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Article

Analysis of Environmental Factors Affecting the Quality of *Neopyropia yezoensis* Cultivated in the Yellow Sea

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Abstract: To investigate the potential influences of nutrients and solar irradiance of the sea area on the laver industry, *Neopyropia yezoensis* samples and the corresponding surface water were collected at different sites in Haizhou Bay and the Jimo aquafarm, and the solar irradiance was recorded on-site. Then the cellular compositions and the nutrients of seawater were determined. A comparative experiment was also designed to investigate the effect of strong light on the cellular composition of *N. yezoensis*. Gray correlation analysis showed that the seawater nutrient levels and solar irradiance had a similar correlation degree of 0.6 to 0.8, which indicated similar effects on algal cellular composition. Compared with those samples collected from Haizhou Bay, the algae cultivated at the Jimo aquafarm had higher contents of total protein and hydrolyzable polysaccharides. In addition, the content of chlorophyll *a* was relatively lower and that of β-carotene higher in the early-stage samples. The results of the comparative experiment showed that the decrease in light intensity on algae promoted the synthesis of chlorophyll *a* and R-phycoerythrin. It is speculated that the nutrient deficiency in the seawater and the resulting high transparency of the water make the algae more exposed to strong light conditions. This may be the reason for the poor glossiness and hardness of the laver products made from the cultivated algae in the north Yellow Sea. Thus, it puts forward specific requirements for the modification of *N. yezoensis* cultivation techniques in the north Yellow Sea.

Keywords: *Neopyropia yezoensis*; environmental factors; nutritional components; nutrient deficiency; solar irradiance

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1. Introduction

Neopyropia is one of the most economically valuable varieties of cultured seaweed and has been cultivated since the 1980s. With the development of cultivation technology and facilities, the scale of *Neopyropia* cultivation in China has expanded rapidly over the past few decades. This has resulted in the continual increase of laver output on a yearly basis. By 2017, China had become the largest producer of laver products globally [1]. *Neopyropia yezoensis*, is mainly cultivated in the coastal areas of Jiangsu Province and south Shandong Province. The annual output of this species is approximately 6000 t, accounting for around 50% of the total output value of laver in China [2].

The northward shift of laver cultivation due to global warming in recent years has become an important trend in China. By the 2021–2022 season, the area of cultivated *N. yezoensis* had reached 700 ha in Qingdao and 1300 ha in Weihai. Although the northward shift of *N. yezoensis* aquaculture prolongs its cultivation duration, the quality of the laver products (food made from *N. yezoensis* blades) is lower than that of traditional products from Jiangsu Province. For example, the laver products of the northern-cultivated *N. yezoensis*

are usually characterized by a poorer glossiness and taste compared with the Jiangsu Province products [3]. Considering the relationship between environmental factors and algal metabolism, fluctuations in environmental conditions may have a large influence on the nutritional components of cultivated laver, either directly or indirectly [3].

Several studies have evaluated algal quality regarding protein, amino acid, and mineral element contents and the environmental influences on the cell development and growth of algae over the cultivation period along the Korean coast [4]. Liang et al. [5] investigated spatial and temporal variations in the chemical composition of *N. yezoensis* and *N. haitanensis* cultured under distinct nutrient conditions. Based on their correlation analysis, typical environmental factors were shown to have a large influence on the laver nutritional composition, such as amino acids, fatty acids, and mineral contents. Moreover, differences in the methods of cultivation, such as periodically drying versus non-drying culture systems, may also influence the nutritional composition [6]. Oceanic conditions can have an important influence on the growth, development, and physiological status of laver. Furthermore, algal quality is greatly influenced by the harvest time and the cultivation area [4,7–10]. However, previous studies have only conducted investigations into the effects of temperature, pH, salinity, and nutrient levels on the spatial and temporal variations in the proximate composition of algae. In addition to these ecological factors, the growth and development of algae will also be inevitably affected by the solar irradiance intensity in the sea area. However, to date, there have been no reports on the effect of light intensity on laver quality over a period longer than a couple of months. The saturation PAR (E_k) of the *Pyropia yezoensis f. narawaensis* gametophyte, which is extensively cultivated in Japan, is 188 μmol m^{-2} s^{-1}, which is much lower than the naturally occurring solar light intensity during its cultivation season [11]. In another study, it was suggested that photoinhibition in *N. yezoensis* usually occurs and the production of reactive oxygen species (ROS) induces a gene expression shift, which causes the accumulation of hydrolyzable polysaccharides, mycosporine-like amino acids (MAAs), and other stress-related molecules [12]. Furthermore, the results of a previous gray correlation analysis implied that nutrients and other environmental factors, including water transparency, also determined laver quality [3]. However, there has been no systematic study investigating the influence of environmental factors, especially light, on the cultivation of laver in Shandong Province.

In this study, *N. yezoensis* cultures from different sites were collected, and the corresponding water samples were analyzed. Changes in solar light intensity were also monitored. Based on the temporal and spatial variations in crude nutrients, correlations between algal cellular compositions and changes in environmental factors were identified. Furthermore, a comparative experiment was conducted to investigate the effect of light conditions directly. The study findings will help to improve our understanding of the effects of different environmental factors on the cellular components and thus contribute to practical support for the improvement of cultivation techniques and facilities in the future.

2. Materials and Methods

2.1. Sampling Sites and Experimental Design

Four sites in the cultivation area of Haizhou Bay were set, and the nutrient levels and solar irradiance were measured. Algal samples were collected simultaneously from the end of December 2021 to the end of February 2022. In the same cultivation season, the nutrient levels at the aquafarm in Jimo, Shandong Province, were monitored and the algal samples were examined. Additionally, at the end of this cultivation season from March to April, a comparative experiment was designed with two algal cultivation nets. One was placed at the surface (SU) and another one at 1 m underwater (UW) at the Jimo aquafarm in order to investigate the effect of strong light on the cellular composition of *N. yezoensis*. SU1 and UW1 represented samples collected at 1 week of the comparative experiment, and SU2 and UW2 represented samples collected at 5 weeks.

2.2. Algal Sampling and Environmental Monitoring

Sampling in Haizhou Bay and the Jimo aquafarm was carried out in December, January, and at the end of February. Algae were randomly collected from the aquaculture nets and thoroughly dried using paper towels. They were then placed into freehold tubes and stored in liquid nitrogen. Upon return to the laboratory, the blades were transferred and preserved at −80 °C until analysis. Surface water samples at each site were collected using rosette samplers, filtered through Whatman filter paper, and analyzed as soon as possible by a nutritional salt analyzer (KRL-Nutrients, Qingdao, China). Solar irradiance was monitored with a HOBO Pendant® Temperature/Light Data Logger (MX2202, Onset, MA, USA) installed on the algae cultivation rafts at site S9. The logger was set to collect light intensity data every 10 min and the daily average irradiance intensity was calculated. A trend line of solar irradiance intensity was obtained using two polynomial curve fitting methods. In the comparative experiment, another HOBO Pendant® Temperature/Light Data Logger (MX2202, Onset) was installed on the algal cultivation net that was placed on the water surface. The algae samples were collected at weeks 1 (SU1 and UW1) and 5 (SU2 and UW2) after the start of the experiment.

2.3. Analysis of the Proximate Composition of N. yezoensis

The total proximate composition of *N. yezoensis*, including soluble carbohydrate, soluble protein, hydrolyzable polysaccharides, and total nitrogen, was determined. Algal samples (~100 mg fresh weight [FW]) collected from the different sites at different cultivation periods were frozen in liquid nitrogen and ground using grinders (Jingxin Technology, Shanghai, China) in 2-mL grinding tubes. An appropriate amount of distilled water was added, and the samples were fully mixed by vortex. The algal homogenates were then centrifuged for 10 min at 12,000× *g* and 4 °C. The supernatant was collected, and another two rounds of extraction were performed. All supernatants from each sample were combined to form the crude extract. The crude extracts were then divided into two parts: one was subjected to soluble carbohydrate determination, and the other was used in the soluble protein assay.

The remaining precipitates were initially hydrolyzed in 0.5 M HCl for 2 h at 50 °C, and the resultant hydrolysates were incubated at approximately 25 °C overnight. The same volume of 0.5 M NaOH was then added to neutralize the hydrolysates. All of the hydrolyzed samples were incubated for 10 min at 95 °C under the conditions of the reaction solution, immediately cooled to room temperature with cold water, and then analyzed using a microplate reader (Infinite® M1000 Pro, Tecan Group Ltd., Männedorf, Switzerland) at 500 nm following the manufacturer's instructions. Meanwhile, an appropriate amount of supernatant from each sample was subjected to the same treatment to determine the concentration of soluble sugar. The absorption values were used to calculate the sugar contents in different samples using a standard curve of glucose.

The soluble protein concentration was determined according to the bicinchoninic acid assay method using spectrophotometry at 562 nm, and the soluble protein content of each sample was calculated by the FW of the algae.

The total nitrogen contents were determined using the Dumas combustion method [13]. Approximately 5 mg of freeze-dried sample was fully ground, weighed, and subjected to an elemental analyzer (vario MACRO cube, Elementar, Hanau, Germany). The sample was combusted under high temperature and high purity oxygen in the combustion tube and then reduced to a stable gas N_2, in the reducing tube. After removal of the halogens and water by the adsorption column, the total N content was quantitatively determined by a thermal conductivity detector. The protein proportions of each sample were automatically calculated by the product of the measured value and the factor of 6.25.

2.4. Pigment Determination

The pigment compositions of *N. yezoensis*, including R-phycoerythrin, chlorophyll *a*, and β-carotene, were also determined. Approximately 100 mg of the FW sample was placed

into a 2.0 mL grinding tube and fragmented as described above. An adequate volume of phosphate buffer solution (pH 6.7) was added sequentially, and the algal homogenates were then centrifuged for 10 min at 12,000× *g* and 4 °C. The supernatant was collected, and another two rounds of extraction were performed. All extracts were combined, and the absorbance of R-phycoerythrin at specified wavelengths was measured using a spectrophotometer (UV-1900, SHIMADZU, Suzhou, China). The total content was calculated based on the following formula [14]:

$$\text{R-phycoerythrin (mg/mL)} = [(A_{564} - A_{592}) - (A_{455} - A_{592})\ 0.2]\ 0.12 \quad (1)$$

where A is the absorbance value at the various wavelengths.

Following this, 1 mL of 80% acetone was added to the remaining precipitates. After the precipitates were fully vortex oscillated, the homogenates were placed at −20 °C overnight. The extracts were then centrifuged for 5 min and 12,000× *g* at 4 °C, and the supernatants were collected for the quantification of chlorophyll *a* and β-carotene using a high-pressure liquid chromatography system (Agilent 1200, Santa Clara, CA, USA). Based on the volume of pigments extracted, the pigment compositions of the samples were obtained. The pigments extracted with acetone were separated through a C18 reversed-phase column (4.6 × 250 mm, DUG-20A5R, Shimadzu, Kyoto, Japan) by linear gradient elution. The mobile phase included A (water), B (methanol), C (acetonitrile), and D (ethyl acetate). The specific chromatographic procedure was: 0–15 min, linear gradient from I (15% A, 30% B, 55% C, 0% D) to II (0% A, 15% B, 85% C, 0% D); 15–17 min, II to III (15% A, 15% B, 35% C, 35% D) linear gradient; 17–40 min, linear gradient from III to IV (0% A, 30% B, 0% C, 70% D). The column temperature was 50 °C, the flow rate was 0.75 mL min^{-1}, and the detection wavelength was 450 nm. Standards for pigment quantification, chlorophyll *a*, and β-carotene were purchased from Sigma-Aldrich. Pigment standard curves were plotted using the peak areas of different concentration standards, and pigment concentrations were calculated from the standard curve.

2.5. Detection of Mycosporine-Like Amino Acids (MAAs)

The homogenates of different samples were obtained through grinding as described above. A 2.4 M methanol solution was then added for 30 min of full extraction at 60 °C. After centrifugation at 12,000× *g* and 4 °C for 20 min, the supernatants were collected, and the absorbance value at 333 nm was determined through a microplate reader (Infinite® M1000 Pro, Tecan Group Ltd.). The relative MAAs content was calculated using the following formula [15]:

$$\text{CMAAs} = A/(\varepsilon \times \text{Cpr}) \quad (2)$$

where A is the absorbance value at 333 nm, ε is the extinction molar coefficient 4 mL M^{-1} cm^{-1}, and C_{pr} is the protein concentration. The MAAs content in each sample was then calculated by dividing the the quotient of the relative MAAs content by the protein concentration.

2.6. $ABTS^+$ Radical-Scavenging Activity

Each 50 mg of FW algae was placed into a 2.0-mL grinding tube and fragmented as described above. Following this, 1 mL of extraction solution was added and mixed intensively with the sample. The algal homogenates were then incubated at 40 °C for 30 min and centrifuged at 10,000× *g* for 10 min. The supernatant was then collected and placed on ice for testing according to the manufacturer's instructions for the ABTS assay kit (Solarbio, Beijing, China). The absorption values were determined using a microplate reader (Infinite® M1000 Pro, Tecan Group Ltd.) at 405 nm, and the $ABTS^+$ radical-scavenging activities were calculated by the following equation:

$$D\% = [(A_{blank} - (A_{measure} - A_{control})]/A_{blank} \times 100\% \quad (3)$$

where A is the absorbance value, and D is the relative value of radical-scavenging activity expressed as a percentage.

2.7. Effects of Nutrients and Solar Irradiance on the Composition of N. yezoensis

Gray correlation analysis between the algal composition and environmental factors was conducted using the pandas and numpy packages of the high-level computer programming language Python 3.7.3 (https://www.Python.org (accessed on 23 January 2023)). Using the same approach, the correlations between light intensity and all of the tested parameters of the samples cultivated in the comparative experiment at the Jimo aquafarm were analyzed.

2.8. Statistical Analysis

All data were presented as the mean ± standard deviation. The three independent measurements were taken as parallels to calculate the average and standard deviations. The data were submitted to a one-way variance analysis using IBM SPSS Statistics 23.0. Multiple comparisons (least significant difference), the Student–Newman–Keuls curve, and the Bonferroni correction were used to determine significant differences between groups ($p < 0.05$).

3. Results

3.1. Dissolved Inorganic N (DIN) and Inorganic P (DIP) in Different Sea Areas

As shown in Table 1, DIN concentrations in December were significantly higher than those in January and February. Regardless of location (Haizhou Bay or Jimo aquafarm), the DIN concentration decreased gradually with increasing of cultivation duration. Moreover, DIN values were relatively lower in the Jimo sea area than in Haizhou Bay in the same month. Among the DIN, NO_3^--N was the dominant form of N and NH_4^+-N was the least dominant in all samples. Of note, the seasonal variation trend of NH_4^+-N in Haizhou Bay was generally the opposite to that of DIN, showing a slight increasing trend with the increasing cultivation duration.

Table 1. Dissolved inorganic nitrogen (DIN), inorganic (DIP) and N:P (the ratio of DIN to DIP) assay in different aquafarm sites.

	NH_4^+-N (μM)	NO_2-N (μM)	NO_3-N (μM)	DIN (μM)	DIP (μM)	N:P
S2-1	0.36 ± 0.04	0.99 ± 0.08	8.15 ± 0.84	9.50 ± 0.94	0.15 ± 0.04	94.78 ± 37.20
S2-2	0.30 ± 0.07	0.99 ± 0.13	4.26 ± 0.36	5.55 ± 0.28	0.21 ± 0.04	26.90 ± 10.10
S2-3	0.45 ± 0.08	1.60 ± 0.10	3.47 ± 0.12	5.52 ± 0.28	0.13 ± 0.01	42.76 ± 11.21
S4-1	0.24 ± 0.06	1.01 ± 0.05	10.81 ± 1.55	12.07 ± 1.55	0.28 ± 0.03	93.79 ± 38.20
S4-2	0.39 ± 0.14	1.07 ± 0.36	4.26 ± 0.49	5.72 ± 0.28	0.13 ± 0.02	47.05 ± 18.99
S4-3	0.31 ± 0.08	1.65 ± 0.74	3.90 ± 0.52	5.87 ± 1.08	0.14 ± 0.02	43.86 ± 8.05
S6-1	0.45 ± 0.10	0.95 ± 0.09	8.56 ± 2.24	9.95 ± 2.24	0.37 ± 0.03	27.13 ± 14.97
S6-2	0.60 ± 0.09	7.08 ± 0.09	0.86 ± 0.59	8.54 ± 0.73	0.36 ± 0.07	25.04 ± 7.58
S6-3	0.57 ± 0.08	5.54 ± 0.23	0.43 ± 0.14	6.54 ± 0.39	0.26 ± 0.02	25.59 ± 7.38
S9-1	0.04 ± 0.03	0.90 ± 0.08	8.81 ± 0.86	9.75 ± 0.95	0.37 ± 0.02	26.83 ± 7.89
S9-2	0.27 ± 0.10	4.95 ± 0.21	0.91 ± 0.23	6.13 ± 0.04	0.36 ± 0.05	17.45 ± 5.20
S9-3	0.36 ± 0.09	3.99 ± 0.99	1.26 ± 0.67	5.60 ± 1.14	0.17 ± 0.05	45.77 ± 8.98
JM-1	0.52 ± 0.07	0.70 ± 0.04	5.96 ± 0.64	7.18 ± 0.63	0.39 ± 0.07	42.67 ± 5.59
JM-2	0.16 ± 0.09	0.78 ± 0.04	3.12 ± 0.37	4.07 ± 0.34	0.26 ± 0.03	35.91 ± 2.34
JM-3	0.19 ± 0.05	0.28 ± 0.04	2.53 ± 0.26	3.00 ± 0.22	0.20 ± 0.03	35.64 ± 6.79

The concentrations of DIP (Table 1) at near-shore sites in Haizhou Bay were significantly lower than those at offshore sites, while concentrations in the Jimo sea area were similar to those at offshore sites in Haizhou Bay. The DIP concentrations at near-shore sites fluctuated at low levels throughout the cultivation season, while those at offshore sites maintained a relatively consistent level of 10 μg L^{-1} in December and January but decreased significantly in February. The variation in DIP concentrations in the Jimo sea

area was similar to that of DIN, showing a continuous decreasing trend with increasing cultivation duration.

The mean ratios of DIN to DIP (N:P) showed a large fluctuation from 25 to 90 at near-shore sites but were relatively stable at offshore sites. In the Jimo sea area, the ratio was maintained at around 40 (Table 1) during the entire cultivation season.

3.2. Solar Irradiance at S9 and Monotoring of Light Variation during the Comparative Experiment at the Jimo Aquafarm

The average light intensity per 10 min at the seawater surface at S9 during the cultivation period is shown in Figure 1a. As illustrated by the fitted light intensity curve, solar irradiance remained roughly stable until the beginning of February, and then it increased continuously until the end of the cultivation season. During the sample collection at week 5 of the comparative experiment, the algal cultivation net originally placed at the seawater surface sank to the same position as that of the underwater cultivation algal net. The monitoring data of light conditions revealed that the sample SU experienced gradually decreasing light conditions, as shown in Figure 1b.

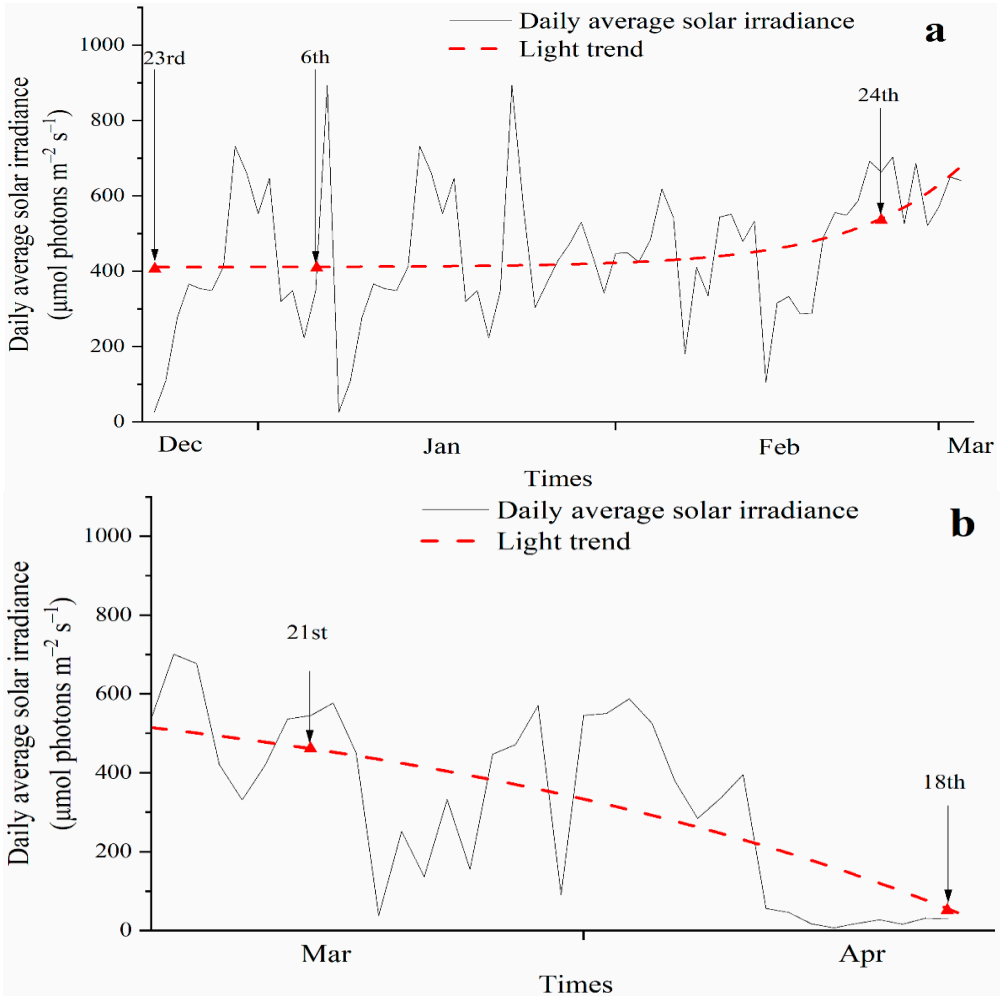

Figure 1. Changes in light intensity during *N. yezoensis* cultivation. (**a**) Haizhou Bay from mid-December to the end of February; (**b**) comparative experiment in Jimo. The intensity values were obtained from the average records collected in the daytime. The dashed line represents the trend line obtained by two polynomial curve fitting methods. The points marked in the figure are the sampling dates.

3.3. Changes in Protein and Polysaccharide Contents under Different Conditions

The soluble protein content continually decreased with the increasing cultivation duration in most samples. As shown in Figure 2a, it reached approximately 10% of algal weight in December and decreased sharply to one-half or one-third of its original value. Compared with soluble protein, the total protein content calculated from the TN determinations in the algae samples decreased more obviously with increasing of cultivation duration (Figure 2b). It is worth noting that in the samples collected on January 6 at S4, S6, and S9, the contents of soluble protein were relatively high (Figure 2a). While in the Jimo sea area, the proportion of structural protein in the January samples was significantly higher than that in samples collected from Haizhou Bay.

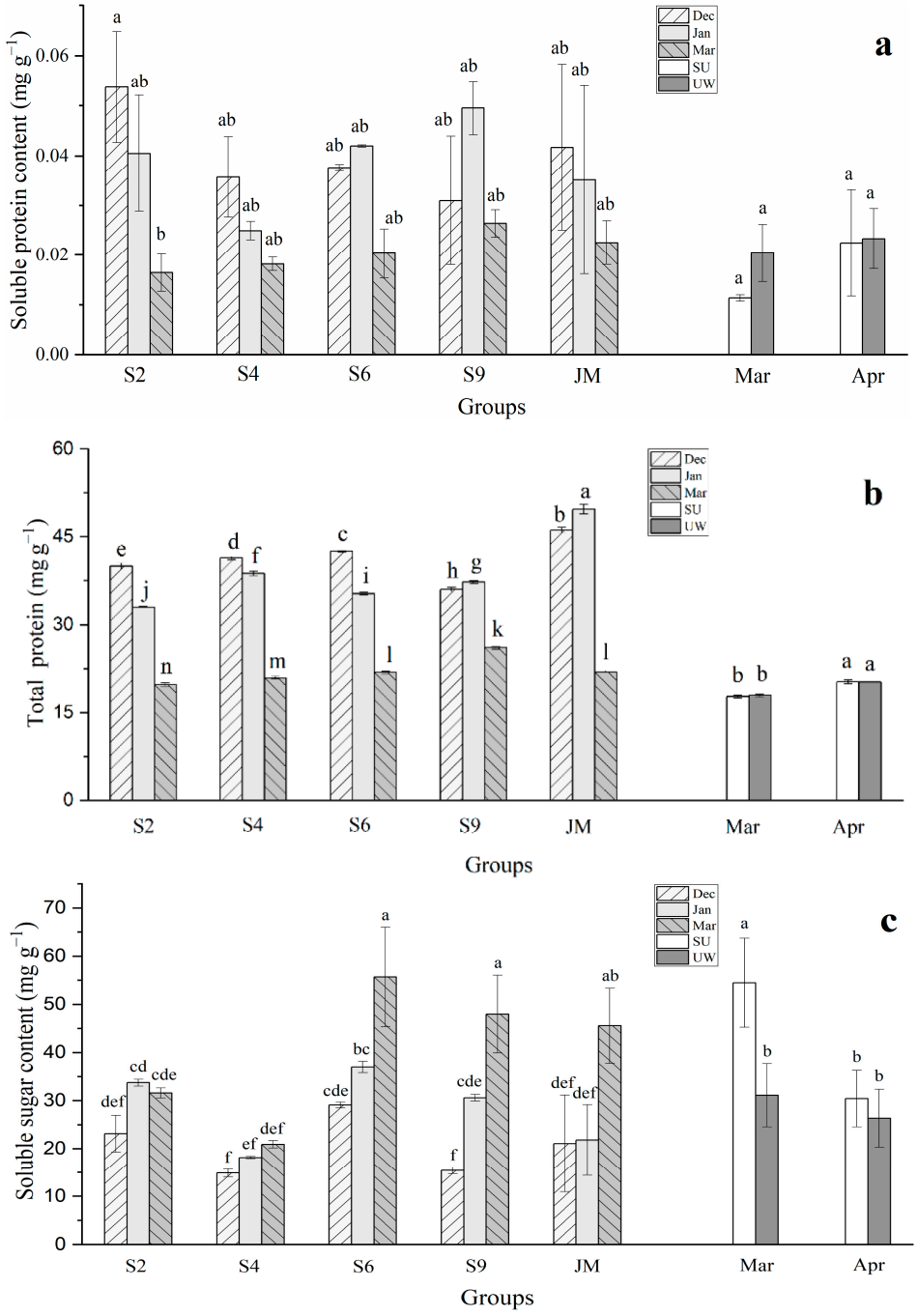

Figure 2. *Cont.*

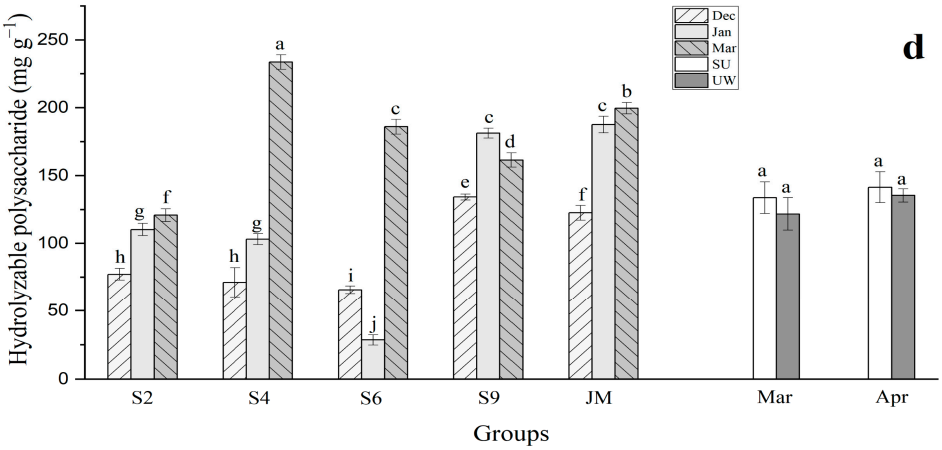

Figure 2. Proximate compositions of different *N. yezoensis* samples. (**a**), Soluble protein content; (**b**), Total protein calculated from the total nitrogen (TN) content; (**c**), Soluble sugar; (**d**), hydrolyzable polysaccharides. S2, S4, S6, and S9 are the different sites in Haizhou Bay. JM is located at Jimo aquafarm in Qingdao. SU, samples cultivated on the water surface during the comparative experiment. UW, samples cultivated at one meter underwater during the comparative experiment. Data are mean ± SD (n = 3). Different letters indicate a significant difference ($p < 0.05$).

The results of the comparative experiment using samples cultivated at different water layers showed that the total protein contents in SU1 and UW1 samples were almost the same (Figure 2b). However, the content of soluble protein in UW1 samples was lower than that in SU1 (Figure 2a). Of note, both soluble protein and the total protein contents in SU2 and UW2 samples were slightly higher than those of UW1 and SU1.

In general, both the contents of soluble sugars and hydrolyzable polysaccharides from the cell walls of algae increased with the extension of cultivation duration (Figure 2c). It should be emphasized that at site S4, the content of hydrolyzable polysaccharides in February samples increased significantly compared with those in January. In addition, the value fluctuated greatly at sites S6 and S9 in January, while it decreased significantly at site S6 in January and decreased slightly at site S9 in February. Compared with the polysaccharide content in algae collected from Haizhou Bay, the samples cultivated in the Jimo sea area showed relatively higher values but were similar at the end of the cultivation season (Figure 2d).

The soluble sugar content of SU1 samples was significantly higher than that of UW1, which was equivalent to SU2 and slightly higher than that of UW2 (Figure 2c). However, the hydrolyzable polysaccharide content was higher in SU1 samples than in UW1 and higher in SU2 samples than in UW2 (Figure 2d).

3.4. Changes in Photosynthetic Pigments under Different Conditions

In contrast to the variations in proteins and polysaccharides with the increasing cultivation duration, the content of chlorophyll *a* decreased, regardless of location. Moreover, the chlorophyll *a* content of different samples collected at the same cultivation time presented similar levels (Figure 3a). Although the variation in β-carotene content was generally consistent with that of chlorophyll *a*, the reduction in β-carotene content (Figure 3b) with the increasing cultivation duration was obviously smaller than that of chlorophyll *a*.

The estimated concentration of R-phycoerythrin in each sample showed a sharp decreasing trend with the increasing cultivation duration (Figure 3c). Specifically, the content of R-phycoerythrin decreased significantly in January in the samples from the Jimo aquafarm and S2. While at S9, R-phycoerythrin also decreased, but the change was very small (Figure 3c).

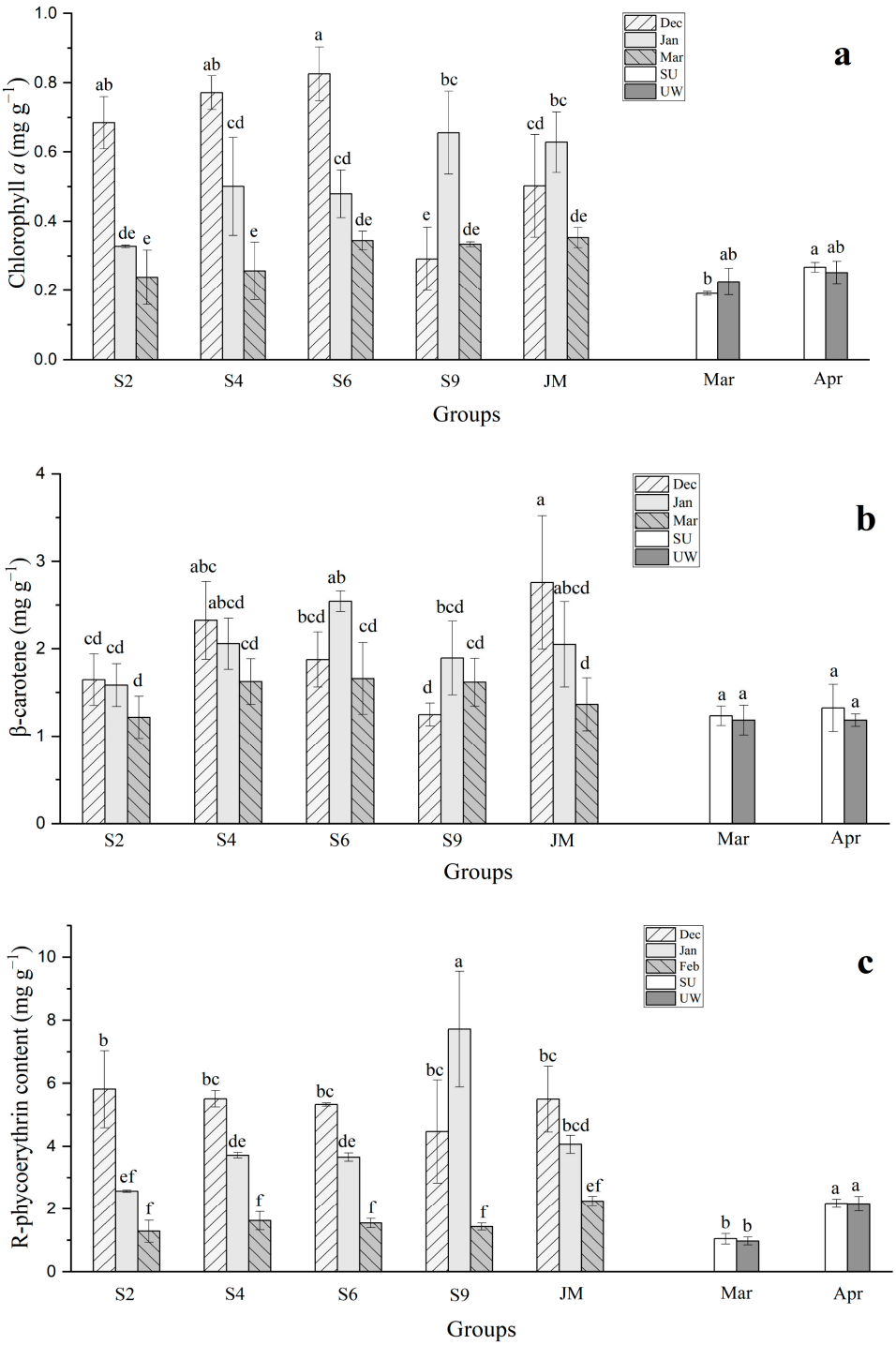

Figure 3. Changes in photosynthetic pigment content of *N. yezoensis* under different conditions. (**a**) chlorophyll *a*; (**b**) β-carotene; (**c**) R-phycoerythrin. Data represent the mean ± SD (n = 3). Different letters indicate a significant difference ($p < 0.05$).

In the field comparative experiment involving different water layer cultivations at the Jimo aquafarm, both chlorophyll *a*, β-carotene, and R-phycoerythrin contents were very slightly higher in UW1 samples than in SU1 (Figure 3). Furthermore, after week 5, these lipid-soluble pigments showed an overall significant increase (Figure 3c).

3.5. Variations in MAAs Content and ABTS$^+$ Radical-Scavenging Activities under Different Conditions

As shown in Figure 4, the relative content of MAAs presented an irregular variation with the increasing cultivation duration. The near-shore samples appeared to have a relatively stable MAAs content, while in offshore samples, the content initially increased followed by a decreasing trend. However, the MAAs content increased continually with increasing of cultivation duration in the Jimo aquafarm. Furthermore, MAAs content remained stable when subjected to decreasing solar irradiance at week 1, and it significantly decreased from week 5 under decreasing solar irradiance (Figure 4).

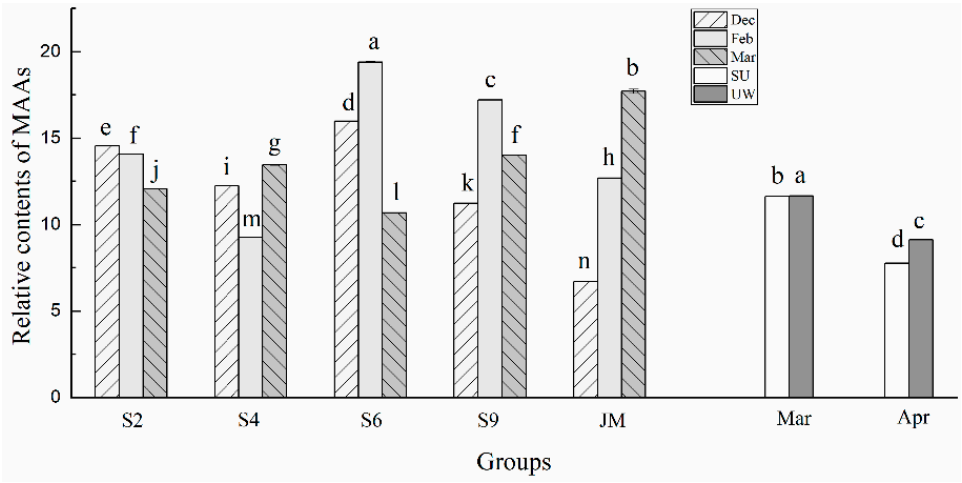

Figure 4. MAAs variations in *N. yezoensis* collected from different sites and cultivation periods. Data represent the mean ± SD (n = 3). Different letters indicate a significant difference ($p < 0.05$).

There was a large variation in ABTS$^+$ radical-scavenging activities among samples (Figure 5). For example, at the S2 and Jimo aquafarm sites, the activity decreased gradually with cultivation duration. However, it was generally up-regulated with an increase in the cultivation duration at S4, while it initially decreased and then increased at S6 and S9.

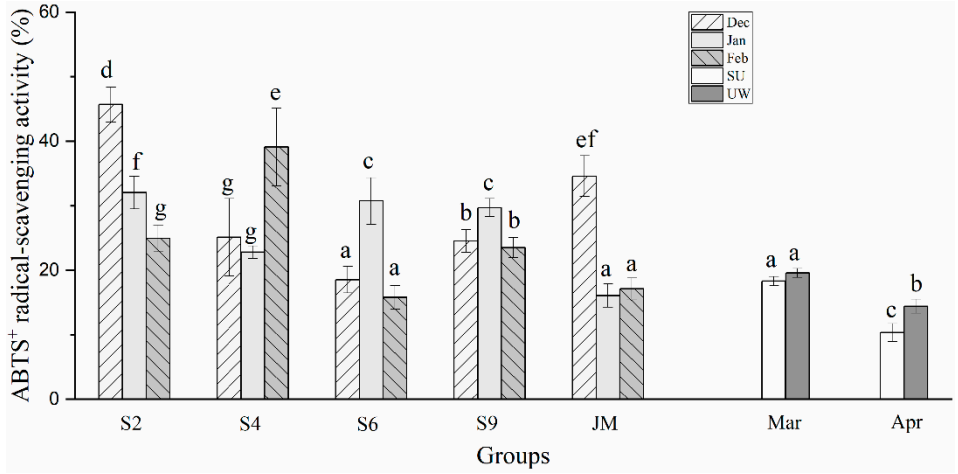

Figure 5. ABTS$^+$ radical-scavenging activity changes in *N. yezoensis* collected from different cultivation sites and periods. Data represent the mean ± SD (n = 3). Different letters indicate a significant difference ($p < 0.05$).

During the first week of the comparative experiment, there was no difference in ABTS$^+$ radical-scavenging activities between the SU and UW samples; however, the activity of the UW2 samples was slightly higher than that of the SU2 sample (Figure 5).

3.6. Effects of Nutrients and Solar Irradiance on the Cellular Components of N. yezoensis

The gray correlation analysis (GCA) results showed that seawater nutrient levels and solar irradiance in the sea area had a similar correlation degree of 0.6 to 0.8, which indicated the same effects on the components of *N. yezoensis*. It should be noted that strong light stress had the most obvious effect on the synthesis of cell sugars and MAAs, while seawater nutrients (including both N and P) had significant positive effects on the synthesis of proteins and pigments (Table 2).

Table 2. Gray correlation coefficients between the contents of crude nutrients, pigments, MAAs, and $ABTS^+$ radical-scavenging activity in *N. yezoensis* and related environmental factors.

Components		Ammonium-N	Nitrate-N	TN	P	N:P	Solar Irradiance
Crude nutrients	Soluble sugar	0.74	0.60	0.71	0.70	0.67	0.81
	hydrolyzable polysaccharides	0.65	0.60	0.66	0.69	0.67	0.79
	Soluble protein	0.61	0.61	0.71	0.78	0.62	0.65
	Total protein calculated from TN	0.68	0.62	0.72	0.73	0.62	0.65
Pigments	Chlorophyll *a*	0.62	0.63	0.76	0.75	0.70	0.64
	R-phycoerythrin	0.67	0.71	0.76	0.77	0.67	0.66
	β-carotene	0.74	0.61	0.73	0.77	0.68	0.72
$ABTS^+$ radical-scavenging activity		0.68	0.67	0.75	0.67	0.72	0.74
Mycosporine-like amino acids (MAAs)		0.70	0.57	0.71	0.67	0.65	0.84

Similarly, in the comparative experiment at the Jimo aquafarm, strong light stress also showed the strongest correlation with the accumulation of sugars and MAAs but had the weakest correlation with the synthesis of soluble proteins and R-phycoerythrin (Table 3).

Table 3. Gray correlation coefficients between light intensity and algal composition components of the samples cultivated in the comparative experiment.

Components		Solar Irradiance
Crude nutrients	Soluble sugar	0.90
	hydrolyzable polysaccharides	0.66
	Soluble protein	0.47
	Total protein calculated from TN	0.62
Pigments	Chlorophyll *a*	0.70
	R-phycoerythrin	0.53
	β-carotene	0.69
$ABTS^+$ radical-scavenging activity		0.71
Mycosporine-like amino acids (MAAs)		0.83

4. Discussion

4.1. Laver Quality Changes with Temporal and Spatial Variations

The quality of the final laver product appears to be related to its protein content and glossiness, and several studies have reported that it is influenced by the cultivation duration and region [4,7,8,16,17]. Recently, laver physicochemical characteristics and antioxidant activities were also investigated, and the results showed that the antioxidant capacity of the algae increased with cultivation duration [12]. Other studies have shown that antioxidant compounds vary greatly according to the cultivation region and duration [18,19]. Furthermore, the nutrient conditions of seawater can have a large influence on the quality of cultivated macroalgae [5]. In the present study, the reductions in algal proteins and pigments with the increasing of cultivation duration were also obtained in both Haizhou

Bay and Jimo sea area. Therefore, algal aging may be one of the most important factors determining changes in cellular components and the decline of laver quality.

4.2. Environmental Stress Modified the Cell Structure and Metabolisms of N. yezoensis

Changes in the abiotic environment occur over scales of days and seasons, and even minutes and seconds. In this constantly changing environment, plants may adopt different strategies, including the adjustment of metabolisms, structural modification, and remodeling of gene expression, to optimize growth and reproductive capacity [20]. Exposure of plants to a mild stress can facilitate efficient environmental adaptability in the future, which is called stress memory [20,21]. The stress memory allows plants to select, at least to a certain extent, the most appropriate mechanisms to shape their future fitness. Thus, it is reasonable to assume that, as an intertidal macroalgae shaped by stress [22], a similar stress tolerance acquirement in *N. yezoensis* may play an important role under natural conditions. During the cultivation process, the *N. yezoensis* nets need to be lifted from the seawater to remove attached fouling organisms. This inevitably exposes the algae to various environmental stress conditions, including dehydration, temperature shock, and high solar irradiance. Therefore, it was speculated that the stress response metabolisms may increase with prolonged cultivation periods, making the algae more resistant to different stress conditions. However, the synthesis of chemical defenses under stress conditions consumes large amounts of intermediates and energy, suggesting a tradeoff from accumulation of assimilated substances in algae cells.

Structural modulation may allow plants to withstand stress caused by environmental conditions. Structural changes can occur across the whole plant, in different tissues and molecules [23]. For example, changes in leaf thickness and molecular complexes such as the photosynthetic apparatus [23]. Regarding *N. yezoensis*, the most direct and obvious structural change is the thickening and hardening of blades due to an increase in cell wall components in the later stage of cultivation. As shown in Figure 2d, the content of hydrolyzable polysaccharides in the samples collected in February was significantly higher than that of those collected in December. Of note, the hydrolyzable polysaccharide content in the Jimo sea area samples was higher than that of samples collected in Haizhou Bay during the same cultivation periods. This indicates that the *N. yezoensis* cultivated in the Jimo aquafarm may suffer more severe environmental stress.

As the most important biochemical process, photosynthesis has been reported to be vulnerable to excess light. Both the sizes of photosystem II (light-harvesting complex II) [24] and the pigment antenna would be reduced [23]. Thus, due to the reduced antenna size, the ROS production was restrained and photo-oxidative stress in chloroplasts would be avoided to a certain extent [23]. The reduction in pigment antenna size caused reduction in the contents of pigments. In the present study, R-phycoerythrin content decreased continuously with the increasing cultivation duration, indicating that the cultivated algae were continuously affected by high light stress and that the decline in the photosynthetic apparatus may ultimately decrease the laver quality and yield. Accordingly, in order to maintain a high quality of laver, special consideration should be paid to avoid an excessive cellular response caused by ROS [12].

Stress conditions can also regulate the kinetics of stress response-related gene expression and thus elicit a rapid and/or intense response to the subsequent stress [22,25,26]. Some metabolic pathways are upregulated, and metabolites are accumulated [27,28]. Indeed, previous reports had proposed that biotic stress could induce dynamic changes in DNA methylation coupled to transcriptional changes of specific genes [29]. During cultivation, *N. yezoensis* experiences significant fluctuations in environmental conditions such as temperature, light intensity, and nutrient availability. This implies the remodeling of global gene expression inevitably and which affected the cellular components finally.

4.3. Low P or N Caused Metabolic Adjustment and Promoted Aging in N. yezoensis

As a key component of the backbone of nucleic acids and membrane phospholipids, P is believed to be one of the most important macronutrients and is essential for energy metabolism. A P limitation leads to lower biomass accumulation and a reduction in plant growth [30,31] due to the negative effect of low cytosol Pi concentrations on the Calvin cycle, reducing the phosphorylated intermediates, or by affecting the required enzymes [30]. Indeed, the photosynthetic efficiency of plants is significantly constrained by low P [30]. Furthermore, this reduction is coordinated with the C status through sophisticated mechanisms [30,32]. Regarding morphology, a reduction in the leaf size of *Arabidopsis* was observed under Pi starvation conditions [33]. As shown in Table 2, the gray correlation coefficients between the content of P and the crude nutrients and pigments reach 0.7 and are higher than most other correlation coefficients. That is, P plays an important role in the accumulation of cell components, and it is of great significance in affecting the yield.

P starvation can also cause alterations in DNA methylation regions, and the altered methylation pattern will change the cell's gene expression [34]. This is particularly important for enabling plants to survive in difficult conditions. For example, an array of stress-related genes, including specific sets of transcription factors and several genes coding for enzymes involved in protein degradation, have been recorded by transcriptome analysis under Pi-limiting conditions [33]. It is worth noting that the upregulation of some genes involved in cell wall metabolism has also been observed [33]. The results of the present study showed that the hydrolyzable polysaccharide content was significantly increased in the samples collected at the end of February when the P level was significantly reduced.

N participates in the synthesis of proteins, nucleic acids, and other molecules. Thus, N limitation influences plant growth, development, and productivity [31,35]. Under mild N stress, the leaf color becomes lighter, and the plant growth rate decreases. Under severe N stress, senescence is activated [36]. Furthermore, the cellular protein level decreases under N starvation [37]. This was corroborated by the present findings regarding the positive correlation between total soluble protein content in the algae collected at different cultivation times (Figure 2a) and the N level. N starvation can damage the photosynthetic proteins and affect both light-dependent and light-independent reactions [38], which ultimately leads to reductions in photosynthetic performance.

N limitation also decreases chlorophyll concentration in cells [39] because N is an important element of chlorophyll and its synthesis is inhibited by NO^{3-} deficiency [40]. Moreover, with the breakdown of chlorophyll, the obvious cell characteristics are chloroplast degradation [41] and changes in the ratio of chlorophyll *a*/*b* [42]. The present results showed that the gray correlation coefficient of chlorophyll *a* content and seawater TN content was 0.76 (Table 2), higher than other environmental factors. Thus, the level of N in seawater can have a large impact on the synthesis of chlorophyll *a* in cultivated *N. yezoensis*.

Some macroalgae have the capacity to store N [43,44], and the protein reservoir is believed to act as an organic N pool for cells when the plant is under stress and responding to nutrient limitation [45]. The blades of *Porphyra*, *Gracilaria*, and other red algae use phycoerythrin initially as a N reserve [46]. Since phycoerythrin concentration usually lags 1 week behind N limitation, it could be used as a rapid and reliable indicator to assess the N status of *N. yezoensis*. As shown in Figure 3c, the decrease in R-phycoerythrin content in *N. yezoensis* from January indicated an N deficiency in the aquafarm, which thus led to the reduced quality and poor color of the final laver products.

When the organic N pool is limited, the mitogen-activated protein kinase signaling pathways will be activated [47]. With the downregulation of photosynthetic genes, the synthesis of carotene increases due to the involvement of the mitogen-activated protein kinase pathway [48]. A down-regulation of genes related to amino acid synthesis has also been reported [49]. In the present study, a decrease in soluble protein content was initially recorded (Figure 2a), followed by a decrease in total protein content (Figure 2b). This indicated that N deficiency initially affected the synthesis of various physiologically relevant proteases and that cellular structural proteins are only significantly affected under

long-term N restriction. Furthermore, the soluble protein content in the Jimo aquafarm samples was lower than that in Haizhou Bay samples during the same cultivation period, while the total protein content in the December and January samples was significantly higher than that in Haizhou Bay samples. This indicates that an N deficiency in the Jimo aquafarm affects the quality and color of the final laver products.

4.4. Strong Light Also Decreased N. yezoensis Laver Yield and Quality

Strong light reduces the number of thylakoids in the chloroplasts and negatively affects the synthesis of chlorophyll [50]. Furthermore, the upregulation of β-carotene has also been reported [51]. The excited energy is transferred to the carotene and dissipated as heat [52]. As presented in Figure 3b, although the content of β-carotene decreased with the increasing cultivation duration, the decreasing trend was significantly smaller than that of chlorophyll *a*. This shows that chlorophyll synthesis decreases with the extension of cultivation time, while carotene synthesis activity may be up-regulated. In addition, the results of the gray correlation analysis indicated β-carotene content was more closely related to light intensity than the content of other pigments (Table 2). Considering the gradual increase in solar irradiance during the cultivation period, strong light could indeed cause changes in the pigment ratios and ultimately cause the blades of *N. yezoensis* become more yellow. This explains why the color of laver cultivated in the north Yellow Sea is generally yellower.

A growing body of literature suggests that photoinhibition usually leads to a reduction in the size of light-harvesting antennae and the inhibition of genes associated with photosynthesis [53,54], or the degradation of existing light-harvesting proteins [55]. The present findings show that the R-phycoerythrin content in all samples decreased with the increase in cultivation duration (Figure 3c), especially in the samples collected in January and February, as it decreased significantly while the nutrients in seawater remained at a constant level. This indicates that the decrease in R-phycoerythrin is also closely related to the increase in solar irradiance. The decrease in R-phycoerythrin not only caused reduced algal color but also led to a decline in laver product quality due to reductions in protein contents. The comparative experiment at different water layers showed that, although the R-phycoerythrin content in the samples did not present a significant increase after 1 week of cultivation, it increased significantly after 5 weeks in the samples cultivated underwater (Figure 4). Unfortunately, the surface-cultured samples gradually sank to a similar position as those placed underwater during the experiment. Nevertheless, compared with the previous results, it could still be concluded that the content of R-phycoerythrin would significantly increase after a period of cultivation under reduced light.

MAAs are believed to play a role in biochemical defense against UV radiation and are accumulated under increased solar irradiance. *Porphyra* species exhibit comparatively high steady-state concentration values of MAAs at all times, and further increases in MAAs are only minor under solar irradiance enhancement [56]. This might be attributed to the adaptation of this genus to the intense intertidal light environments. Since MAAs are nitrogenous compounds, their synthesis and accumulation are closely related to the supply of N, and N limitation has been shown to increase the sensitivity of photoinhibition by UV radiation [57]. Although the results of the present gray correlation analysis revealed that the content of MAAs had the strongest correlation with TN compared with the other parameters, the MAAs content in the Jimo sea area showed the opposite trend with TN. In addition, the comparative experiment revealed that the samples cultivated at the surface had relatively higher MAA contents (Figure 4). This indicated that the synthesis of MAAs in *N. yezoensis* was also significantly influenced by the level of solar irradiance.

The saturated solar intensity at the surface of seawater can reach 2000 μ E/(m^2 s), or even higher at times [58]. In addition, regular and continuous waves resulting in bright bands or patches, which cause an amplification of irradiance of up to five times the mean subsurface intensity, result in the algae being frequently exposed to fluctuating irradiance environments [59]. Fortunately, the mutual occlusion of the algal body under waves

reduces its exposure to excessive irradiances for a long period. This might explain why algae farming can be carried out successfully in the field where the actual light intensity is significantly higher than the saturated light intensity of cultivated algae. Indeed, we measured the light intensity one meter under water at midday in the north Yellow Sea and found that it reduced to about 40% of that at the surface. Compared with the coastal environments of Jiangsu Province, the nutrient level of the northern Yellow Sea is lower, which results in a high transparency and makes the cultivated algae more exposed to strong light stress. Consequently, corresponding stress responses are more avtive which causes the decrease in algae yield and quality.

5. Conclusions

During the 2021 to 2022 cultivation season, nutrient levels in the Yellow Sea were relatively low, which increased the exposure of the cultivated algae to solar irradiation. The present results show that the contents of soluble protein, R-phycoerythrin, and chlorophyll *a* in *N. yezoensis* decreased with the increasing cultivation duration in both the Haizhou Bay and the Jimo sea area. The gray correlation analysis revealed that nutrients and solar irradiance affected all algal parameters at a similar level. It is proposed that nutrient deficiency or strong light stress caused structural modulation and the remodeling of gene expression for algae survival. At the same time, stress-related molecules accumulated, resulting in the thickening of the cell wall. This might be the main reason for the poor quality and light color of the laver products derived from algae cultured in the north Yellow Sea. Moreover, the comparative experiment in the Jimo aquafarm showed that a reduction in light intensity increases the synthesis of R-phycoerythrin, chlorophyll *a*, and soluble proteins in *N. yezoensis*, although the range of the increase appears to be very limited. This indicates that reducing the stress light intensity would appropriately reduce the response metabolisms and increase in soluble protein synthesis. Considering the characteristics of low nutrient levels in the North Yellow Sea, reducing excessive irradiance during the cultivation of *N. yezoensis* appears to be important.

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Abbreviations

FW, fresh weight; DIN, dissolved inorganic N; DIP, dissolved inorganic P; MAAs, mycosporine-like amino acids; N_2, nitrogen; NH_4^+-N, Ammonium-N; NO_2-N, Nitrite-N; NO_3-N, Nitrate-N; TN, the total N content; ROS, reactive oxygen species.

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