · min^{-1} and the detection wavelength 254 nm.

Lipid and fatty acid spectrum analysis.

Lipid content was determined using a standard Soxhlet extraction method. A 0.5 g sample was ground and then extracted with petroleum ether in a Soxhlet apparatus for 8 h. The petroleum ether was then volatilized under air flow. For the fatty acid profile analysis, 20 mg of each lyophilized sample were added to a conical flask with 30 mL petroleum ether. The solution was placed in an ultrasound bath (40.0 kHz, 600 W) at 50℃ for 30 min, and this operation was repeated twice. Then, the solvent was removed with a rotary vacuum evaporator at 50ºC. Glycerophospholipid FAs were transmethylated to fatty acid methyl esters (FAMEs) with 0.4 M

KOH-methanol (v/v) at room temperature (25℃). Analysis of the resulting FAMEs was carried out using a Finnigan Trace GC-MS. FAMEs were identified by comparison with authentic standards (Sigma Chemicals Co., USA) and peaks were integrated with DPS software Version 7.05 (Zhejiang University, China).

Elemental composition.

A sample of 0.2 g dry seaweed was placed in the digestion vessel and 5 mL of concentrated nitric acid was added. The vessels were capped and placed in a microwave pressure digestor Speedwave WX-8000 (Preekem) and subjected to microwave-mediated heating according to the following program: 100ºC for 3 min, 140ºC for 3 min, 160ºC for 3 min, 180ºC for 3 min, 190ºC for 15 min. After cooling, acid digests were made up to 20 mL with Milli-Q water. Three replicates were performed for each seaweed sample as well as blanks. The elemental composition was determined using an inductively coupled plasma (ICP) optical emission spectrometer model Optima™ 8000 DV ICP-OES (Dual View, PerkinElmer Life and Analytical Sciences, Shelton, CT, USA).

Carbohydrate analysis.

The total soluble carbohydrate was extracted from samples with 2.5 N HCl and the concentration determined by the phenol-sulfuric acid colorimetric method. Percent soluble carbohydrate was calculated based on absorptions at 490 nm in a PERSEE T6 UV/VIS spectrophotometer. The alginate contents were analysed colorimetrically after alkali extraction according to Schiener et al. (2015). Laminarin and mannitol contents were analysed using a HPLC method according to Schiener et al (2015). Cellulose (CLL) contents were determined using a Cellulose Quantification Assay kit (Comin Biotechnology, China) based on anthrone colorimetry method and the cellulose content was determined by subtracting the non-structural laminarin content from the total glucan content. Fucoidan contents were determined using HPLC based on the precolumn derivatization method (Zhang et al. 2009).

Analysis of enzyme activities.

Frozen algal samples were ground to powders in liquid nitrogen and the enzyme activities were measured using different Enzyme Activity Assay kits (Comin Biotechnology, China) based on the colorimetry methods. Pyruvate kinase, Glutamate synthase, isocitrate dehydrogenase activities were measured using the NADH-coupled colorimetry method (Groat and Vance 1981, Igamberdiev and Gardeström 2003, Lepper et al. 2010). Glutamine synthetase activity was measured based on the γ-glutamyl-transfer reaction (Yu et al. 2012).

Transcriptome sequencing and analysis

To understand the transcriptional responses to the eCO_2 effect, genome wide expression profiling was carried out. Three *Saccharina japonica* sporophyte samples from the control and three from the eCO_2 condition were selected after cultivation for 3 months. The samples were cleaned with sterile seawater, frozen in liquid nitrogen and stored at -80°C for subsequent transcriptomic analysis in triplicate.

Total RNA was extracted from *Saccharina japonica* sporophyte samples according to Ye et al. (2015). Sequencing libraries were generated with the rRNA-depleted RNA using the NEBNext® UltraTM Directional RNA Library Prep Kit for Illumina® (NEB, USA) following the manufacturer's recommendations. The library preparations were sequenced on an Illumina Hiseq 4000 platform (Gene Denovo, Guangzhou, China) and 150 bp paired-end reads were generated.

Raw data in fastq format were firstly processed using in-house perl scripts. In this step, clean data were obtained by removing reads containing adapter, reads containing poly-N and low-quality reads. Clean data were mapped to the *Saccharina japonica* reference genome using Bowtie v2.0.6 and TopHat v2.0.9. The mapped reads of each sample were assembled by both Scripture (beta2; Guttman et al. 2010) and Cufflinks (v2.1.1; Trapnel et al. 2010) using a reference-based approach. Gene abundances were quantified using the software RSEM (Li et al. 2011). Gene expression level was normalized using the FPKM (Fragments Per Kilobase of transcript per Million mapped reads) method. A gene was considered differentially regulated when a 1.5-fold change in gene expression (q-value < 0.01) was observed.

RESULTS

Effects of eCO_2 on growth

Elevated CO_2 levels significantly increased blade length, width and weight compared to the controls over the cultivation periods of 1, 2 and 3 months (Fig. 1). After the 3-month cultivation, the average blade length, width and weight under eCO_2 were 49.9%, 40.3% and 70.1% higher relative to the controls, respectively.

Effects of eCO_2 on the elemental composition

Elevated CO_2 significantly lowered nitrogen (N) content by 21.7% and 23.2% after 1 and 2-months of cultivation respectively, but the statistical significance disappeared in the third month (Fig. 2). The effect of eCO_2 on carbon (C) content sharply differed from its effect on N content. Elevated CO_2 significantly increased C content by 8.5% after 3-months but had no significant effect after 1 or 2-months (Fig. 2). Over the entire cultivation course, eCO_2 significantly increased the C/N ratio (Fig. 2).

Elevated CO_2 had no statistically significant effect on zinc or iron contents at either time point (Fig. 3). The phosphorus and potassium contents were significantly increased in eCO_2 by 14.11% and 14.09% respectively after 3-month cultivation, but did not show any difference between treatments after 2 months. On the contrary, eCO_2 significantly decreased contents of magnesium, sodium and calcium by 12.6%, 18.7% and 16.4%, respectively after 3-month cultivation and lowered sodium content by 11.7% after 2 months (Fig. 3).

Effects of eCO_2 on protein and amino acid

Elevated CO_2 significantly decreased the crude protein and free amino acid contents by 18.7% and 36.4% respectively after cultivation for 2 months, but had no significant effect on them after 3 months (Fig. 3). To assess whether eCO_2 affected the nutritional value of protein, we analysed 17 types of hydrolytic amino acid components of proteins and the proportion of the amino acids

essential to humans (EAAs) to the total amino acids (TAAs; Fig. 4). The EAA/TAA ratio was 54.1% higher under eCO_2 compared with the control after cultivation for 2 months but 5.4% lower after cultivation for 3 months. This relative decrease in EAA/TAA was caused by an increase in non-essential amino acids, such as Asp, Glu, Cys and Arg (Fig. 4). This means that even when eCO_2 does not affect the overall protein content, it can still alter protein quality.

Effects of eCO_2 on carbohydrate content

The total carbohydrate content of *Saccharina japonica* had no significant difference between the eCO_2 and control treatments at either time point (Fig. 3). Cultivation under eCO_2 did not have any significant effect on the levels of any of the three dietary fibres, cellulose, alginates and fucoidans, at either time point (Figs. 3 and 4). For storage carbohydrate compounds laminarin and mannitol, eCO_2 significantly increased mannitol content by 33% after 3-month cultivation but had no significant effect on laminarin content at either 2 or 3 months (Figs. 3 and 4).

Effects of eCO_2 on lipid and fatty acids

Elevated CO_2 had a significantly negative effect on lipid content (Fig. 3). Lipid content decreased by 26.7% and 18.8% after cultivation for 2 and 3 months under eCO_2, respectively (Fig. 3). Contents of total polyunsaturated fatty acid (PUFA), ω6 and ω3 PUFA were significantly higher by 45.7%, 43.93% and 47.58% under eCO_2 after cultivation for 2 months but showed no significant difference after cultivation for 3 months (Fig. 3). The effects of eCO_2 on PUFA were diverse (Fig. 4). After cultivation for 2 months, contents of all PUFAs were significantly higher under eCO_2 than in control condition, except for linolenic acid (ALA, 18:3 ω3). However, only the linoleic acid (LA, 18:2ω6) level was higher under eCO_2 compared with control after cultivation for 3 months. There was no significant effect of eCO_2 on the ω6/ω3 ratio compared to the control at any of the cultivation times (Fig. 4).

Variation of expression pattern of carbon and nitrogen metabolism genes

For the carbon fixing genes, there are 13 CA encoding genes in *Saccharina japonica* genome, but

just one was significantly up-regulated by 2.3-fold under eCO_2 compared to the control. Among the 17 expressed Calvin cycle genes, only one phosphoglycerate kinase gene was up-regulated significantly (fold change > 2), and 5 other genes increased modestly (1.5 < fold change < 2) by eCO_2 (Fig. 5, Table S1 in the Supporting Information).

For the nitrogen metabolism and amino acid synthesis genes, one out of the two nitrate transporter genes and all of the five Glu synthase (GOGAT) genes were modestly up-regulated by eCO_2 (Table S1). The nitrogen assimilation genes nitrite reductase (NR) did not change significantly, however, two nitrite reductase (NiR) genes were modestly down-regulated (0.55 and 0.79 fold). Elevated CO_2 also modestly increased the expression of some enzymes that synthesize amino acids using intermediate metabolites of glycolysis, such as the 3-isopropylmalate dehydrogenase and dihydroxy-acid dehydratase which synthesize Ile and Val using pyruvate, and bifunctional 3-dehydroquinate dehydratase which synthesize Tyr using phosphoenolpyruvate (Table S1).

For the energy metabolism related genes, most glycolysis genes was up-regulated expressed, however, most of the genes in Krebs cycle were not changed significantly with the exception of one dihydrolipoamide dehydrogenase, one fumarate hydratase and one malate dehydrogenase encoding genes.

Two OUC genes, ornithine transcarbamylase (OTC) and argininosuccinate synthetase (ASS) genes, were modestly increased by 1.53 and1.56 under eCO_2 (Fig. 5). The OUC are associated with TCA cycles through the Asp–argininosuccinate shunt (AAS). In this shunt, fumarate, a product of the OUC product is converted to malate and oxaloacetate, which can be transported to mitochondria acting as substrates of TCA cycle. At the same time, aspartate aminotransferase (AST) catalyzes the conversion of oxaloacetate to aspartate feeding back to the OUC. In line with this, two of three cytoplasmic malate dehydrogenase encoding genes were up-regulated significantly by 1.97 and 1.55-fold respectively and the AST encoding gene was up-regulated by 1.76-fold under eCO_2.

Analysis of the activity of key enzymes and contents of metabolic intermediates

The transcriptional changes of key enzymes were largely confirmed by enzymatic activity measurements. Generally, eCO_2 did not affect the activity of NR but increased the activity of NiR by 28.5%. Elevated CO_2 generally did not affect the activity of GS but significantly increased the activity of NADH-GOGAT by 80.3% (Fig. 6). However, contents of free Glu did not change significantly (Fig. 7).

Pyruvate kinase (PK) catalyzes the final step of glycolysis by producing pyruvate and ATP from phosphoenolpyruvate (PEP). Although eCO_2 did not increase the transcription of PK, it increased the activity of PK by 24.0% and the pyruvate contents by 22.5% (Figs. 6 and 7). Elevated CO_2 significantly increased the activity of ICDHm by 27.2% and the 2-OXG content by 53.0%. Contents of most TCA metabolic intermediates were significantly increased except succinate showing no significant variation. Contents of both free Asp and Arg, which are directly associated with OUC, were significantly increased by eCO_2 by 34.9% and 36.9%, respectively.

DISCUSSION

Kelp is predicted to benefit from eCO_2 under global change and therefore will thrive in natural pCO_2 rich environments (Enochs et al. 2015, Xu et al. 2017). However, studies regarding macroalgal responses to eCO_2 are mostly limited to short-term laboratory experiments in controlled conditions (Kumar et al. 2017). In this study, we constructed stable coastal mesocosm settings and studied the long-term eCO_2 effects on nutrient metabolism in cultivation *Saccharina japonica* population. We found that eCO_2 decreased the lipid and sodium contents but increased Arg, ω6LA contents and C/N-ration during the whole cultivation period. Although the decreased N, lipid contents and increased the C/N-ratio were in accordance with previous studies in *S. latissima* that conducted in short-term experiments (Olischläger et al. 2014), statistical significance disappeared in the third cultivation month. In addition, eCO_2 had a diverse effect on other nutrient contents, such as protein, amino acid, Ca, Mg and PUFA contents. Altogether, short term laboratory experiments still have their limitations to predict how macroalgae will respond to eCO_2 in natural ecosystems.

The effects of eCO_2 on the photosynthetic performance and growth of CCM-type macroalgae depend on their CCM responses (Giordano et al. 2005, Fernández et al. 2015). In most algae, both external carbonic anhydrase (CA_{ext}) and internal CAs (CA_{int}) were crucial components of CCM (Bi et al. 2020). The CAext, located in periplasmic space, dehydrates HCO_3^- to enhance the supply of CO_2 to the cell surface, and the CA_{int}, localized at the mitochondria, chloroplast thylakoid, and cytoplasm, interconverts HCO_3^- and CO_2 to maintain the inorganic carbon (Ci) pool and supply CO_2 to RuBisCO (Fernández et al. 2018). For kelp, CA_{int} activities are much higher than CA_{ext} activities both in *S. japonica* and *Macrocystis pyrifera* (Fernández et al. 2015, 2018, Bi et al. 2020). Moreover, *M. pyrifera* mainly uptake HCO_3^- via the anion exchange (AE) mechanism with CA_{ext} making little contribution (Fernández et al. 2014). So, it is predicted that kelp CA_{int} has more effects on kelp physiological responses than CA_{ext} when they are in repsonse to eCO_2 and OA conditions. This is proved in *M. pyrifera*, in which OA significantly increased CA_{ext} activity while has no effect not only on CA_{int} activity but also on photosynthesis and growth rates (Fernández et al., 2015). However, in *S. japonica*, high CO_2 and HCO_3^- conditions decreased external CA (CA_{ext}) significantly, while they promoted CA_{int} and total CA activities significantly (Bi et al., 2020). In our study, one α-CA gene was significantly up-regulated under eCO_2 condition. This gene has been proved associated with the chloroplast envelopes and thylakoid membranes by gold immunolocalization, and was suggested to provide Ci for chloroplast carbon assimilation (Ye et al. 2014). So, we predicted that the up-regulation of this α-CA_{int} in our study was correlated with the promoted growth and biomass yield of *S. japonica* under the eCO_2 conditions.

Increased CO_2 did not enhance the total carbohydrate content and the main carbohydrate components in *Saccharina japonica* significantly with the exception of mannitol. This differs from land plants which typically showed a marked increase in carbohydrate content in foliar tissues under eCO_2 (Poorter et al. 1997). Nitrogen assimilation is known tightly regulated by respiration which provides energy and C skeletons for N assimilation (Vicente et al. 2016). Although the carbohydrate content was not significantly changed in *S. japonica* by eCO_2, the expression of some genes involved in glycolysis and amino acid synthesis, the activity of PK, and the content of

pyruvate were all up-regulated significantly (Figs. 5-7). We proposed that carbohydrates in *S. japonica* were mainly used for respiration to fuel increased growth and maintain cellular processes, such as nitrogen assimilation and amino acid metabolism, instead of energy storage under eCO_2 conditions. This prediction was also confirmed by the decreased lipid content which is another important carbon and energy storage.

In our study, eCO_2 had no effect on total nitrogen and protein contents at the end of cultivation period. Moreover, both of the gene expression levels and the enzymatic activities of GOGAT were increased in *Saccharina japonica*, which indicated that eCO_2 did not inhibit the assimilation of nitrate. This result was consistent with the recent studies which confirmed that eCO_2 does not inhibit $NO3^-$ assimilation and growth of C3 vascular plants (Andrews et al. 2019, 2020). Our results was also in accordance with the enhanced nitrogen assimilation of brown seaweed *Hizikia fusiforme* in eCO_2 (Zou et al. 2005) and the increased protein content in the giant kelp *Macrocystis pyrifera* (Fern ά ndez et al. 2015). In *M. pyrifera*, future eCO_2 will not affect inorganic nitrogen uptake by metabolically modifying the surface microenvironment (Fernandez et al. 2017). The availability of nitrogenous nutrients in marine habitats is one of the main regulating and limiting factors for growth and biochemical composition in seaweeds (Roleda and Hurd 2019). In our systems, the inorganic nitrogen concentration of sea water was far below the saturation point for *S. japonica* (Xu et al. 2019). So, we predicted that increasing eutrophication of coastal seawater combined with elevated CO_2 may benefit the growth of *S. japonica* in future.

Kelps are phylogenetically distant from the green lineages (e.g., land plants and green algae), and arose from a secondary endosymbiosis and belong to the stramenopiles (Ye et al. 2015). They were found to hold several unique metabolism pathways such as the OUC, which is similar to that of metazoans but is absent in green lineages (Allen et al. 2011). In stramenopiles, OUC was initially found in diatoms, and functions as a key pathway for anaplerotic carbon fixation into nitrogenous compounds. It is also linked directly to both the TCA cycle and the glutamine synthetase-glutamate synthase cycle (GS-GOGAT cycle), making it a distribution and repackaging hub, connecting carbon metabolism and nitrogen fixation/remobilization (Allen et al. 2011). In addition to diatoms, the haptophyte *Emiliania huxleyi* uses the OUC for an efficient metabolic

budgeting to rapidly turn over cellular nitrogen (Rokitta et al. 2014). The complete OUC cycle was also found in brown algae (Cock et al. 2010, Ye et al. 2015). In our study, when *S. japonica* was cultured under eCO_2 conditions, we predicted that the OUC was strengthened by the increased gene expression and metabolic intermediates. This likely generated fumarate and arginine as additional carbon skeletons for energy generation and for cytoplasmic N assimilation (Fig. 5). Thus, it might be reasoned that under eCO_2, more carbon could be fixed in precursors like 2-OXG by the coupled operation of the OUC and the TCA cycle in *S. japonica*.

There are four genes encoding malate dehydrogenases in the *Saccharina japonica* genome, but only one of them has signal peptides and participated in TCA cycle and the other three were supposed to participate in the cytoplasmic AAS which convert malate into both oxaloacetate and reduced power of NADH (Fig. 5). We predicted that the CO_2-enhanced function of the OUC and AAS in *S. japonica* not only recycles the CO_2 molecules to produce 2-OXG, but also could generate more reduced NADH by the cytoplasmic MDH in AAS to support nitrogen assimilation. This prediction was in accordance with the results in C4 plants, which has also been shown that NO_3^- assimilation is relatively independent of CO_2 concentrations in C4 plants because the C4 carbon fixation pathway could generate ample amounts of malate and NADH in the cytoplasm of mesophyll cells (Bloom et al. 2010, Bloom et al. 2012). The unique OUC and AAS pathways may allow kelp to balance carbon and nitrogen metabolism and reduce the nitrogen metabolism stress to increased CO_2 more efficiently than green algae and land plants. This prediction was also confirmed by other kelp species. For example, eCO_2 reduced C:N ratios in *Fucus vesiculosu*s (Gutow et al. 2014) and in small turf algae of the Australian kelp forest (Falkenberg et al. 2013); eCO_2 had no effect on *Sargassum linearifolium* (Poore et al. 2013), and increased protein content in the giant kelp *M. pyrifera* (Fernández et al. 2015).

Moreover, eCO_2 induced the variation of algal nutritional quality could be amplified towards higher trophic levels, such as reducing energy and mass transfer efficiency to aquatic herbivores (Urabe et al. 2003). It has been shown that lower N contents and higher C:N ratios of seaweeds significantly decreased the growth rate of grazers (Duarte et al. 2015). In our results, though there was no significant variation of relative N contents, as well as protein and amino acid contents in

Saccharina japonica between control and eCO_2 condition after cultivation for three months, eCO_2 significantly decreased the N products after cultivation for 1 and 2 months. In natural marine ecosystem, nutritional variation at any stage of seaweed thallus development could be transferred upwards through food chain. Moreover, during the entire period of cultivation, lipid and mineral contents were significantly decreased by eCO_2, which exacerbated the negative effects of eCO_2 on *S. japonica* nutritional quality. It has also been shown that ocean acidification can elevate consumers' metabolic costs and increase the need for more intensive foraging (Duarte et al. 2015, Pistevos et al. 2017). The decreasing primary-producer nutrient quality will intensify foraging stress on consumers, reduce energy flow to higher trophic levels and may induce changes in the structure and stability of marine food webs.

The results here reported improve our knowledge about the nutrient response and molecular mechanisms underpinning the adaptation of *Saccharina japonica* population to future eCO_2. Elevated CO_2 altered protein (EAA/TAA) quality, and differentially affected mineral contents, including Ca, K, Mg, Na, and P, depending on the period of the exposure to eCO_2. Such diverse responses of mineral (Ca, K, Mg, Na, and P) contents to eCO_2 are in line with individual CO_2 studies on land crops that used relatively small sample sizes and replicate numbers, as in our study (3 to 10). However, when averaged over multiple studies spanning various latitudes and plant species, mineral contents show rather consistent declines in C3 land crops and wild plants exposed to eCO_2 (Loladze 2014). While it is plausible that minerals (and the overall ionome) would exhibit broad-based declines in aquatic crops exposed eCO_2 (as they do in land C3 crops), it would take many more data points to obtain a high statistical power to either confirm or refute this hypothesis.

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Author contributions

N.Y. conceived and designed the experiments. D.X. performed the cultivation experiment. X.Z., Y.W. and Y.Z. analyzed the nutrient contents and enzyme activities. X.Z., W.H., and X.F. performed transcriptome experiment and analyzed the data. X.Z. I.L.G.G and S.T. wrote the paper. All authors read and approved the final manuscript.

Data accessibility: Clean sequence data were generated by the Illumina pipeline and are available in NCBI's SRA database (http://www.ncbi.nlm.nih.gov/Traces/sra/sra.cgi) under accession number PRJNA423137, SRP127301.

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Fig. 1 Growth of *Saccharina japonica* under conditions of control (400 μatm, aCO_2) and elevated CO_2 (1000 μatm, eCO_2) for 3 month periods. n = 9 per month per CO_2 level. The columns in a, b and c separated by dashed lines represent cultivation time, and the columns in d represent growth features. The left and right boxes in each column show the data from samples cultured under aCO_2 and eCO_2, respectively. Asterisks indicate significant differences between treatments (* $P < 0.05$, ** $P < 0.01$, wilcoxon test).

Fig. 2 Variation in N content, C content and C/N in *Saccharina japonica* during cultivation under control (aCO_2) and eCO_2 conditions. n = 3 for cultivation periods of 1 and 2 months, and n = 10 for cultivation periods of 3 months. The columns separated by dashed lines represent cultivation time, and the left and right boxes in each column show the data from samples cultured under aCO_2 and eCO_2, respectively. Asterisks indicate significant differences between treatments (* $P < 0.05$, ** $P < 0.01$, wilcoxon test).

Fig. 3 Percentage change in nutrients at eCO_2 relative to control during cultivation for 2 and 3 months. The variance was calculated by standard deviation. n = 3 and n = 10 for cultivation periods of 2 and 3 months respectively. Contents of N3-PUFA, N6-PUFA and total PUFA were significantly increased, and contents of N, Pro, AA, Lipid and Na were significantly decreased at eCO_2 after 2 months ($p<0.05$, wilcoxon test). Contents of C, Man, Pi and K were significantly increased, and contents of Lipid, Ca, Mg and Na were significantly decreased at eCO_2 after 3 months ($p<0.05$, wilcoxon test). N, total nitrogen; Pro, crude protein; AA, amino acid; C, total carbon; TC, total carbohydrate; CLL, cellulose; Alg, alginate; Fuc, fucoidan; Man, mannitol; Lam, laminarin; PUFA, polyunsaturated fatty acid; N3, total ω3 PUFAs; N6, total ω6 PUFAs; Zn, zinc; Fe, iron; K, potassium; P, phosphorus; Mg, magnesium; Na, sodium; Ca, calcium.

Fig. 4 Variation in carbohydrate, hydrolytic amino acids and PUFA contents of *Saccharina japonica* after cultivation for 2 and 3 months under control and eCO_2 conditions. n = 3 for

cultivation periods of 2 months. n = 10 for carbohydrate and PUFA analysis and n=8 for hydrolytic amino acid analysis after cultivation for 3 months. The asterisk above the column means a significant difference between control and eCO_2 condition compared between the same cultivation time. The columns separated by dashed lines represent types of nutrient of carbohydrate, PUFA, EAA and NEAA. The boxes from left to right in each column show the data from samples after cultivation under aCO_2 for 2 months, eCO_2 for 2 months, aCO_2 for 3 months and eCO_2 for 3 months, respectively. EAA, essential amino acids; NEAA, non-essential amino acids; PUFA, polyunsaturated fatty acids. Asterisks indicate significant differences between treatments (* $P < 0.05$, wilcoxon test).

Fig. 5 Graphical representation of the main *Saccharina japonica* metabolic pathways differentially affected by cultivation for 3 months under control and eCO_2 conditions. Expression levels of OTC, Ass, MDH, Fum, GOGAT encoding genes were significantly up-regulated at eCO_2. Enzyme activities of GOGAT and PK were increased and activities of GS were unchanged under eCO_2. Intermediate metabolite contents of Arg, Asp, OAA, malate, fumarate, pyruvate, citrate, 2-OXG were increased at eCO_2 condition. AAS, aspartate-argininosuccinate shunt; NR, Nitrate reductase; NiR, Nitrite reductase; GS, Glutamine synthetase; GOGAT, Glutamate synthase; RubisCO, Ribulose-1,5-bisphosphate carboxylase/oxygenase; PK, Pyruvate kinase; ICDHm, Isocitrate dehydrogenase; GDH, Glutamate dehydrogenase; 2-OXG, 2-oxoglutarate; CA, carbonic anhydrase; GAP, Glyceraldehyde 3-phosphate; DHAP, Dihydroxyacetonephosphate; F6P, Fructose 6-phosphate; PEP, Phosphoenolpyruvate; Pyr, Pyruvate; OAA, oxaloacetate; CPS, Carbamoyl-phosphate synthase; OTC, ornithine transcarbamoylase; ASS, argininosuccinate synthetase; ASL, argininosuccinate lyase; ARG, arginase; Ure, urease; MDH, malate dehydrogenase, Fum, fumarate hydratase.

Fig. 6 Variation of metabolic intermediate contents of *Saccharina japonica* after cultivation for 3 months under control (aCO_2) and eCO_2 conditions. n = 9 for both aCO_2 and eCO_2 levels.

Asterisks indicate significant differences between treatments (* $P < 0.05$, ** $P < 0.01$, wilcoxon test).

Fig. 7 Variation of the enzymatic activities of *Saccharina japonica* after cultivation for 3 months under control (aCO_2) and eCO_2 conditions. n = 9 for both aCO_2 and eCO_2 levels. GS, Glutamine synthetase; GOGAT, Glutamate synthase; PK, Pyruvate kinase; ICDHm, Isocitrate dehydrogenase. Asterisks indicate significant differences between treatments (* $P < 0.05$, ** $P < 0.01$, wilcoxon test).

Table S1. Some differently expressed genes under eCO_2 condition compared to control in *Saccharina japonica*.

Fig. S1. Photograph of net cages and CO_2 enrichlor used for ocean acidification experiment.

Fig. S2. Variation of seawater carbonate chemistry (mean ± standard error) under ambient (400 ppm bubbled with air) or elevated pCO_2 (1,000 ppm bubbled with air/CO_2 premixed gas using a CO_2 Enrichlor) conditions for more than 5 months in the coastal field experiment. (a) pH; (b) Total alkalinity (TA); (c) DIC; (d)HCO_3^-; (e) CO_3^{2-}; (f) CO_2. The shaded areas indicate the standard deviation of three replicates.