Nitrogen enrichment mediates the effects of high temperature on the growth, photosynthesis, and biochemical constituents of Gracilaria blodgettii and Gracilaria lemaneiformis

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Abstract
Gracilaria blodgettii and Gracilaria lemaneiformis are often adopted as tools to purify aquaculture tail water. However, there has been such phenomenon that high temperature in summer restricts the process of aquaculture. To explore the adaptive capacity of G. blodgettii and G. lemaneiformis, we experimented them and cultured for 12 days under three temperatures (20, 25, and 30 °C) and three levels of multiple nitrogen sources (0.12, 0.6 and 4.4 mg L⁻¹). Their growth, photosynthetic characteristics, and biochemical compositions including the contents of pigments and soluble protein were determined to investigate the single and interactive effects of temperatures and nitrogen levels on these two species. The results showed that in terms of G. blodgettii, the higher growth rate and more pigment (chlorophyll a and carotenoids) contents were observed at 25 and 30 °C in comparison to 20 °C, and the pigments showed maximum contents at 25 °C. More nitrogen improved the growth rate, net photosynthetic rate (Pn) at 25 and 30 °C, Fv/Fm at 20 °C, maximal photosynthetic electron transfer rate (ETRm), as well as soluble protein content at 20 and 25 °C. Additionally, the growth rate, Pn, and ETRm of G. lemaneiformis all showed a decline as increasing temperature; analogously high nitrogen concentration increased the growth rate at 25 and 30 °C, Fv/Fm at each temperature, ETRm, and pigments contents at 20 °C, as well as soluble protein content at 20 and 25 °C. Conclusions indicated that high temperature restricted the growth rate, inhibited photosynthetic characteristics, and decreased the soluble protein content of G. lemaneiformis. The reduced photosynthetic performance, pigments, and soluble protein contents of G. blodgettii were noted under similar conditions. However, nitrogen enrichment induced the greater resistant level to high temperature, and G. blodgettii showed better response. These findings suggested that these two Gracilaria species possessed a certain adaptability to tail water from aquaculture at high temperature and G. blodgettii can resist more to. Therefore, it seems to be an alternative and workable scheme to adopt some suitable macroalgae to optimize the solution to present purification of aquaculture wastewater or eutrophic waters under high temperature.

Keywords
Aquaculture tail water · Nitrogen · Temperature · Macroalgae · Photosynthetic characteristic · Adaptability

Introduction
There has been always nutrient enrichment in aquaculture caused by both low assimilation ratio and conversion efficiency of bait as well as the decomposition of metabolic sediment.

The phenomenon leads to self-purification and recovery abilities destroyed; consequently environment load increases and water quality presents a deteriorating trend (Smith et al. 1999). Intensive emissions of wastewater result in excessive nitrogen and phosphorus in coastal areas, serious coastal pollution, and ecological balance destruction (Kaspar et al. 1985; Cao et al. 2007). The China Ecology and Environment Bulletin in 2018 reported that inorganic nitrogen and active phosphate were the major pollution indexes in the coastal waters of China, where nitrogen levels in most areas were higher than those in other coastal regions of the world (Valiela et al. 2018). Red or green tides outbreaks thrive by part reason of nutrient supplementation for the proliferation of aquatic plants (Kessing et al. 2011; Chen et al. 2016), and the survival environment of human being and other creatures’ habitat are obviously affected and

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inhibited (Tett et al. 2007). As large-scale and intensive development of aquaculture, excess feed and then masses of effluent bring a toxic impact of the eutrophication on the circulating production, fundamentally influencing benefits of industry (Frances et al. 1998; Gao et al. 2005; Klaoudatos et al. 2006).

Over the years, traditional physical and chemical measures have been to deal with effluent (Boley et al. 2000; Ridha and Cruz 2001; Mook et al. 2012). However, high cost and side effect limit its practical application and progress (Tango and Gagnon 2003). A viable alternative is bioremediation taking advantage of algae to remove nutrients from aquaculture wastewater (Neori et al. 2004; Marinho-Soriano et al. 2009). The project not only achieves purification as well as the improvement in utilization rates of nitrogen and phosphorus within aquaculture system (Shpigel et al. 1993) but also recycles nutrient to harvest biomass and by-products with potential revenue (Buschmann et al. 1996; Ross et al. 2018).

Great economic values are possessed by *Gracilaria blodgettii* and *Gracilaria lemaneiformis*, as the key ingredients for food, medicine, and main biological bait of factory rearing of abalone (Yu and Yang 2008). Moreover, these species exert such capacities to absorb nutrient and resist stresses and then adopted for treatment of aquaculture tail water (Liu et al. 2005; Yang et al. 2006). However, the aquaculture with increasing temperature in season remains the unresolved issue. Previous studies noted that high nitrogen could enhance certain resistances to high irradiance and salinity (Huovinen et al. 2006; Zheng et al. 2019). Therefore, we hypothesized that more nitrogen could improve the resistant ability of these two *Gracilaria* species to high temperature. Because the main inorganic nitrogen in wastewater are NH$_4^+$-N, NO$_3^-$-N, and NO$_2^-$-N, and NO$_3^-$-N, in addition that algae are able to prioritized utilization of ammoniacal nitrogen, nitrates, and nitrates (Przytocck-Jusiak et al. 1984), and thus multiple nitrogen sources at different concentrations but fixed proportions were added to this experiment. After 12 days treatment experiment under three temperatures, the growth, photosynthesis, pigments, and soluble protein contents in the thalli were measured. The probable goals on present research were to confirm the aforesaid assumption and then explore the variable effects of aquaculture wastewater or eutrophic waters at high temperature on these two *Gracilaria* species and corresponding adaptability, as well as the selection of suitable species under different conditions and the matters paid attention to.

**Materials and methods**

**Materials and precultured conditions**

*G. blodgettii* and *G. lemaneiformis* were adopted in July 2019 from the mariculture zone of Xuwen, Guangdong (N 20° 19′ 43.46″, E 110° 10′ 14.32″) and Rongcheng, Shandong (N 37° 08′ 41.26″, E 122° 24′ 9.12″), respectively. These thalli were stored in an ice box and brought back within 24 h, immediately precultured in daily-changed seawater (Salinity 30) for 3 days at 25 ± 1 °C and 100 μmol photons m$^{-2}$s$^{-1}$ (L/D = 12:12) by aerating with ambient air.

**Culture conditions**

In this study, there were three temperatures 20, 25, and 30 °C set up. Three nitrogen levels including 0.1235 (LN), 0.6 (MN), and 4.4 mg L$^{-1}$ (HN) with 18:74:8 ratios for NH$_4^+$-N, NO$_3^-$-N, and NO$_2^-$-N, were applied to these two species in 100 μmol photons m$^{-2}$s$^{-1}$ light conditions (L/D = 12:12), which were respectively based upon the China Offshore Sea Environmental Quality Bulletin 2016, and the total concentration of N in shrimp pond aquaculture wastewater (Zheng et al. 2019). Approximately 1 g of healthy thalli in same part for further treatment was selected to proceed. Each treatment respectively combined different temperatures with nitrogen levels was set as three replicates. The thalli were cultured in 500 ml conical flask of constant temperature incubators, daily measurement of fresh weight and renewed f/2 medium without N and Si were done (Guillard and Ryther 1962), and 12-day culture later the evaluation went ahead.

**Growth rate**

We recorded the daily fresh weight and calculated the growth rate. The relative growth rate (RGR) was averaged over the last 6 days to show the algal growth that was calculated as follows: RGR (%·day$^{-1}$) = $100 \times \ln(W_{t2}/W_{t1})/(t_{2}-t_{1})$, where $W_{t1}$ represents the fresh weight measured on previous day and $W_{t2}$ means that of present day.

**Dark respiration and net photosynthetic rate**

The Clark-type oxygen electrode (Hansatech Instruments Ltd., UK) was used to measure dark respiration rate ($R_{d}$) and net photosynthetic rate ($P_{n}$) of the weighed fresh thalli (0.025–0.03 g). These samples were put into the sample cup of Clark-type oxygen electrode containing 2 ml fresh seawater at cultured temperature. We measured the $R_{d}$ in the dark and the $P_{n}$ under 100 μmol photons m$^{-2}$s$^{-1}$ treatment, respectively.

**Chlorophyll fluorescence in vivo**

The $F_{v}/F_{m}$ reflects maximal quantum yield of photosystem II (PS II) after undergoing dark adaptation under optimal conditions, equal to $(F_{m}\cdot F_{o})/F_{m}$, where $F_{o}$ and $F_{m}$ stand for minimum fluorescence and maximum fluorescence of dark-adapted samples. And the $ETR_{m}$ represents maximal photosynthetic electron transfer rate. Moreover, the $NPQ$ that fully
named as non-photochemical quenching means chlorophyll fluorescence yield decreased due to heat dissipation, calculated by \( (F_m' - F_m^\prime)/F_m^\prime \), where \( F_m' \) stands for maximum fluorescence of light-adapted samples. The above parameters of thalli could be all measured by the pulse amplitude modulated fluorometer (Diving-PAM, Germany) after dark adaptation (15 min), and the actinic light level was set in 104 μmol photons m\(^{-2}\) s\(^{-1}\).

**Biochemical constituents**

Prior to scanning the absorption spectrum of samples’ extraction solution using a spectrophotometer, all thalli prepared for extracting pigments with 3 ml methanol were saved in darkness overnight at 4 °C. On the basis of Porra (2002) as well as Parsons and Strickland (1963), respectively, there was the absorbance of the methanol extracts at 480, 510, 652, 665, and 750 nm scanned to calculate chlorophyll \( \text{a} \) (Chla) and carotenoid (Car) concentrations. Bradford (1976) indicated that Coomassie Brilliant Blue G-250 dye was able to measure soluble protein content. The homogenized and extracted thalli (ca. 0.1 g) in phosphate buffer solution (PBS: 0.05 mol l\(^{-1}\), pH 7.8) were centrifuged at 5000 g and 4 °C for 15 min, and these solutions were to measure protein content.

**Data analysis**

The statistical analyses and plotting adopted SPSS 26 and Origin 2018 to accomplish, respectively. All these data of three replicates of each treatment was dealt with as the mean and standard deviation, and the normal distribution and homogeneity of variance were determined severally by the Shapiro-Wilk test and Levene’s test \((P > 0.05)\). A two-way analysis of variance and the Tukey post hoc test were able to be performed to assess separately these effects of single factor and their interactions as well as differences between temperature conditions and nitrogen levels, where significance levels were set at \( P < 0.05 \).

**Results**

**Effects of N and T on growth**

The greater growth rates of *G. blodgettii* were observed at higher temperature, and there was no significant difference at 25 °C under any N level. As the enriching nitrogen concentration \( C_{(\text{N})} \), an increasing trend of the growth was observed among three temperatures, especially significant improvements under HN levels (Fig. 1a). Thus, the effects of N and T were assessed as significant (T, \( P = 0.003 \); N, \( P = 0.010 \); Table 1).

For *G. lemaneiformis*, there were downward directions in terms of the growth as increasing temperature. Although the growth at 20 °C showed no significant effect of N, other temperatures that have higher growth rates were observed significantly with more \( C_{(\text{N})} \) (Fig. 1b). Consequently, the effects of T and N and their interactions were all assessed as significance (T, \( P = 0.000 \); N, \( P = 0.000 \); T*N, \( P = 0.019 \); Table 1).

**Effects of N and T on respiration and photosynthesis**

Higher temperature strengthened gradually the dark respiration rates \( (R_d) \) of *G. blodgettii* under three N levels. When getting to HT condition, the \( R_d \) exceeded significantly than the others. And there were relatively high values at 20 and 25 °C under MN level, respectively (Table 2). Higher temperature weakened the \( P_n \) under LN level, and a clear improvement of \( P_n \) was observed with the increasing \( C_{(\text{N})} \) at 25 and 30 °C (Fig. 2a). Moreover, there were higher \( P_n/R_d \) at 20 and 25 °C than those at 30 °C. As the \( C_{(\text{N})} \) rose, significant increase of the value was found at 30 °C (Table 2).

The maximal quantum yield of PS II \( (F_{v}/F_m) \) showed no significant effect of N at 25 and 30 °C. However, the increasing \( C_{(\text{N})} \) would enhance the value (a lowest) at 20 °C (Fig. 2b).
Table 1 The two-way analysis of variance about both effects of temperature (T) and nitrogen (N) on all the measured indexes for *G. blodgettii* and *G. lemaneiformis* under different temperatures and nitrogen levels

| Source  | df  | F     | p value | Source  | df  | F     | p value |
|---------|-----|-------|---------|---------|-----|-------|---------|
| **G. blodgettii** |   |       |         |         |     |       |         |
| RGR     | T 2 | 4.655 | 0.030   |         | N 2 | 6.802 | 0.010   |         | T*N 4 | 1.788 | 0.191 | 63.200 | 0.000  |         |         |         |
|         | T 2 | 123.413 | 0.000   |         | N 2 | 143.108 | 0.000   |         | T*N 4 | 122.170 | 0.000   |         |         |         |         |
| Pn      | T 2 | 123.413 | 0.000   |         | N 2 | 143.108 | 0.000   |         | T*N 4 | 122.170 | 0.000   |         |         |         |         |
|         | T 2 | 132.750 | 0.000   |         | N 2 | 7.994   | 0.008   |         | T*N 4 | 26.255    | 0.000   |         |         |         |         |
| NPQ     | T 2 | 10.307 | 0.005   |         | N 2 | 3.481   | 0.076   |         | T*N 4 | 9.191     | 0.003   |         |         |         |         |
|         | T 2 | 9.191   | 0.003   |         | N 2 | 26.255  | 0.000   |         | T*N 4 | 49.083    | 0.000   |         |         |         |         |
| **G. lemaneiformis** |   |       |         |         |     |       |         |         |         |         |         |         |         |         |         |
| RGR     | T 2 | 53.414 | 0.000   |         | N 2 | 27.709 | 0.000   |         | T*N 4 | 4.907     | 0.019   |         |         |         |         |
|         | T 2 | 124.404 | 0.000   |         | N 2 | 0.873  | 0.447   |         | T*N 4 | 6.962     | 0.006   |         |         |         |         |
| Pn      | T 2 | 124.404 | 0.000   |         | N 2 | 0.873  | 0.447   |         | T*N 4 | 6.962     | 0.006   |         |         |         |         |
|         | T 2 | 47.596  | 0.000   |         | N 2 | 7.139  | 0.000   |         | T*N 4 | 8.191     | 0.003   |         |         |         |         |
| NPQ     | T 2 | 118.281 | 0.000   |         | N 2 | 0.161  | 0.853   |         | T*N 4 | 0.148     | 0.959   |         |         |         |         |
|         | T 2 | 0.873   | 0.447   |         | N 2 | 0.713  | 0.000   |         | T*N 4 | 0.148     | 0.959   |         |         |         |         |

regard to maximal photosynthetic electron transfer rate (*ETR*<sub>m</sub>), HN levels showed higher values (Fig. 2c). Only significant effect of T on the non-photochemical quenching (*NPQ*) was observed (*P* = 0.005; Table 1), and higher temperature conditions improve the *NPQ* under MN and HN levels (Table 2).

Therefore, the significant effects of the single and interactive of these two factors on the above six parameters were all assessed as significant excluding the *NPQ* (*R*<sub>d</sub>, *P*<sub>n</sub>, and *P*<sub>n</sub>/*R*<sub>d</sub>; T, N and T*N, *P* = 0.000; *F*/*F*<sub>m</sub>; T, *P* = 0.002. N, *P* = 0.007. T*N, *P* = 0.003; *ETR*<sub>m</sub>; T, *P* = 0.001. N, *P* = 0.000. T*N, *P* = 0.031; Table 1).

In terms of *G. lemaneiformis*, there were lower *R*<sub>d</sub> owing to the increasing *C*<sub>(N)</sub> at 25 and 30 °C. And high temperature condition increased the *R*<sub>d</sub> of LN level, and maximum one was observed (Table 2). Then the significant effects of T and N and their interaction on the *R*<sub>d</sub> were found (T, *P* = 0.014. N, *P* = 0.000. T*N, *P* = 0.000; Table 1). Differently, only the *P*<sub>n</sub> of 20 °C showed no significant distinction under whatever N levels and was highest *P*<sub>n</sub> under each N level (Fig. 2d). Then, the N level affected the *P*<sub>n</sub> insignificantly (*P* = 0.000; Table 1). Under each N level, although no significant difference of the *P*<sub>n</sub>*R*<sub>d</sub> was found at 20 °C, these values were significantly higher than those of other temperature excluding HN level, owing to the higher values under higher N levels at 25 and 30 °C (Table 2). Therefore, the effects of T and N and their interaction on the *P*<sub>n</sub>*R*<sub>d</sub> were assessed as significant (T, *P* = 0.000. N, *P* = 0.009. T*N, *P* = 0.001; Table 1).

Similarly, the *F*/*F*<sub>m</sub> at 30 °C condition was lower than those of others under every N level. And the maximum *F*/*F*<sub>m</sub> was observed at 20 and 25 °C under HN level. As the increase of the *C*<sub>(N)</sub>, the *F*/*F*<sub>m</sub> at all temperature conditions showed a higher value especially when HN level was reached. (Fig. 2e). Additionally, the *ETR*<sub>m</sub> would decrease with improving temperature under the same N level. On the contrary, only the value of 20 °C noted an increasing trend with more *C*<sub>(N)</sub> (Fig. 2f). Thus, there were the significant effects of T and N and their interaction on both of them (*F*/*F*<sub>m</sub>; T, *P* = 0.000. N, *P* = 0.000. T*N, *P* = 0.003; *ETR*<sub>m</sub>; T, *P* = 0.000. N, *P* = 0.017. T*N, *P* = 0.003; Table 1). About the *NPQ*, there were high values at higher temperature (Table 2), but only effect of T was statistically significant (*P* = 0.000; Table 1).

Effects of N and T on pigments

The higher pigment contents of *G. blodgettii* including Chla and Car, especially that of LN and MN levels at 25 °C, were observed. Even if there was not much regularity between all the N levels and temperature conditions, both pigments showed an identical trend (Table 2). And there were significant effects of T and N and their interaction on each pigment (Chla: T, *P* = 0.000. N, *P* = 0.008. T*N, *P* = 0.000; Car: T, *P* = 0.000. N, *P* = 0.000. T*N, *P* = 0.000; Table 1).

Only LN level showed a relative improvement about Chla and Car contents of *G. lemaneiformis* with the rise of temperature. Difference from the higher temperature...
conditions that no significant effect of N were observed, high N levels facilitated the increase of the pigment contents at 20 °C (Table 2). Then there was a significant interaction effect of T and N on them (Chla: T*N, $P = 0.002$; T*N, $P = 0.004$; Table 1).

**Effects of N and T on soluble protein**

The soluble protein contents of *G. blodgettii* and *G. lemaneiformis* at 20 and 25 °C conditions under higher C(N) were more than those under the low N level, while the content at 30 °C was no significant difference. Furthermore, the contents of these two species at 30 °C and HN level were significantly less than those of lower temperature (Fig. 3). Thus, except for temperature on *G. lemaneiformis*, the single and interactive effects of these two factors on the soluble protein contents were all evaluated as significant (*G. blodgettii*: T, $P = 0.000$. N, $P = 0.002$. T*N, $P = 0.008$; *G. lemaneiformis*: N, $P = 0.000$. T*N, $P = 0.000$; Table 1).

**Discussion**

As noted above, some situations like nutrient enrichment and high temperature appear in aquaculture. These ecological factors impact upon the physiological characteristics and biochemical components of macroalgae (Yu and Yang 2008; Chen et al. 2016; Zheng et al. 2019). In order to explore the adaptability of two *Gracilaria* to aquaculture tail water, especially in high temperature season, this study tried to assess the single and reciprocal effects of multiple nitrogen sources and high temperatures.

The results indicated that there was a highest growth rate of *G. lemaneiformis* at 20 °C but *G. blodgettii* at 30 °C and showed the latter tolerance to high temperature more than that of the former. One evident phenomenon was that high nitrogen concentration turned the growth of *G. blodgettii* at 20 and 30 °C better and also reflected similarly in *G. lemaneiformis* of 25 and 30 °C. The positive observations were similarly found on these studies of *G. lemaneiformis*, *Ulva lactuca*, and *U. prolifera* with the supplement of various nitrogen sources at different concentrations (Yu and Yang 2008; Xu et al. 2014; Van Alstyne 2018). However, algal respiration was enhanced, and then net photosynthesis significantly decreased at high temperature. The growing affinity of rubisco for oxygen caused extra respiration, and the heat stress inhibited the fixation capacity of enzymatic reaction for CO$_2$ and heat stability of reaction site connected with photosynthesis (Weis 1981; Feller et al. 1998; Vona et al. 2004). High N level inhibited the respiration of *G. lemaneiformis* and improved the photosynthesis of *G. blodgettii* respectively.

| Treat       | $R_d$ (μmol O$_2$ g$^{-1}$ h$^{-1}$) | $P_n/R_d$ | NPQ | Chla (μg ml$^{-1}$) | Car (μg ml$^{-1}$) |
|-------------|------------------------------------|-----------|-----|---------------------|-------------------|
| *G. blodgettii* |                                    |           |     |                     |                   |
| 20-LN       | 18.79 ± 0.09$^{Au}$                | 11.82 ± 0.14$^{Au}$ | 0.164 ± 0.035$^{Au}$ | 46.01 ± 3.97$^{Au}$ | 12.13 ± 0.75$^{Au}$ |
| 20-MN       | 41.11 ± 0.16$^{Ba}$                | 4.90 ± 0.27$^{Ba}$ | 0.186 ± 0.011$^{Ba}$ | 43.17 ± 0.13$^{Ba}$ | 13.31 ± 0.24$^{Ba}$ |
| 20-HN       | 24.83 ± 0.53$^{Ca}$                | 8.28 ± 0.22$^{Ca}$ | 0.133 ± 0.016$^{Ca}$ | 40.09 ± 1.18$^{Ca}$ | 10.00 ± 0.03$^{Ba}$ |
| 25-LN       | 23.54 ± 1.49$^{Ab}$                | 8.40 ± 0.52$^{Ab}$ | 0.217 ± 0.035$^{Ab}$ | 85.60 ± 0.62$^{Ab}$ | 27.51 ± 0.20$^{Ab}$ |
| 25-MN       | 55.72 ± 2.76$^{Bb}$                | 4.72 ± 0.04$^{Ba}$ | 0.249 ± 0.043$^{Ab}$ | 81.84 ± 3.13$^{Ab}$ | 24.31 ± 0.74$^{Ab}$ |
| 25-HN       | 45.01 ± 0.16$^{Bb}$                | 6.10 ± 0.07$^{Bb}$ | 0.192 ± 0.030$^{Ab}$ | 57.17 ± 4.15$^{Bb}$ | 12.72 ± 1.01$^{Bb}$ |
| 30-LN       | 64.99 ± 3.80$^{Ac}$                | 2.80 ± 0.23$^{Ac}$ | 0.228 ± 0.041$^{Ac}$ | 49.68 ± 6.17$^{Ac}$ | 13.16 ± 1.19$^{Ac}$ |
| 30-MN       | 60.51 ± 2.05$^{Ac}$                | 4.04 ± 0.14$^{Ac}$ | 0.261 ± 0.032$^{Ac}$ | 64.61 ± 4.82$^{Ac}$ | 20.03 ± 0.73$^{Bc}$ |
| 30-HN       | 67.55 ± 0.34$^{Ac}$                | 3.99 ± 0.01$^{Bc}$ | 0.230 ± 0.020$^{Bc}$ | 68.77 ± 2.15$^{Bc}$ | 16.31 ± 0.24$^{Bc}$ |
| *G. lemaneiformis* |                                |           |     |                     |                   |
| 20-LN       | 54.90 ± 7.88$^{Au}$                | 7.58 ± 1.45$^{Au}$ | 0.056 ± 0.019$^{Au}$ | 62.70 ± 1.52$^{Au}$ | 12.32 ± 1.31$^{Au}$ |
| 20-MN       | 59.04 ± 2.03$^{Au}$                | 6.70 ± 0.13$^{Au}$ | 0.056 ± 0.007$^{Au}$ | 80.39 ± 0.20$^{Ba}$ | 18.45 ± 0.43$^{Ba}$ |
| 20-HN       | 64.61 ± 0.08$^{Bb}$                | 6.51 ± 0.20$^{Bb}$ | 0.051 ± 0.007$^{Bb}$ | 87.08 ± 1.37$^{Bb}$ | 20.68 ± 1.60$^{Bb}$ |
| 25-LN       | 94.72 ± 8.58$^{Ab}$                | 3.39 ± 0.42$^{Ab}$ | 0.058 ± 0.006$^{Ab}$ | 79.83 ± 5.65$^{Ab}$ | 20.96 ± 2.71$^{Ab}$ |
| 25-MN       | 61.45 ± 1.09$^{Ab}$                | 5.63 ± 0.09$^{Ab}$ | 0.075 ± 0.004$^{Ab}$ | 81.88 ± 4.14$^{Ab}$ | 21.30 ± 2.52$^{Ab}$ |
| 25-HN       | 54.04 ± 0.69$^{Bb}$                | 5.35 ± 0.16$^{Bb}$ | 0.067 ± 0.028$^{Bb}$ | 72.90 ± 2.16$^{Bb}$ | 15.48 ± 0.66$^{Bb}$ |
| 30-LN       | 98.99 ± 7.25$^{Ac}$                | 2.95 ± 0.24$^{Ac}$ | 0.243 ± 0.021$^{Ac}$ | 87.91 ± 7.60$^{Ac}$ | 24.36 ± 2.55$^{Ac}$ |
| 30-MN       | 62.30 ± 0.72$^{Bb}$                | 3.70 ± 0.08$^{Bb}$ | 0.240 ± 0.001$^{Bb}$ | 76.05 ± 2.00$^{Bb}$ | 21.22 ± 0.70$^{Bb}$ |
| 30-HN       | 37.20 ± 2.32$^{Cc}$                | 5.22 ± 0.53$^{Cc}$ | 0.230 ± 0.055$^{Cc}$ | 81.27 ± 10.15$^{Cc}$ | 21.36 ± 3.13$^{Cc}$ |

Different uppercase letters mean significant differences between different nitrogen levels at the same temperature, and different lowercase letters mean significant differences between different temperature conditions in the same nitrogen level ($P < 0.05$).
equal results to make net photosynthetic rate rebounded. Similar observation in published papers also noted high N concentrations lower the respiration but enhanced the photosynthetic rate of *U. prolifera* (Zheng et al. 2019). Additionally, the supplement of nutrients especially the N increased the levels of certain key enzymes for photosynthesis including rubisco and carbonic anhydrase (Jiménez del Río et al. 1995). Photosynthetic parameters were able to reflect the important physiological activities of algae, and the photosynthetic/respiration ratio could evaluate the algal ability to accumulate materials (Humphrey 1975). These two species showed the higher ratio owing to more material synthesis as well as less metabolism at 20 °C, especially under lower N level, which indicated that there were greater photosynthetic activities under this condition that could be the optimally environmental level to adaptation and preservation of algae.

Moreover, high temperature restrained their photosynthetic activity, but more nitrogen improved *G. lemaneiformis* or *G. blodgettii* at 30 °C. Such an interesting tendency was observed that the *Pn* of *G. blodgettii* at 20 °C under low N level recorded higher than those at 25 and 30 °C but the growth was decreased; the nitrate reductase (NR) activity for nitrogen assimilation may be the limiting factor. Previous reports have shown that nitrate reductase had its optimum at 25 °C in the cryophilic algae *Koliella antarctica* and green algae *U. prolifera*, at 30 °C in red algae *G. lemaneiformis* as well as at 35 °C in the mesophilic *Chlorella sorokiniana* (Vona et al. 2004; Gao et al. 2016; Zhong et al. 2020). The assimilation of nitrate and the synthesis of enzymes especially nitrate reductase are linked fundamentally with photosynthesis in alga (Thomas et al. 1976; Xu et al. 2017). Therefore, the assimilations of carbon and nitrogen synergistically

Fig. 2 The net photosynthetic rate (*Pn*), maximal quantum yield of PS II (*Fv/Fm*), and maximal photosynthetic electron transfer rate (*ETRm*) of *G. blodgettii* (a, b, c) and *G. lemaneiformis* (d, e, f). Different uppercase letters mean significant differences between different nitrogen levels at the same temperature, and different lowercase letters mean significant differences between different temperature conditions in the same nitrogen level (*P* < 0.05)
act on algal growth, and then the decreased NR activity at low temperature may lead to the restricted growth despite the relatively high \( P_r \). However, the supplement of nitrogen may increase the activity and concentration of substrate for NR (Navarro et al. 1996; Chow and Oliveira 2008; Zhu et al. 2016), which enhanced the assimilation of nitrogen and eased the limitation of growth.

Chlorophyll fluorescence is an effective probe for photosynthesis (Schreiber 2004). The non-photochemical quenching (NPQ) represents the suffered stress level and the ability to dissipate surplus light energy into heat, also means photoprotection (Schreiber et al. 1995). Although there was no significant effect of nitrogen level on the NPQ, this study showed that high temperature increased the stress and enhanced photoprotective ability. Furthermore, the maximal quantum yield of PS II \( (F_/F_m) \) reflects the potentially maximum photosynthetic capacity, and its reduction stands for the organism suffering ambient pressures (Schreiber et al. 1995). The maximally photosynthetic electron transfer rate \( (ETR_m) \) is the expression of the potentially maximum ability of pigment molecules to transfer electrons into the photosynthetic reaction chain through PS II (Schreiber et al. 1995). They could also indirectly mean the level of photochemical activity of PS II higher or lower. In this study, the \( F_/F_m \) as well as \( ETR_m \) of *G. lemaneiformis* at 30 °C were lower than those at 20 and 25 °C, and the \( ETR_m \) of *G. blodgettii* at 25 °C was higher than other values at 20 and 30 °C under identical temperature. However, more nitrogen increased the \( F_/F_m \) of *G. blodgettii* and \( ETR_m \) of *G. lemaneiformis* at 20 °C, as well as the \( F_/F_m \) of *G. lemaneiformis* and \( ETR_m \) of *G. blodgettii* regardless of the temperature. These above phenomena indicated that PS II responded to heat stress sensitively, and a series of photosynthetic chain reactions and corresponding electron transfer were bound up with temperature (Davison 2004). Heat stress could stimulate photoinhibition of PS II and even inactivate the oxygen-evolving complex directly and also accelerate the production of \( \text{H}_2\text{O}_2 \) to induce oxidative stress and then inhibit D1 protein synthesis and the repair of PS II (Murata et al. 2007). Algae by means of rapidly adjusting the open and close status of PS II reaction center to adapt ambient temperature (Lincoln and Eduardo 2010), associated with significant pressures from temperature on *G. lemaneiformis* at 30 °C and *G. blodgettii* at 20 °C. Whereas sufficient nitrogen mitigated heat stress reflecting in increasing potential capacities of maximum photosynthesis and electron transfer for the improvement of photochemical activity, which would contribute to facilitating the conversion of captured light energy into chemical energy at a higher rate and efficiency, to provide more energy for assimilation in photosynthesis.

Photosynthetic pigments provide the light energy for photosynthesis (Xia and Gao 2005) and then enhance the photosynthetic capacity of algae (Dawes and Koch 1990; Crawford 1995). Rising temperature enhanced the synthesis of some algal pigment contents of *G. blodgettii* and *G. lemaneiformis* under low N level. However, the pigment contents of *G. blodgettii* at 25 °C showed the maximum under low and middle N level, which may be owing to the part degradation of pigments at 30 °C, whereas more nitrogen improved the contents of *G. lemaneiformis* at 20 °C even up to the state of higher temperature. Additionally, the content of soluble protein could stand for the algal matter accumulation to a certain extent. And certain proteins possess great hydrophilic competent to enhance the water-retained capacity and respond to thermal shock against cellular damage as a symbol of the stress tolerance (Lobban and Harrison 1997). Nitrogen supply increased the amount of protein of two species at 20 and 25 °C, which could be explained that the lacking nitrogen supply limited synthesis of protein (Pettersson and McDonald 1994), and the decreasing content under low nitrogen may be caused by nitrogen source required for growth (Young et al. 2009). However, the 30 °C condition inhibited the extra influence of nitrogen owing to high temperature that may restrain activities of protein synthesis and certain reactions related to synthesis. The improvements of nutrient supply on biochemical composition of *Chondrus crispus*,

![Fig. 3](image_url) The soluble protein contents of *G. blodgettii* (a) and *G. lemaneiformis* (b) under different treatments. Different uppercase letters mean significant differences between different nitrogen levels at the same temperature, and different lowercase letters mean significant differences between different temperature conditions in the same nitrogen level \( (P < 0.05) \).
Gracilaria gracilis, and Cladophora parriaudii were verified to enable substance accumulation under N-deprivation with a shift towards the synthesis of proteins and pigments (Chopin et al. 1995; Smits et al. 1997; Ross et al. 2018). Thus, high temperature and nitrogen deficiency induced certain pressures on the accumulation of pigments and soluble protein of two Gracilaria, and the supplementation of nitrogen source brought the positive effect on the ability to withstand.

To sum up, G. blodgettii and G. lemaneiformis respectively possessed different optimum temperatures for growth and high temperature tolerance. Temperature affects the related physiological and biochemical processes and then controls algal growth. Abundant nitrogen source signals to the algae for growth and is capable of being in these ways partly by improving photosynthesis and promoting the synthesis of photosynthetic pigments as well as soluble protein to reduce this effect caused by temperature stress. These findings thus support our hypothesis that more nitrogen was able to induce the resistance to high temperature within a certain range. In the future in terms of aquaculture wastewater problem especially under high temperature, suitable macroalgae can be selected for optimal treatment on the basis of different environmental conditions. Additionally, in our areas where sea cucumbers are farmed in seaside ponds, there are such problems that the sea cucumbers are against the high temperature, reduction of dissolved oxygen, excreta, etc. The applications of macroalgae on the surface of the pond seem to block the part sun, enhance the interchange of air, and consume some contaminants owing to their size, strong photosynthesis, and absorption capacity. Therefore, we put forward a good choice using the macroalgae as the shading and cooling of creatures. Ultimately, the study as regards nutrient enrichment and temperature stress on two Gracilaria may be the foundation of optimization of both aquaculture structure and wastewater treatment.

Authors’ contributions CM, YW, and ZZ conducted all the experiment. CM and ZZ analyzed and interpreted all the data and were major contributors in writing the manuscript. ZL, SQ, HC, and LZ performed the examination of the manuscript. All authors read and approved the final manuscript.

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References

Boley A, Müller WR, Haider G (2000) Biodegradable polymers as solid substrate and biofilm carrier for denitrification in recirculated aquaculture systems. Aquac Eng 22:75–85
Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248–254
Buschmann AH, Troell M, Kautsky N, Kautsky L (1996) Integrated tank cultivation of salmonids and Gracilaria chilensis (Gracilariaceae, Rhodophyta). Hydrobiologia 326:75–82
Cao L, Wang W, Yang Y et al (2007) Environmental impact of aquaculture and countermeasures to aquaculture pollution in China. Environ Sci Pollut R 14:452–462.
Chen BB, Zou DH, Ma JH (2016) Interactive effects of elevated CO2 and nitrogen-phosphorus supply on the physiological properties of Pyropia haitanensis (Bangiales, Rhodophyta). J Appl Phycol 28:1235–1243
Chopin T, Gallant T, Davison I (1995) Phosphorus and nitrogen nutrition in Chondrus crispus (Rhodophyta): effects on total phosphorus and nitrogen content, carrageenan production, and photosynthetic pigments and metabolism. J Phycol 31:283–293
Chow F, Oliveira MC (2008) Rapid and slow modulation of nitrate reductase activity in the red macroalga Gracilaria chilensis (Gracilariaceae, Rhodophyta): influence of different nitrogen sources. J Appl Phycol 20:775–782
Crawford NM (1995) Nitrate: nutrient and signal for plant growth. Plant Cell 7:859–868
Davison IR (2004) Environmental effects on photosynthesis. J Temperature. J Phycol 27:2–8
Dawes CJ, Koch EW (1990) Physiological responses of the red algae Gracilaria verrucosa and G. tikvahiae before and after nutrient enrichment. B Mar Sci 46:335–344
Feller U, Crafts-Brandner SJ, Salvucci ME (1998) Moderately high temperatures inhibit ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) activase-mediated activation of Rubisco. Plant Physiol 116:539–546
Frances J, Allan GL, Nowak BF (1998) The effects of nitrite on the short-term growth of silver perch (Bidyanus bidyanus). Aquaculture 163:63–72
Gao QF, Cheung K, Cheung SG et al (2005) Effects of nutrient enrichment derived from fish farming activities on macroinvertebrate assemblages in a subtropical region of Hong Kong. Mar Pollut Bull 51:994–1002
Gao G, Zhong ZH, Zhou XH, Xu JT (2016) Changes in morphological plasticity of Ulva prolifera under different environmental conditions: a laboratory experiment. Harmful Algae 59:51–58
Guillard RRL, Ryther JH (1962) Studies of marine planktonic diatoms. I. Cyclotella nana Hustdevt, and Detonula confervacea (Cleve) Gran. Can J Microbiol 8:229–239
Humphrey GF (1975) The photosynthesis: respiration ratio of some unicellular marine algae. J Exp Mar Biol Ecol 18:111–119
Huovinen P, Matos J, Pinto IS, Figueroa FL (2006) The role of ammonium in photoprotection against high irradiance in the red alga Grateloupia lanceolata. Aquat Bot 84:308–316
Jiménez del Rio M, Ramazanov Z, García Reina G (1995) Effects of nitrogen supply on photosynthesis and carbonic anhydrase activity in the green seaweed Ulva rigida (Chlorophyta). Mar Biol 123:687–691
Kaspar HF, Gillespie PA, Boyer IC, MacKenzie AL (1985) Effects of mussel aquaculture on the nitrogen cycle and benthic communities in Kenepuru Sound, Marlborough Sounds, New Zealand. Mar Biol 85:127–136

Kessing JK, Liu DY, Feamis P, Garcia R (2011) Inter-and intra-annual patterns of Ulva prolifera green tides in the Yellow Sea during 2007-2009, their origin and relationship to the expansion of coastal seaweed aquaculture in China. Mar Pollut Bull 62:1169–1182

Klaoudatos SD, Klaoudatos DS, Smith J et al (2006) Assessment of site specific benthic impact of floating cage farming in the eastern Hios Island, Eastern Aegean Sea, Greece. J Exp Mar Biol Ecol 338:96–111

Lincoln T, Eduardo Z (2010) Plant physiology. Oxford University Press, US

Liu Y, Zhou Y, Yang HS et al (2005) Growth characters and photosynthetic capacity of Gracilaria lemaneiformis as a biofilter in a shellfish farming area in Sanggou Bay, China. J Appl Phycol 17:199–206

Lobban CS, Harrison PJ (1997) Seaweed ecology and physiology. Cambridge University Press, UK

Marinho-Soriano E, Nunes SO, Carneiro MAA, Pereira DC (2009) Nutrients’ removal from aquaculture wastewater using the macroalgae Gracilaria birdiae. Biomass Bioenergy 33:327–331

Mook WT, Chakrabarti MH, Aroua MK, Khan GMA, Ali BS, Islam MS, Abu Hassan MA (2012) Removal of total ammonia nitrogen (TAN), nitrate and total organic carbon (TOC) from aquaculture wastewater using electrochemical technology: a review. Desalination 285:1–13

Murata N, Takahashi S, Nishiyama Y, Allakhverdiev SI (2007) Theoretical determination of marine-plant pigments, with revised equations for ascertaining chlorophylls and carotenoids. J Biolog Enegmetics 1767:414–421

Navarro MT, Prieto R, Fernandez E, Galvan A (1996) Constitutive expression of nitrate reductase changes the regulation of nitrate and nitrate transporters in Chlamydomonas reinhardtii. Plant J 9:819–827

Neori A, Chopin T, Troell M, Buschmann AH, Kraemer GP, Halling C, Young EB, Berges JA, Dring MJ (2009) Physiological responses of intertidal marine brown algae to nitrogen deprivation and resupply of nitrate and ammonium. Physiol Plantarum 135:400–411

Parsons TR, Strickland JDH (1963) Discussion of spectrophotometric determination of marine-plant pigments, with revised equations for ascertaining chlorophylls and carotenoids. J Mar Res 21:155–163

Petersson R, McDonald AJ (1994) Effects of nitrogen supply on the acclimation of photosynthesis to elevated CO2. Photosynth Res 39:389–400

Porra RJ (2002) The checked history of the development and use of simultaneous equations for the accurate determination of chlorophylls a and b. Photosynth Res 73:149–156

Przytocka-Jusiak M, Duszota M, Matusiak K, Mycielski R (1984) Intensive culture of Chlorella vulgaris/AA as the second stage of land-based culture of fish, bivalves and seaweeds. Aquaculture 254:248–255

Ridha MT, Cruz EM (2001) Effect of biofilter media on water quality and biological performance of the Nile tilapia Oreochromis niloticus L. reared in a simple recirculating system. Aquac Eng 24:157–166

Ross ME, Davis K, McColl R, Stanley MS, Day JG, Semiao AJC (2018) Nitrogen uptake by the macro-algae Cladophora coelothrix and Cladophora parrauidii: influence on growth, nitrogen preference and biochemical composition. Algal Res 30:1–10

Schreiber U (2004) Pulse-amplitude-modulation (PAM) fluorometry and saturation pulse method: an overview. In: Papageorgiou GC, Govindjee (eds) chlorophyll a fluorescence. Advances in photosynthesis and respiration. Springer, Dordrecht, pp 279–319

Schreiber U, Bilger W, Neubauer C (1995) Chlorophyll fluorescence as a noninvasive indicator for rapid assessment of in vivo photosynthesis. In: Schulze ED, Caldwell MM (eds) Ecophysiology of photosynthesis. Springer, Berlin, Heidelberg, pp 49–70

Shipgel M, Neori A, Popper DM, Gordin H (1993) A proposed model for “environmentally clean” land-based culture of fish, bivalves and seaweeds. Aquaculture 117:115–128

Smit AJ, Robertson BL, du Preez DR (1997) Influence of ammonium-N pulse concentrations and frequency, tank condition and nitrogen starvation on growth rate and biochemical composition of Gracilaria gracilis. J Appl Phycol 8:473–481

Smith VH, Tilman GD, Nekola JC (1999) Eutrophication: impacts of excess nutrient inputs on freshwater, marine, and terrestrial ecosytems. Environ Pollut 100:179–196

Tango MS, Gagnon GA (2003) Impact of ozonation on water quality in marine recirculation systems. Aquac Eng 29:125–137

Tett P, Gowen R, Mills D, Fernandes T, Gilpin L, Huxham M, Kennington K, Read P, Service M, Wilkinson M, Malcolm S (2007) Defining and detecting undesirable disturbance in the context of marine eutrophication. Mar Pollut Bull 55:282–297

Thomas RJ, Hipkin CR, Syrett PJ (1976) The interaction of nitrogen assimilation with photosynthesis in nitrogen deficient cells of Chlorella. Planta 133:9–13

Valllela I, Liu DY, Lloret J, Chenoweth K, Hanacek D (2018) Stable isotopic evidence of nitrogen sources and C4 metabolism driving the world’s largest macroalgal green tides in the Yellow Sea. Sci Rep-UK 8:17437

Van Alstyne KL (2018) Seawater nitrogen concentration and light independently alter performance, growth, and resource allocation in the bloom-forming seaweeds Ulva lactuca and Ulvaria obscura (Chlorophyta), Harmful Algae 78:27–35

Vona V, Rigano VDM, Lobosco O et al (2004) Temperature responses of growth, photosynthesis, respiration and NADH: nitrate reductase in cryophylic and mesophylic algae. New Phyto:163:325–331

Weis E (1981) Reversible heat inactivation of the Calvin cycle: a possible mechanism of the temperature regulation of photosynthesis. Planta 151:33–39

Xia JR, Gao KS (2005) Impacts of elevated CO2 concentration on biochemical composition, carbonic anhydrase, and nitrate reductase activity of freshwater green algae. J Integr Plant Biol 47:668–675

Xu ZG, Wu HY, Zhan DM, Sun FX, Sun JZ, Wang GC (2014) Combined effects of light intensity and NH4+-enrichment on growth, pigmentation, and photosynthetic performance of Ulva prolifera (Chlorophyta). Chin J Oceanol Limnol 2:1016–1023

Xu ZG, Gao G, Xu JT, Wu HY (2017) Physiological response of a golden tide alga (Sargassum muticum) to the interaction of ocean acidification and phosphorus enrichment. Biogeosciences 14:671–681

Yang YF, Fei XG, Song JM, Hu HY, Wang GC, Chung JK (2006) Growth of Gracilaria lemaneiformis under different cultivation conditions and its effects on nutrient removal in Chinese coastal waters. Aquaculture 254:248–255

Young EB, Berges JA, Dring MJ (2009) Physiological responses of intertidal marine brown algae to nitrogen deprivation and resupply of nitrate and ammonium. Physiol Plantarum 135:400–411

Yu J, Yang YF (2008) Physiological and biochemical response of seaweed Gracilaria lemaneiformis to concentration changes of N and P. J Exp Mar Biol Ecol 367:142–148

Zheng MS, Lin JJ, Zhou SD, Zhong J, Li Y, Xu N (2019) Salinity mediates the effects of nitrogen enrichment on the growth, photosynthesis, and biochemical composition of Ulva prolifera. Environ Sci Pollut Res 26:19982–19990

Zhong ZH, Liu ZY, Zhuang LC et al (2020) Effects of temperature on photosynthetic performance and nitrate reductase activity in vivo assay in Gracilariotopsis lemaneiformis (Rhodophyta)*. J Oceanol Limnol 39:362–371
Zhu M, Liu ZP, Shao HB, Jin Y (2016) Effects of nitrogen and phosphate enrichment on the activity of nitrate reductase of *Ulva prolifera* in coastal zone. Acta Physiol Plant 38:169

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