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Differential Physiological Response of *Pyropia haitanensis* Conchocelis to Nitrate and Ammonium

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Abstract

The effects of different nitrogen sources (nitrate, nitrate-ammonium, and ammonium) and concentrations (1 and 5mg L^{-1}) on *Pyropia haitanensis* conchocelis were investigated. Ammonium as nitrogen source yielded higher growth rates, chlorophyll a, carotenoid, phycoerythrin, phycocyanin, and elemental nitrogen content of *P. haitanensis* than with nitrate. 5 mg L^{-1} nitrogen resulted in increase of elemental nitrogen content, decrease of growth rates and triglycerides content with all nitrogen sources. The expression of glutamine synthetase 1 and glutamine synthetase 2 genes were higher with nitrate as nitrogen source than with ammonium, while expression of glutamate synthase gene was higher with ammonium as nitrogen source than with nitrate. Higher total contents of monogalactosyldiacylglycerols, digalactosyldiacylglycerols, sulfoquinovosyldiacylglycerols, and phosphatidylethanolamines of *P. haitanensis* were found at 5 mg L^{-1} than at 1 mg L^{-1} nitrogen with nitrate as nitrogen source. However, the total content of lipids (except phosphatidic acids) of *P. haitanensis* was less at 5 mg L^{-1} than at 1 mg L^{-1} nitrogen with ammonium as nitrogen source. In summary, the study indicated that ammonium was a favorable nitrogen source to *P. haitanensis*, as it promoted the growth rates and pigments content. 5 mg L^{-1} nitrogen with nitrate as nitrogen source was benefit for lipids production.

Introduction

Nitrogen (N) is an important factor controlling algal growth (Li et al., 2022; Naldi & Wheeler, 2010; Wang et al., 2024), because nitrogen is an essential component for various macromolecules (e.g. proteins, chlorophyll and phycoerithrin (Gao et al., 2018a; Jiang et al., 2020; Navarro-Angulo & Robledo, 1999). The process of photosynthesis and growth of algae are typically stimulated by the enrichment of nitrogen (Gao et al., 2018b; Traugott et al., 2020). For example, nitrogen played a beneficial role in the growth of *Gracilaria lemaneiformis* (Duan et al., 2019). The increase in nitrogen source utilization efficiency in seawater promoted growth and increased the photosynthetic pigment content of *Pyropia yezoensis* (Yu et al., 2022). Supplememtation of nitrate (NO_3^-) in artificial seawater increased pigments, monogalactosyldiacylglycerols, polyunsaturated fatty acids and nitrate reductase of *Ulva lactuca* (Kumari et al., 2014). Nitrogen also increased amino acid content of *Porphyra yezoensis* (Li et al., 2017).

Nitrate and ammonium (NH_4^+) are the two principal forms of inorganic nitrogen that facilitate the growth of marine algae (Cohen & Fong, 2004; Copertino et al., 2009; Mansilla et al., 2008; Roleda & Hurd, 2019). The utilization of nitrate and ammonium as nitrogen source by algae exhibits interspecies variability, with the assimilation of these nutrients affects their growth (Ale et al., 2011; Carmona et al., 2006). For instance, *Laminaria japonica* exhibited a preference for nitrate over ammonium as the nitrogen source (Xu et al., 2011), whereas *Ulva lactuca* demonstrated a robust growth response to ammonium as nitrogen source (Ale et al., 2011). No significant differences were observed in the growth rate of *Gracilaria cornea* when nitrate and ammonium were used as nitrogen source (Navarro-Angulo & Robledo, 1999). Nitrate reductase (NR) and nitrite reductase (NiR) are capable of reducing nitrate to ammonium, which is subsequently reduced directly to amino acids by glutamine synthetase-glutamine: 2-oxoglutarate amidotransferase (GS-GOGAT) pathway (Yu et al., 2022).

Pyropia haitanensis (previously named *Porphyra haitanensis*, *Neoporphyra haitanensis*) is a marine crop that is mainly cultivated in the southern coast of China (Xie et al., 2009). The majority of previous studies regarding *P. haitanensis* have focused on the physiological stress response of this species. For example, high temperature affected photosynthesis, conchospore germination and early seedling development of *P. haitanensis* (Ai et al., 2022; Yang et al., 2022), and salicylic acid protected *P. haitanensis* during *Vibrio mediterranei* 117-T6 infection (Zhu et al., 2023). Nevertheless, a few studies have been undertaken on physiological properties of *P. haitanensis* conchocelis under different nitrogen sources. Therefore, the objective of this study is to evaluate the physiological response of *P.haitanensis* conchocelis to different nitrogen sources and concentrations. The results will contribute to understanding the key nutritional factors supporting the growth of *P. haitanensis*.

Materials and Methods

Culture Conditions and Experimental Design

The *P. haitanensis* conchocelis were cultured by the method of Wang et al (2020, 2022). At first, the *P. haitanensis* conchocelis containing 800 mL basic culture medium (without adding nitrogen) were cultured for 7 days in a 2000-mL plastic box (20×14×10.5 cm). Afterwards a 3×2 factorial layout with three nitrogen source and two nitrogen concentration (1 and 5mg L^{-1}) in medium were designed, which correspondingly created seven treatment groups abbreviated as N1-NO3, N1-NO3-NH4, N1-NH4, N5-NO3, N5-NO3-NH4 and N5-NH4, each treatment had three replicates. $NaNO_3$ (5 mg mL^{-1}) and NH_4Cl (5 mg mL^{-1}) were supplemented into the culture medium to generate the target concentrations of nitrogen. The medium changes were every 5 days. The cultures were at kept 23°C, with a light intensity of 20 μmol m^{-2} s^{-1} and a light-dark (12: 12 h). Samples were collected after culture 10 d, and at last, the samples were frozen in liquid nitrogen immediately after collection and stored at −80°C for future use.

Growth Measurements

The relative growth rate (RGR) was estimated by the method of Wang et al (2020), and the formula RGR (% day^{-1}) = (ln W_t − ln W_0)/t × 100 was used. W_t (g) represented the fresh weight of *P. haitanensis* conchocelis after the end of the experiment, W_0 (g) represented the fresh weight of *P. haitanensis* conchocelis before the start of the experiment and t represented the experiment period. The initial fresh weight was about 0.12 ± 0.02 g. The samples were gently blotted using filter paper to remove surface water before weighing.

Pigment Analysis

The pigments chlorophyll a and carotenoids were calculated by the method of Parsonset and Strickland (1963), Porra (2002) and Wang et al. (2020). Briefly, chlorophyll a and carotenoids were extracted by 90% acetone. And we measured the absorbance value of chlorophyll a and carotenoids at 480, 510, 652, 665 and 750 nm. Phycoerythrin and phycocyanin contents were extracted in buffer (0.1 mol L^{-1} potassium phosphate, pH=6.8), and the amount was calculated by the method of Beer and Eshel (1985), and Wang et al. (2020). We measured the absorbance value of phycoerythrin and phycocyanin at 455, 564, 592, 618 and 645nm.

Soluble Protein Analysis

Total protein was extracted by phosphate buffer (0.1 mol L^{-1}, pH=6.8), and soluble protein contents were determined using the Coomassie Brilliant Blue G-250 dye and bovine albumin (BSA) was used as the stand (Bradford, 1976).

Determination of Carbon (C) Elements and N Elements

The total C and N contents were determined with an elemental analyzer Vario EL cube (Elementar Analysensysteme GmbH, Frankfurt, Germany). And 5 mg (dry weight) sample was weighed accurately.

RNA Extraction and the Quantitative Reverse Transcriptionpolymerase Chain Reaction Assay

RNA was extracted using Trizol (Takara Bio Inc, Shiga, Japan). RNA (1 μg) was treated with DNase I and reverse-transcribed into cDNA using a Prime ScriptTM RT reagent kit with gDNA eraser (Takara Bio Inc, Shiga,

Japan). The resulting cDNA sequences were used as template for gene expression analysis by real time-quantitive PCR. All RT-qPCR assays were performed using TranStart Tip Geen qPCR SuperMix (TransGen Biotech, Beijing, China) on the QuantStudioTM 7 Flex Real-Time PCR System (Thermo Fisher Scientific, Waltham, USA). Table S1 lists the gene-specific primers. Actin (ACT) gene was used as an internal reference gene (Li et al., 2014a). PCR conditions were: 95°C for 10 min, 40 cycles of 95°C for 10 s, Tm for 10 s and 72°C for 15 s, and with a melting program from 65°C to 95°C. The relative gene expression was quantified by the comparative $2^{-\Delta\Delta Ct}$ method (Livak & Schmittgen, 2001). The nitrate reductase (NR), nitrite reductase (NiR), glutamine synthetase (GS) and glutamate synthase (GOGAT) gene sequences of *Pyropia haitanensis* were acrooding to Chen et al. (2022).

Lipid Analysis

The lipids of *Pyropia haitanensis* conchocelis were extracted by the method of Bligh and Dyer (1959), and the identification of lipids and the semi-quantitative analysis of lipids were according the methods of Li et al. (2014b), and Wang et al. (2020).

Statistical Analyses

The homogeneity and normal distribution of the data were examined with Levene's test and KolmogorovSmirov test. One-way, Two-way analysis of variance (ANOVA) and the Duncan's test were used to analyze differences between different treatments (P≤0.05). All data are displayed as mean ± SD for three measurements.

Results

Growth Rates of *P. haitanensis*

Nitrogen source and concentration had a significant effect on the relative growth rate of *P. haitanensis* conchocelis (P<0.05), while the reciprocal action between nitrogen source and nitrogen concentration had no significant influence on *P.haitanensis* conchocelis (Figure 1, Table 1). The relative growth rate was higher with ammonium as nitrogen source than with nitrate and nitrate-ammonium (P<0.05). Increase in nitrogen concentration decreased the relative growth rate of *P. haitanensis* conchocelis with nitrate, nitrate-ammonium or ammonium as nitrogen source (P<0.05).

Contents of Pigment, Soluble Protein, Elemental C and Elemental N of *P. haitanensis*

Ammonium as nitrogen source showed an increase in chlorophyll a and carotenoid (P<0.05), and no significant differences were found between nitrate and nitrate -ammonium as nitrogen source in carotenoid (P>0.05, Figure 2, Table 1). The contents of carotenoids increased with the increase of nitrogen concentration with nitrate- ammonium and ammonium as nitrogen source (P<0.05), while no significant difference was found in chlorophyll a with the increase of nitrogen concentration with nitrate and ammonium as nitrogen source (P>0.05, Figure 2, Table 1). 5 mg L^{-1} nitrogen concentration increased contents of phycocyanin and phycoerythrin with nitrate and ammonium as nitrogen source (P<0.05), while 5 mg L^{-1} nitrogen concentration had no significant influence on contents of phycocyanin

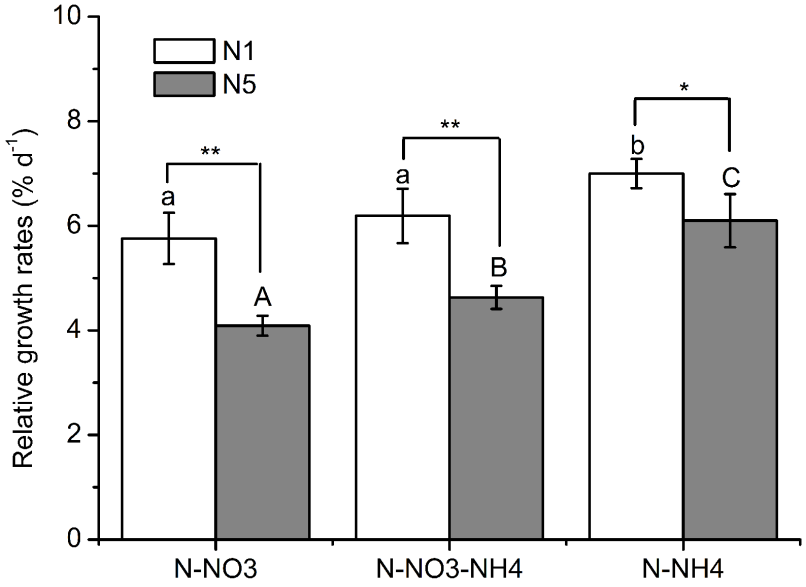

Figure 1. Relative growth rates of *Pyropia haitanensis* conchocelis cultivated in different nitrogen sources and nitrogen concentrations (mean ± standard errors, n=3). * denoted P<0.05, ** denoted P<0.01. Different letters represent significant differences at P<0.05 among different nitrogen sources (lowercase for 1 mg L^{-1} nitrogen sources and capitalized for 5 mg L^{-1} nitrogen sources). N-NO3: nitrate as nitrogen source, N-NO3-NH4: nitrate-ammonium as nitrogen source, N-NH4: ammonium as nitrogen source. N1: 1 mg L^{-1} nitrogen; N5: 5 mg L^{-1} nitrogen.

Table 1. Analysis of variance of relative growth rates, pigments, soluble protein, elemental carbon (C) content, elemental nitrogen (N) content, gene expression of nitrite reductase (NiR), glutamine synthetase (GS), glutamate synthase (GOGAT), and lipids of *Pyropia haitanensis* conchocelis cultivated in different nitrogen sources and nitrogen concentrations

	N sources		N concentrations		N sources×N concentrations	
	F value	P value	F value	P value	F value	P value
Relative growth rates	17.655	0.003	35.856	0.001	1.083	0.397
Chlorophyll a	17.201	0	7.472	0.015	1.93	0.177
Carotenoid	9.632	0.002	21.619	0	0.669	0.526
Phycoerythrin	11.205	0.001	26.934	0	7.509	0.005
Phycocyanin	16.47	0	48.247	0	8.306	0.003
Soluble protein	9.34	0.002	19.835	0	4.515	0.027
Elemental C content	3.806	0.052	4.661	0.052	4.653	0.032
Elemental N content	60.144	0	2610.37	0	7.963	0.006
NiR	340.007	0	1425.783	0	198.744	0
GS1	150.583	0	48.982	0	10.032	0.003
GS2	17.644	0	107.544	0	0.245	0.786
GOGAT	96.542	0	17.195	0.001	52.961	0
MGDGs	37.639	0	15.344	0.002	52.042	0
DGDGs	28.954	0	0.223	0.645	32.678	0
SQDGs	13.119	0.001	0.116	0.739	12.564	0.001
PAs	0.977	0.405	1.892	0.194	0.728	0.503
PCs	5.479	0.02	1.096	0.316	8.748	0.005
PEs	7.771	0.007	15.845	0.002	15.501	0
PGs	12.171	0.001	22.652	0	5.521	0.02
PIs	10.599	0.002	0.002	0.962	9.721	0.003
TGs	22.793	0	308.625	0	23.619	0

[1]MGDGs: monogalactosyldiacylglycerols; DGDGs: digalactosyldiacylglycerols; SQDGs: sulfoquinovosyldiacylglycerols; PAs: phosphatidic acids; PCs: phosphatidylcholins; PEs: phosphatidylethanolamines; PGs: phosphatidylglycerols; PIs: phosphatidylinositols; TGs: Triglycerides.

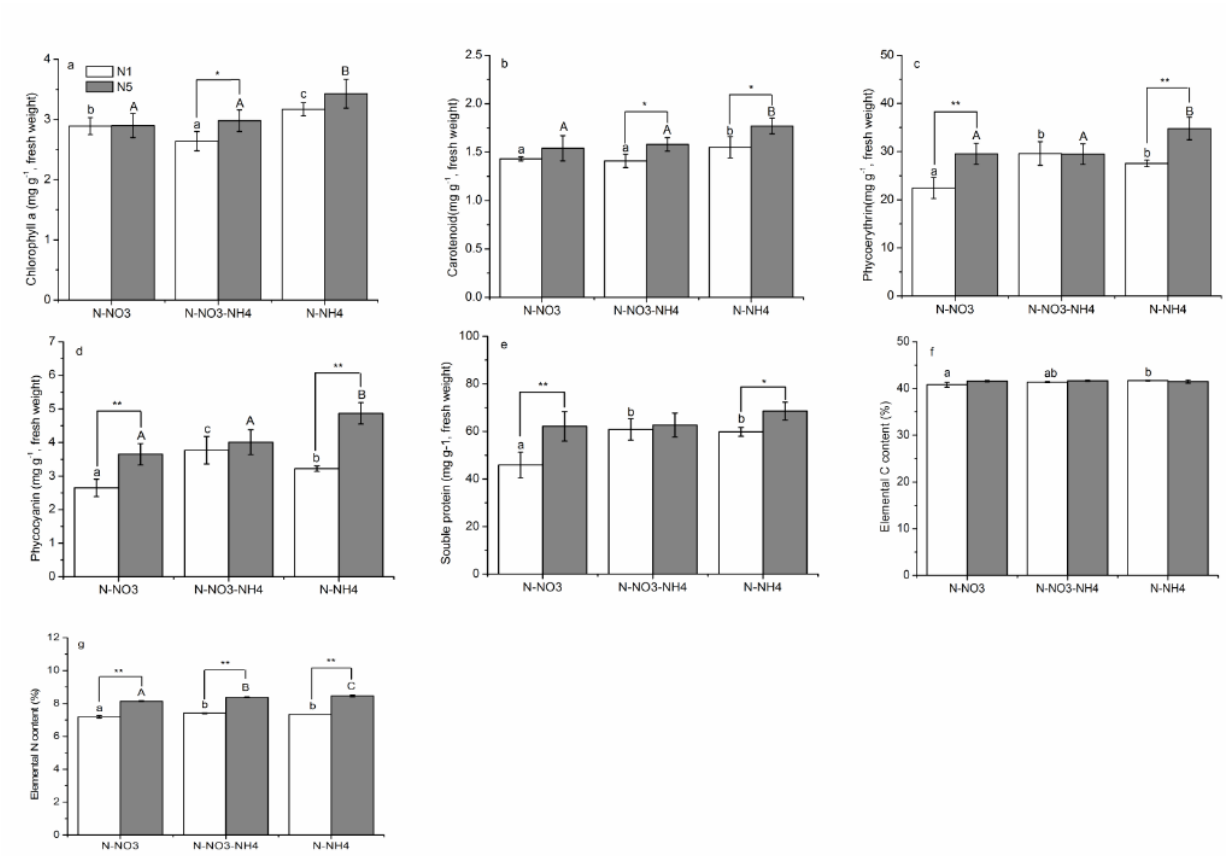

Figure 2. Analysis of variance of pigments, soluble protein, elemental carbon (C) content, elemental nitrogen (N) content of *Pyropia haitanensis* conchocelis cultivated in different nitrogen source and nitrogen concentration (mean ± standard errors, n=3). * denoted P<0.05, ** denoted P<0.01. Different letters represent significant differences at P<0.05 among different nitrogen sources (lowercase for 1 mg L^{-1} nitrogen and capitalized for 5 mg L^{-1} nitrogen). N-NO3: nitrate as nitrogen source, N-NO3-NH4: nitrate-ammonium as nitrogen source, N-NH4: ammonium as nitrogen source. N1: 1 mg L^{-1} nitrogen; N5: 5 mg L^{-1} nitrogen.

and phycoerythrin with nitrate-ammonium as nitrogen source (P>0.05, Figure 2, Table 1). In comparison with ammonium and nitrate-ammonium as nitrogen source, the ammonium as nitrogen source showed an increase in phycoerythrin and phycocyanin at 5 mg L^{-1} (P<0.05, Figure 2, Table 1). Nitrogen source, nitrogen concentration and the reciprocal action between nitrogen concentration and nitrogen source had a significant effect on soluble protein (P<0.05, Figure 2, Table 1). Increase in nitrogen concentration increased the soluble protein with nitrate and ammonium as nitrogen source (P<0.05). Neither nitrogen source nor nitrogen concentration showed a significant effect on elemental C content (P>0.05), however, the reciprocal action between nitrogen source and nitrogen concentration had a significant effect on elemental C content (P<0.05, Figure 2, Table 1). Nitrogen concentration had a significant effect on elemental N content, and increase in nitrogen concentration increased elemental N content in all nitrogen sources (P<0.05). The reciprocal action between nitrogen concentration and nitrogen source had a significant effect on elemental N content (P<0.05, Figure 2, Table 1).

Expression of NR, NiR, GS, and GOGAT in *P. haitanensis*

The expression of NR gene of *P. haitanensis* conchocelis was not found among all the treatments (data not shown). The expression of NiR gene was higher with nitrate and nitrate-ammonium as nitrogen source than with ammonium at 1 or 5 mg L^{-1} (P<0.05, Figure 3, Table 1). The expression of NiR, GS1 and GS2 genes were higher at 1 mg L^{-1} nitrogen concentration than at 5 mg L^{-1} nitrogen concentration (P<0.05, 5 mg L^{-1}) with all nitrogen source (Figure 3, Table 1). The expression of GS1 gene was higher with nitrate as nitrogen source than with nitrate-ammonium and ammonium as nitrogen source at both nitrogen concentrations (P<0.05). The expression of GS2 gene was higher with nitrate and nitrate-ammonium as nitrogen source than with ammonium at both nitrogen concentrations (P<0.05, Figure 3, Table 1). The expression of GOGAT gene was higher at 1 mg L^{-1} nitrogen concentration both with nitrate-ammonium and ammonium as nitrogen source, while the expression of GOGAT gene was lower at 1 mg L^{-1} nitrogen concentration than at 5 mg L^{-1} nitrogen with nitrate as nitrogen source (P<0.05, Figure 3, Table 1).

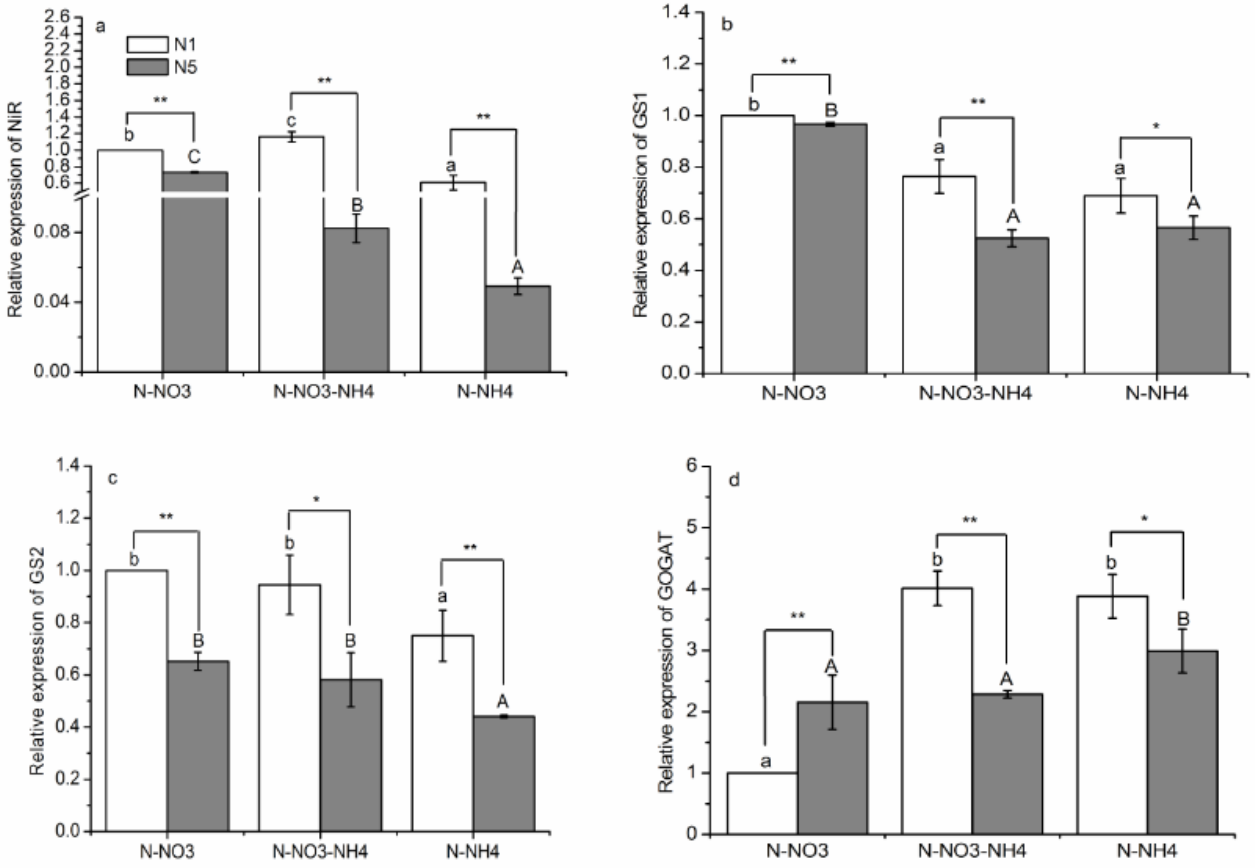

Figure 3. Expression level of nitrite reductase (NiR) gene, glutamine synthetase (GS) gene, and glutamate synthase (GOGAT) gene in *Pyropia haitanensis* conchocelis cultured in different nitrogen source and nitrogen concentration (mean ± standard errors, n = 3). * denoted P < 0.05, ** denoted P < 0.01. Different letters represent significant differences at P < 0.05 among different nitrogen sources (lowercase for 1 mg L^{-1} nitrogen and capitalized for 5 mg L^{-1} nitrogen). N-NO3: nitrate as nitrogen source, N-NO3-NH4: nitrate-ammonium as nitrogen source, N-NH4: ammonium as nitrogen source. N1: 1 mg L^{-1} nitrogen; N5: 5 mg L^{-1} nitrogen.

Changes of Lipids of *P. haitanensis*

Nitrogen source had a significant effect on monogalactosyldiacylglycerols (MGDGs), digalactosyldiacylglycerols (DGDGs), sulfoquinovosyldiacylglycerols (SQDGs), phosphatidylethanolamines (PEs), phosphatidylglycerols (PGs), phosphatidylinositols (PIs) and triglycerides (TGs) of *P. haitanensis* conchocelis (P<0.05, Figure 4, Table 1). Higher MGDGs and DGDGs were found with nitrate as nitrogen source at 5 mg L^{-1} (P<0.05). Higher SQDGs, PCs, PEs PGs and PIs were found with nitrate and nitrate- ammonium as nitrogen source at 5 mg L^{-1} (P<0.05). Nitrogen concentration had a significant effect on MGDGs and TGs of *Pyropia haitanensis* conchocelis (P<0.05), and increase in nitrogen concentrations decreased the content of TGs (P<0.05, Figure 4, Table 1). Increase in nitrogen concentrations decreased the content of MGDGs with ammonium as nitrogen source, while increase in nitrogen concentrations increased the content of MGDGs with nitrate and nitrate-ammonium as nitrogen source (P<0.05, Figure 4, Table 1). The reciprocal action between nitrogen source and nitrogen concentrations had a significant effect on MGDGs, DGDGs, SQDGs, PCs, PEs, PGs, PIs and TGs (P<0.05, Figure 4, Table 1).

Discussion

The Effects of Nitrogen Sources on *P. haitanensis*

Nitrate and ammonium are the primary sources of nitrogen for algae (Copertino et al., 2009; Roleda & Hurd, 2019). Many algae demonstrate a higher uptake rate with ammonium ($NH4^+$) as nitrogen source than with nitrate (NO_3^-) (De Boer, 1981). For example, a greater quantity of NH_4^+ was taken up and assimilated in *Enteromorpha intestinalis* (L.) Link (Cohen & Fong, 2004). *Ulva lactuca* exhibited a good growth response when ammonium was used as a nitrogen source (Ale et al., 2011). Nitrogen sources containing ammonium produced the highest phycoerythrin and protein content than that contained nitrate-grown cultures of *Gracilaria cornea* (Navarro-Angulo & Robledo, 1999). In this study, the relative growth rates of *P. haitanensis* conchocelis were found to be higher when ammonium

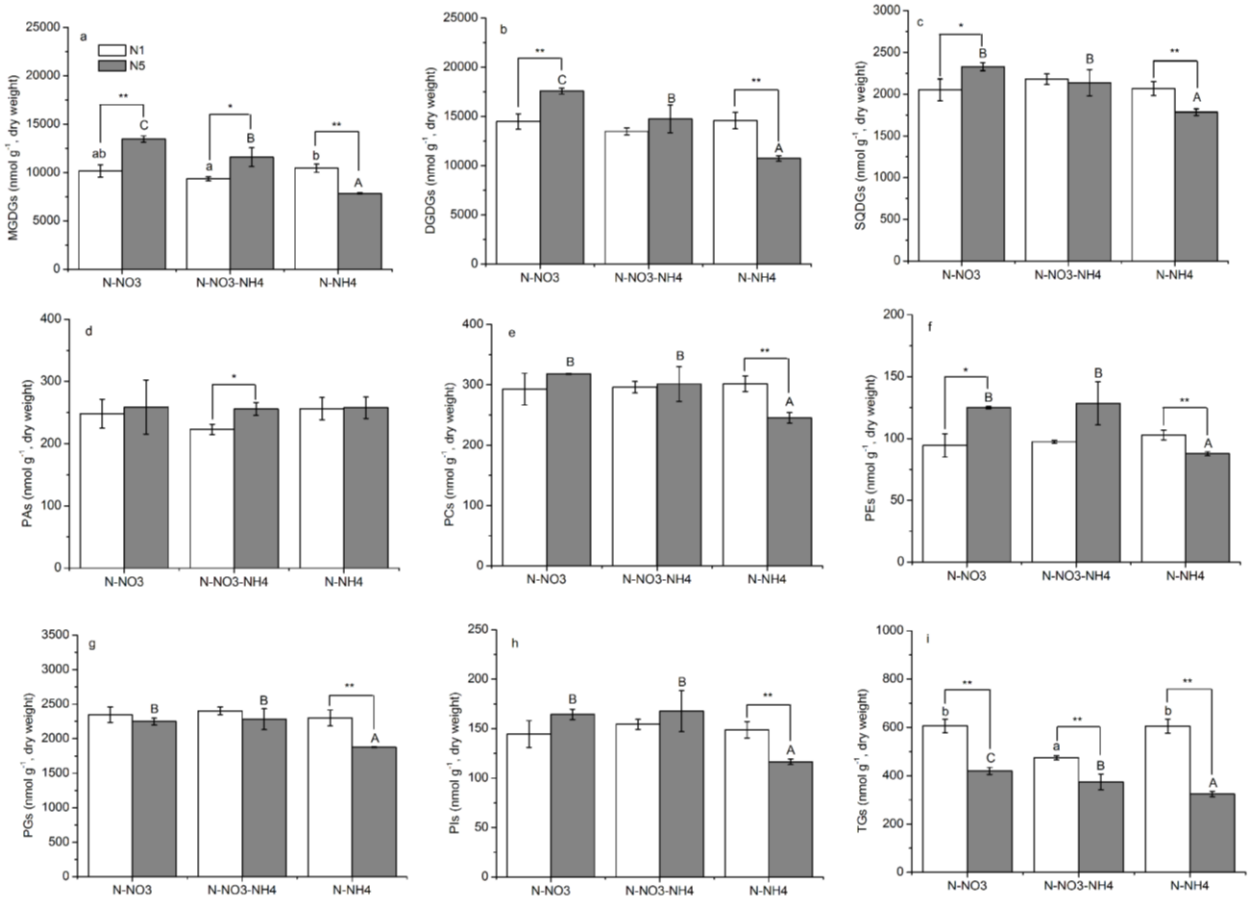

Figure 4. Contents of lipids of *Pyropia haitanensis* conchocelis cultivated in different nitrogen source and nitrogen concentration (mean ± standard errors, n=3). * denoted P<0.05, ** denoted P<0.01. Different letters represent significant differences at P<0.05 among different nitrogen sources (lowercase for 1 mg L^{-1} nitrogen and capitalized for 5 mg L^{-1} nitrogen). N-NO3: nitrate as nitrogen source, N-NO3-NH4: nitrate-ammonium as nitrogen source, N-NH4: ammonium as nitrogen source. N1: 1 mg L^{-1} nitrogen; N5: 5 mg L^{-1} nitrogen. MGDGs: monogalactosyldiacylglycerols, DGDGs: digalactosyldiacylglycerols, SQDGs: sulfoquinovosyldiacylglycerols, PAs: phosphatidic acids, PCs: phosphatidylcholins, PEs: phosphatidylethanolamines, PGs: phosphatidylglycerols, PIs: phosphatidylinositols, TGs: Triglycerides.

was used as the nitrogen source, in comparison to nitrate. Furthermore, the exposure of *P. haitanensis* conchocelis to ammonium resulted in elevated levels of Chlorophyll a, carotenoid, phycoerythrin and phycocyanin, in comparison to nitrate treatment. Meanwhile, the expression of NiR, GS1 and GS2 genes were found to be higher with nitrate as the nitrogen source than with ammonium, while the GOGAT gene displayed the opposite response in the present study. The GS mainly participated in ammonia assimilation, with the process being initiated by nitrate reduction or photorespiration (Yu et al., 2022). Based on these results, we proposed a hypothesis that nitrogen in the form of ammonia is often absorbed and assimilated faster than nitrate in *Pyropia haitanensis* conchocelis (Abreu et al., 2011). However, nitrogen sources containing nitrate resulted in higher levels of MGDGs, DGDGs, SQDGs, PGs and PIs content than those containing ammonium of *P. haitanensis* conchocelis in this study.

The Effects of Nitrogen Concentrations (1 and 5 mg mL^{-1}) on *P. haitanensis*

The results of previous studies indicated that the enhancement of nitrate and ammonium will result in a significant increase in total nitrogen within algae (Naldi & Wheeler, 2010). A nitrogen-rich environment is conductive to the synthesis of phycobiliprotein (Kursar & Alberte, 1983). The enrichment of nitrogen can also lead to an increase in the accumulation of algal nitrogen assimilates, which are mainly stored as soluble protein (Jiang et al. 2020; Li et al., 2022; Ribeiro et al., 2013). In the present study, with nitrate or ammonium as a nitrogen source, higher phycoerythrin, phycocyanin and soluble proteins, while lower relative growth rate were observed in *P. haitanensis* conchocelis cultured at 5 mg L^{-1} nitrogen concentration than at 1 mg L^{-1} nitrogen concentration. These results suggested that excessive nitrogen in the 5 mg L^{-1} nitrogen culture may be stored in cells of *P. haitanensis* conchocelis as nitrogen pools in the forms of phycocyanin and soluble proteins. The low growth rate may be due to the fact that all the required nutrient resources are used for higher nutrient supply to produce soluble proteins, etc., which do not contribute to growth under of 5 mg L^{-1} nitrogen conditions.

Nitrate concentration strongly affects the lipid content and fatty acid composition of algae (Xu et al., 2001; Kumari et al., 2014). In the present study, 5 mg L^{-1} nitrogen concentration increased contents of MGDGs, DGDGs, SQDGs and PEs of *P. haitanensis* conchocelis with nitrate as nitrogen source. The result was in agreement with some reports, for example, the lipid content of *Ulva pertusa* Kjellman (Floreto et al., 1996). And nitrogen concentration of ammonium also affects the lipid content of algae. An excessive nitrogen concentration of ammonium did not benefit for the lipid content of *P. haitanensis* conchocelis. The total contents of MGDGs, DGDGs, SQDGs, PEs, PGs, PIs and TGs in *P. haitanensis* conchocelis decreased under higher (5 mg L^{-1}) nitrogen with ammonium as nitrogen source in the present study. Triglycerides are considered to be storage lipids for algae (Sukenik & Carmeli, 1990). The N-limited cells showed an increase in triglycerides (Lynn et al. 2000). Similar to this response, the higher contents TGs were found at all nitrogen sources under lower nitrogen concentration (1 mg L^{-1}) in the present study.

Conclusions

In conclusion, relative growth rate, pigments content and lipids content of *P. haitanensis* conchocelis were affected by the nitrogen type. The use of ammonium as nitrogen source was beneficial for the growth of *P. haitanensis* conchocelis, resulting in higher relative growth rates and pigments content of *P. haitanensis* conchocelis. Furthermore, the addition of 5 mg L^{-1} nitrogen with nitrate as nitrogen source was benefit to lipids production, while the use of 5 mg L^{-1} nitrogen with ammonium as nitrogen source did not promote the accumulation of lipids.

Ethical Statement

Not applicable.

Funding Information

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Author Contribution

Peng Zhang: Writing–original draft, data analysis, formal analysis; Xiujuan Wang: data analysis, manuscript writing and editing; Yubo Wu: manuscript writing and editing.

Conflict of Interest

The authors declare that they have no known competing financial or non-financial, professional, or personal conflicts that could have appeared to influence the work reported in this paper.

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