



Response of energy microalgae *Chlamydomonas reinhardtii* to nitrogen and phosphorus stress

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Abstract

Microalgae can effectively absorb nitrogen (N) and phosphorus (P) in wastewater, while growth characteristics can be affected by such nutrients. The influences of the N and P concentration on growth, biomass yield, protein yield, and cell ultrastructure of *Chlamydomonas reinhardtii* (*C. reinhardtii*) were investigated in this study. The results showed that, in the optimum conditions (24–72 mg/L for N and 4.5–13.5 mg/L for P), the final biomass and protein content of *C. reinhardtii* could reach maximum value, and the cell organelles (chloroplast, mitochondria, etc.) showed good structures with larger chloroplasts, and more and neater thylakoids. However, if the concentration of nutrients was much higher or lower than the optimal value, it would cause adverse effects on the growth of *C. reinhardtii*, especially in high nitrogen (1000 mg/L) and low phosphorus (0.5 mg/L) conditions. Under these extreme conditions, the ultrastructure of the cells was also damaged significantly as follows: the majority of the organelles were deformed, the chloroplast membrane became shrunken, and the mitochondria became swollen, even partial disintegrated (differing slightly under high-N and low-P conditions); furthermore, it is found that *C. reinhardtii* was more sensitive to low-P stress. On the basis of these results, our findings have general implications in the application of wastewater treatment.

Keywords *Chlamydomonas reinhardtii* · Environmental stress · Nitrogen · Phosphorus · Removal efficiency · Ultrastructure

Introduction

Nowadays, eutrophication caused by nitrogen and phosphorus from wastewater treatment plant effluents frequently occurs. The development of effective and practical technologies for nitrogen and phosphorus removal has received tremendous interest, and the key point lies in exploring and studying biological species which have strong absorption and degradation capability of nitrogen and phosphorus.

Algae, as a kind of potential renewable resource, are not only used for biofuels (Suganya et al. 2016) but also for human health, animal and aquatic nutrition, environmental

applications such as CO₂ mitigation (Nordlander et al. 2017; Sarat Chandra et al. 2016), wastewater treatment (Phuong Thu and Michele 2016; Wang et al. 2016), biofertilizers (Doğan-Subaşı and Demirer 2016), high-value compounds (Chew et al. 2017), etc. Although the idea of using microalgae for wastewater treatment is not new, it has received renewed attention recently in the search for sustainable energy. The use of microalgae in wastewater treatment can not only remove nitrogen (N) and phosphorus (P) from wastewater, but also recycle these nutrients in biomass that can be converted into energy, raw chemicals, or other products (Mata et al. 2010). The use of this technology is also driven by microalgal biomass generation, which may be suitable for conversion into biofuel and supportive of the development of sustainable environmental solutions (Chisti and Yan 2011; Prajapati et al. 2013). As a kind of energy microalgae, *Chlamydomonas reinhardtii*, known as “photosynthetic yeast”, is used as a model organism due to a number of advantages such as its short growth period, fast growth rate, simple cultivation mode, and high photosynthetic efficiency (Siaut et al. 2011), which make it have very broad application potential.

Till now, factors that impact on *C. reinhardtii*'s removal efficiency of N and P have been investigated. It has been

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reported that, with L/D ratios of 12 h:12 h, 100% removal of $\text{NH}_4^+\text{-N}$ and TP could be achieved by *C. reinhardtii*. The optimal pH range for *C. reinhardtii* to remove $\text{NH}_4^+\text{-N}$ and TP was 6–7, and after immobilization, the ability of *C. reinhardtii* to remove $\text{NH}_4^+\text{-N}$ was significantly enhanced (Deng et al. 2010). However, such studies mainly focused on the effect of temperature, algal density, illumination, pH, and cell immobilization. Few studies on the physiological characteristics of *C. reinhardtii* under different N and P concentrations, and its tolerance to N and P, have been reported.

This work aimed at studying the influence of N and P concentrations on the growth characteristics of *C. reinhardtii*. By using the WC medium, which was enriched N and P, separately or simultaneously in different concentration gradients, the growth rate, protein production, and the ultrastructure of *C. reinhardtii* under different nutrition conditions were investigated. The optimum concentration of TN, TP, and the N&P mixed concentration for the growth of *C. reinhardtii* was found. The response of *C. reinhardtii* to enriching N and P individually or simultaneously was analyzed, which could provide a scientific basis for interpreting bioremediation mechanism of *C. reinhardtii*.

Materials and methods

Microalgae culture

The *C. reinhardtii* used in this study was provided by the College of Life Sciences, Sichuan University.

The culture medium of microalgae was WC medium, in which TN and TP concentration was 14 and 1.55 mg/L, respectively. And the composition of the WC medium was showed in Table 1.

Table 1 Composition of the WC medium

| Components | Concentration (g/L) | Volume (mL) |
|---|---------------------|-------------|
| 1. $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ | 36.80 | 1 |
| 2. $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ | 37.00 | 1 |
| 3. NaHCO_3 | 12.60 | 1 |
| 4. $\text{K}_2\text{HPO}_4 \cdot 3\text{H}_2\text{O}$ | 11.40 | 1 |
| 5. NaNO_3 | 85.00 | 1 |
| 6. Trace elements | | |
| Na_2EDTA | 4.36 | 1 |
| $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ | 3.15 | |
| $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ | 0.01 | |
| $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ | 0.022 | |
| $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ | 0.01 | |
| $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$ | 0.18 | |
| NaMoO_4 | 0.006 | |
| H_3BO_3 | 1.00 | |

Pre-culture

A 50-mL conical flask (in total ten flasks) was used as a reactor, and was filled with 20 mL of WC medium which contained 10% (V/V) *C. reinhardtii* as inoculum. The conditions were controlled as follows: temperature of 25 ± 1 °C, light intensity of 2000 lx, light period of 12L:12D, and statically cultured. Daily exchanging the conical flask positions and artificially shaking it to ensure microalgae were suspended in the medium evenly. For the logarithmic growth period of *C. reinhardtii*, we chose the optimal liquid group as the inoculum of next experiment.

Experimental program

(1) Enriching N and P separately Based on the initial WC medium, we enriched the TN concentration, kept the initial TP concentration (1.55 mg/L) stable and enriched the TN concentration with 5, 14, 24, 72, 216, and 1000 mg/L respectively. To enrich the TP concentration, we kept the initial TN concentration (14 mg/L) stable and enriched the TP concentration with 0.5, 1.55, 4.5, 13.5, 40.5, and 200 mg/L, respectively. A TN concentration of 14 mg/L (and a TP concentration of 1.55 mg/L) was set as the control group. Each group was conducted in triplicates.

(2) Enriching N and P simultaneously Based on the WC medium, enriching TN and TP simultaneously (reference to the Redfield ratio as N: P = 16:1 (Redfield 1960)) was carried out. Generally, the absorption of N&P is consistent with the Redfield ratio, that is, in accordance with the 16:1 microalgae absorb N&P in the media, and that was why we chose this ratio. The concentrations of N:P in the experimental group were set as: 12:0.75, 24:1.5, 48:3, 72:4.5, 96:6, 960:60 mg/L, with a control group using WC as the medium (TN 14 mg/L, TP 1.55 mg/L). Each group was conducted in triplicates.

Both 150- and 250-mL conical flasks were used, filled with 50 mL culture medium and 50 μL inoculum, and 130 mL of culture medium and 50 μL inoculum, respectively. And the biomass concentration of *C. reinhardtii* in the inoculum was 460.46 mg/L. The culture conditions were: temperature 25 ± 1 °C, light intensity 2000 lx, light period 12L:12D, and statically cultured. Daily changing of the conical flask position and shaking the flask artificially enabled the microalgae to be uniformly suspended in the culture medium. The experimental period was 17 days.

To ensure the nutrient supply of microalgae cultured in, the 150-mL conical flask was adequate with a 10-mL culture solution (20% of the microalgae metabolic capacity), and was replaced with 10 mL of the original medium every other day. The removal cell solution (cultured 3, 5, 7, 9, 11, 13, 15, and 17 days) was used to determine the algae OD_{750} (UV spectrophotometric, Alpha-1506 type), microalgae protein content

(Coomassie brilliant blue G-250), and other indicators. For the 250-mL conical flask samples, algal samples were collected on days 3, 5, 7, 9, 11, 13, 15, and 17 (eight samples). Determining the TN and TP of samples was by only taking the algae liquid, but not adding the original conical flask culture medium.

Growth parameters determination

Determination of biomass and specific growth rate (μ_m)

C. reinhardtii algae in the liquid stayed in logarithmic growth and was diluted tenfolds by deionized water before determination. The absorbance of the diluted solution was determined at a wavelength of 750 nm in order to measure OD₇₅₀ (Harris 1989). Then, they were centrifuged (about 6000 g) for 5 min, and the supernatant was wiped off. The algal cells were deposited at 80 °C to a constant weight, and the dry weight was measured after being naturally cooled. The regression equation of *C. reinhardtii*'s dry weight (W_{dry}) and OD₇₅₀ was established, as shown in Eq. (1).

The OD₇₅₀ of the algae liquid in the 150-mL conical flask was also determined. The biomass of *C. reinhardtii* (W_{dry}) was calculated using Eq. (1), and the specific growth rate (μ_m) was calculated using Eq. (2), where μ_m = specific growth rate (d^{-1}), X_1 and X_2 are the biomass concentrations of the cells (unit: mL^{-1} or $mg L^{-1}$) at time t_1 (d) and t_2 (d) (Shi and Li 2014).

$$W_{dry}(g/L) = 0.846030 D_{750} - 0.0025575, R^2 = 0.9992 \quad (1)$$

$$\mu_m = \frac{\ln X_2 - \ln X_1}{t_2 - t_1} \quad (2)$$

Determination of N and P removal efficiency

The experiment lasted 17 days, and concentrations of TN and TP in the 250-mL experimental group were determined, respectively, on the last day. TN and TP were determined according to the Chinese National Standards (HJ636-2012 (2012) and GB11893-89 (1989), respectively). The removal efficiency of TN and TP was calculated using Eq. (3), where r = removal efficiency, C_0 and C_t are the concentrations of TN and TP at culture times 0 and t , respectively.

$$r = \frac{C_0 - C_t}{C_0} \times 100\% \quad (3)$$

Determination of protein yield

The protein extraction from fresh thalli was performed as described by Hurkman and Tanaka (1986). One gram of *C. reinhardtii* was homogenized in 20 mL of extraction buffer

[0.5 M Tris–HCl (pH 8.0) containing 0.7 M sucrose, 50 mM ethylenediaminetetraacetic acid (EDTA), 0.1 M KCl, 2% (v/v) 2-mercaptoethanol, and 2 mM phenylmethylsulfonyl fluoride]. After incubation for 10 min at 4 °C, an equal volume of 1 M Tris–HCl (pH 7.5)-saturated phenol was added. The sample was shaken for 10 min and centrifuged at 5000 g for 30 min. The phenol and middle phases were recovered and reextracted with an equal volume of the extraction buffer. Proteins were precipitated by addition of five volumes of 0.1 M ammonium acetate in methanol and incubated at –20 °C overnight. After centrifugation at 13,000 g for 30 min, the precipitate was washed again with the ammonium acetate in methanol and acetone. The pellet obtained was dried and resuspended in 6 mL of lysis buffer [125 mM Tris–HCl (pH 6.8) containing 9 M urea, 4% (v/v) 10% triton X-100, 5% (v/v) 2-mercaptoethanol, and 2% (v/v) of 40% ampholine]. The total protein concentration was then determined by the method of Bradford (1976) using bovine serum albumin as the standard. The results indicated the protein content of the microalgae (by fresh weight).

Ultrastructure observation methods

Changes in the ultrastructure of *C. reinhardtii* were determined by using transmission electron microscopy (TEM). After treatment with different concentrations of N and P for 17 days, samples from the same part of the species were fixed with 2.5% (V/V) glutaraldehyde and 1% (W/V) osmium tetroxide and then dehydrated through an ethanol series and embedded in Spur resin. Ultra-thin sections were cut using a diamond knife and a microtome (LEICA EM6/UC6). The sections were stained with uranyl acetate and lead citrate and examined with a transmission electron microscope (TECNAI G2 F20TEM) (Yu 2004).

Statistical analysis

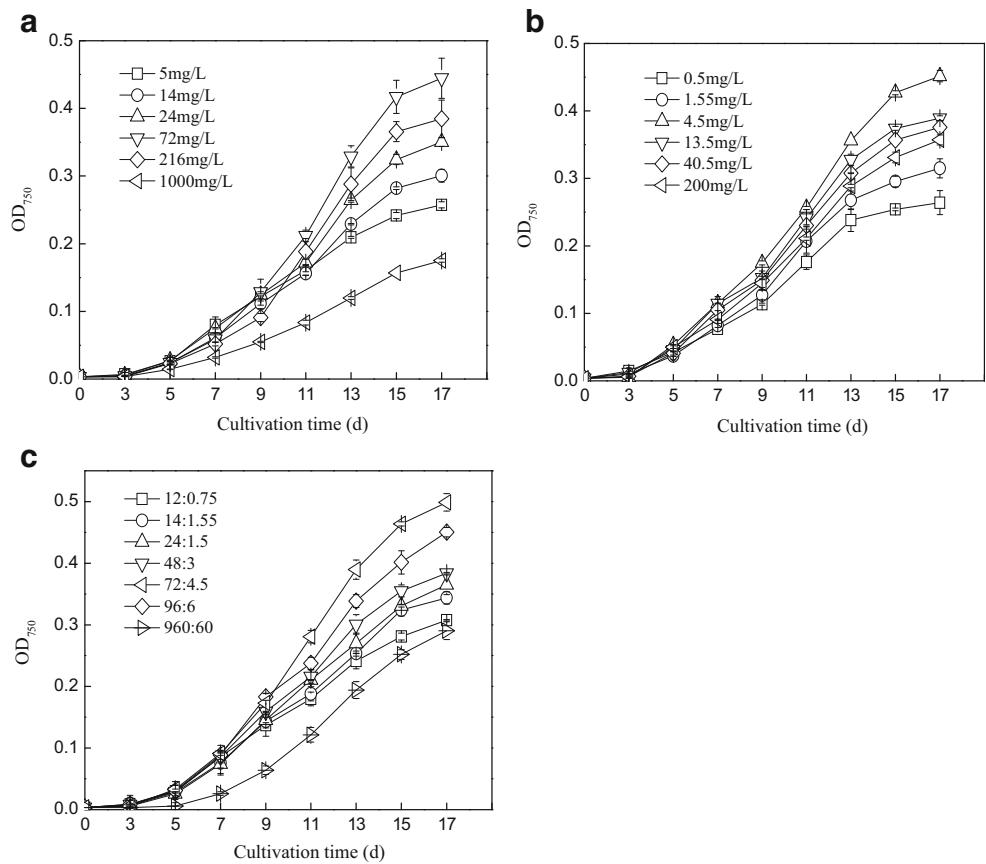
Statistical analyses were done with the software SPSS version 16.0 for Windows. All data are reported as the means (standard deviation) of three replicates. A value of $P < 0.05$ was considered statistically significant.

Results and discussion

Effects of different N and P on the growth of *C. reinhardtii*

Figure 1a illustrates that when enriching N, the group in which TN concentration was 72 mg/L showed the highest growth. At the end of the experiment, the OD₇₅₀, final biomass (Table 2) and μ_m (Table 3) reached 0.45, 367.89 mg/L, and 0.43 d^{-1} , respectively. The 1000 mg/L TN group was always the worst

Fig. 1 OD₇₅₀ of *C. reinhardtii* at different conditions: **a** enriching N only and the TP concentration was stabilized at 1.55 mg/L; **b** enriching P only and the TN concentration was stabilized at 14 mg/L; **c** enriching N and P simultaneously with the N:P ratio at 16:1



one, which indicated that the growth of microalgae was significantly inhibited in this group, the algal cells absorbed TN excessively, and was not conducive to the algal cell division (Song et al. 2010; Wang et al. 2011). Under this situation, the algal biomass was much lower than in the other groups.

When enriching P, the growth curve which corresponded to the group of 4.5 mg/L TP was the highest among all ($P < 0.05$), and the OD₇₅₀ value (Fig. 1b) and the final biomass (Table 2) was 0.45 and 373.56 mg/L, respectively. And it was also pretty

good for the 13.5 mg/L group. Among all experimental groups, the growth curve of the TP concentration of 0.5 mg/L was the lowest and even worse than the 200 mg/L group. This might be because that the 0.5 mg/L TP group was the P-limited group ($N:P = 28$). The phosphorus limitation stress led to ATP (adenosine triphosphate) synthesis, restricting the activity of H⁺-ATP enzyme, low cell metabolic activity, so the growth was constrained (Levy and Gantt 1990; Rasdi and Qin 2015; Feng et al. 2008). Thus, this group of *C. reinhardtii* exhibited a growth inflection

Table 2 Biomass of *C. reinhardtii* under different conditions: (A) enriching N only with the TP concentration stabilized at 1.55 mg/L; (B) enriching P only with the TN concentration stabilized at 14 mg/L; (C) enriching N and P simultaneously with the N:P ratio at 16:1. Results shown are averages and (standard deviation)

| A | TN concentration (mg/L) | | | | | | |
|----------------|-------------------------|----------------|---------------|---------------|----------------|---------------|---------------|
| | 5.00 | 14.00 | 24.00 | 72.00 | 216.00 | 1000.00 | -- |
| Biomass (mg/L) | 212.00 (3.75) | 247.90 (7.91) | 289.30 (5.41) | 367.89 (15.0) | 317.89 (13.33) | 143.37 (2.08) | -- |
| B | TP concentration (mg/L) | | | | | | |
| | 0.50 | 1.55 | 4.50 | 13.50 | 40.50 | 200.00 | -- |
| Biomass (mg/L) | 217.42 (15.0) | 259.80 (12.09) | 373.56 (7.08) | 321.78 (2.91) | 310.33 (0.0) | 294.90 (6.25) | -- |
| C | TN:TP ratio | | | | | | |
| | 12:0.75 | 14:1.55 | 24:1.5 | 48:3 | 72:4.5 | 96:6 | 960:60 |
| Biomass (mg/L) | 253.70 (4.12) | 283.90 (3.41) | 301.60 (2.16) | 317.30 (5.27) | 389.40 (13.11) | 372.80 (7.31) | 239.40 (2.69) |

The symbol of “--” represents the experiment which was not conducted

Table 3 μ_m of *C. reinhardtii* under different conditions: (A) enriching N only with TP concentration stabilized at 1.55 mg/L; (B) enriching P only with TN concentration stabilized at 14 mg/L; (C) enriching N and P simultaneously with the N:P ratio at 16:1

| A | | TN concentration (mg/L) | | | | | | |
|----------------------------|--|-------------------------|---------|--------|--------|--------|---------|--------|
| | | 5.00 | 14.00 | 24.00 | 72.00 | 216.00 | 1000.00 | -- |
| μ_m (d ⁻¹) | | 0.3943 | 0.4035 | 0.4126 | 0.4268 | 0.4182 | 0.3713 | -- |
| B | | TP concentration (mg/L) | | | | | | |
| | | 0.50 | 1.55 | 4.50 | 13.50 | 40.50 | 200.00 | -- |
| μ_m (d ⁻¹) | | 0.3617 | 0.3722 | 0.3936 | 0.3848 | 0.3827 | 0.3797 | -- |
| C | | TN:TP ratio | | | | | | |
| | | 12:0.75 | 14:1.55 | 24:1.5 | 48:3 | 72:4.5 | 96:6 | 960:60 |
| μ_m (d ⁻¹) | | 0.3941 | 0.4007 | 0.4043 | 0.4073 | 0.4207 | 0.4167 | 0.3907 |

The symbol of “--” represents the experiment which was not conducted

point earlier than other groups, and the final biomass was also lower than that of the other groups. All data showed that the growth of *C. reinhardtii* in a phosphorus limited condition was far from that under an adequate phosphorus condition. Compared with the high phosphorus concentration, *C. reinhardtii* might be more sensitive in low phosphorus concentrations.

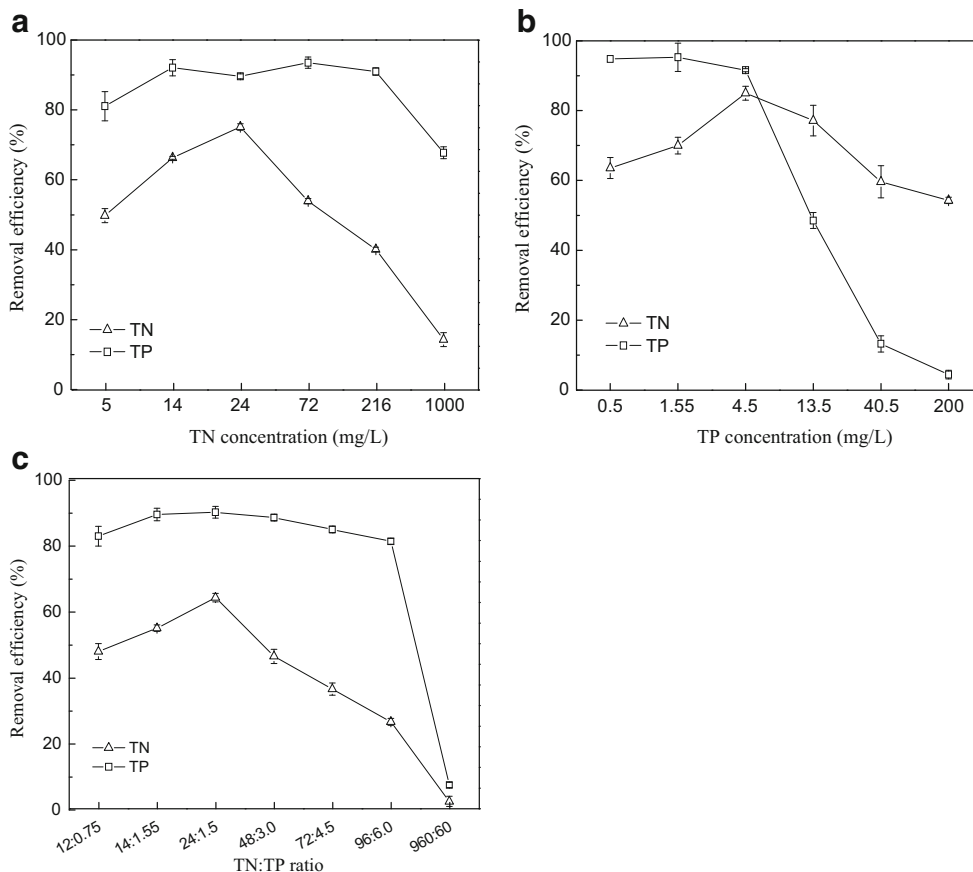
When enriching N and P simultaneously, the final biomass of 72:4.5 group reached 389.4 mg/L, 37.16% higher than the control group (Table 2). The 12:0.75 group was a low nitrogen

and phosphorus group. With the culture time proceeding, *C. reinhardtii* went into the steady growth phase due to nutritional deficiencies, and also had an earlier decreasing phase than the other groups. From the beginning, the OD₇₅₀ value of the 960:60 group was lower than other groups, and the gap in the growth curve was large ($P < 0.05$). Obviously, the growth of *C. reinhardtii* was severely inhibited by levels of high nitrogen and phosphorus, and this situation was more serious than that in the low nitrogen and phosphorus group (the 12:0.75 group). This is because the algae of this typical “high-N and high-P” group was significantly affected by high nitrogen and phosphorus stress. When remaining in a high nitrogen and high phosphorus environment, the quantity of the initial microalgae was insufficient, which indicated that main reason for the poor growth of *C. reinhardtii* was in the medium of high concentration of nitrogen and phosphorus (Li et al. 2010). Table 3 showed that either enriching P only or the N and P simultaneously had little impact on the specific growth rate.

Effects of different N and P on removal efficiency of *C. reinhardtii*

From Fig. 2a, with the TN concentration rising from 5 to 72 mg/L, the removal efficiency of TN and TP were above 50 and 80%, respectively. The highest efficiency of TN was

Fig. 2 N and P removal efficiency by *C. reinhardtii* under different conditions: **a** enriching N only with TP concentration stabilized at 1.55 mg/L; **b** enriching P only with TN concentration stabilized at 14 mg/L; **c** enriching N and P simultaneously with the N:P ratio at 16:1



75.11% in the 24 mg/L group, whereas the efficiency of TP was 89.59%. However, the highest efficiency of TP was 93.51% in the 72 mg/L group where the corresponding efficiency of TN was 53.90%. When enriching P concentration, the removal efficiency of TN ranged from 54.29 to 85%. The highest efficiency of TN appeared in the 4.5 mg/L group, at 91.57% for TP (Fig. 2b).

Combining Table 2 and Fig. 2, it was found that an appropriately high-P concentration and a low-N concentration (compared to the WC medium) are conducive to enhance that the growth of microalgae and the removal efficiency. This illustrates the response of microalgae to high nitrogen and low phosphorus stress. In addition, the removal efficiency of microalgae to P was higher than that of N, and the removal of P was less affected by the concentration of N under the above three conditions. This indicated that the efficiency of P by *C. reinhardtii* was better than that of N, so *C. reinhardtii* was more suitable for the treatment of wastewater with high P concentration. When enriching P, the phenomenon in which the best removal efficiency of N and P appeared in the 4.5 mg/L TP group with the biggest biomass also confirmed this view. When enriching N, the best removal efficiency occurred in the 24 mg/L TN group, where the biomass was relatively high, and the N:P ratio was just close to 16:1, which indicated that the growth of microalgae was in line with the Redfeild ratio.

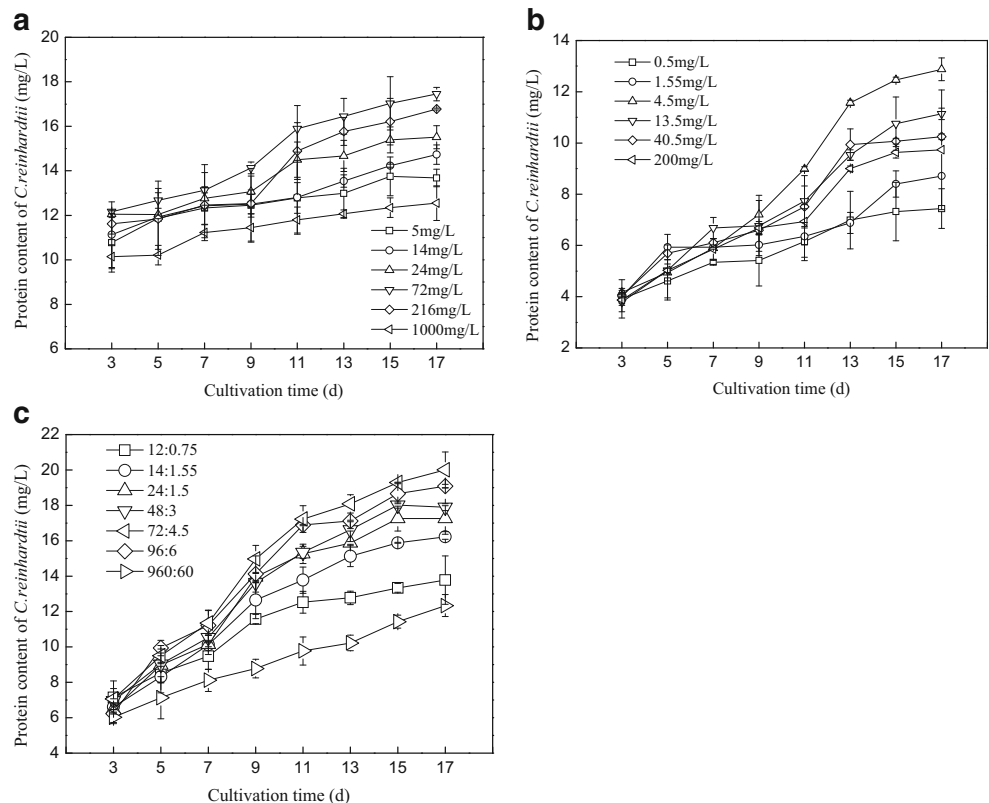
Interestingly, when enriching N and P simultaneously, the best removal efficiency occurred in the 24:1.5 group (Fig. 2c),

with the efficiency for both TN and TP, 64.40 and 90.27%, respectively, and was the same as that under the enriching N condition. However, the biomass of this group was not very high. The removal efficiency of high concentration group (960:60) was really low, only 2.60 and 7.58% for TN and TP, respectively. It was also found that the efficiency of TP always stayed around 85% except for the 960:60 group. Thus, considering the growth of microalgae and the removal efficiency of nitrogen and phosphorus, 24–72 mg/L TN and 4.5–13.5 mg/L TP were the best concentration ranges for *C. reinhardtii*.

Effects of different N and P on the protein content in *C. reinhardtii*

In the experimental period, with different concentrations of N enriched culture, the protein content of each group rose gradually with the culture time (Fig. 3a), but the difference between each group was not very significant. The condition of protein synthesis in the 72 mg/L group was the best, and the final protein content was 17.45 mg/L, whereas the protein content of the 24 mg/L group was 15.51 mg/L which was second only to the best group in the first 10 days of culture. The protein content of the 5 and 1000 mg/L groups were both lower than that of the control group (TN concentration of 14 mg/L), and the final protein content was 13.68 and 12.55 mg/L, respectively. And the 5 mg/L TN group was not

Fig. 3 Protein content of *C. reinhardtii* under different conditions: **a** enriching N only with TP concentration stabled at 1.55 mg/L; **b** enriching P only with TN concentration stabled at 14 mg/L; **c** enriching N and P simultaneously with the N:P ratio at 16:1



high as it probably was a N restriction group (N: $P < 5$). As culture time went on, *C. reinhardtii* grew in nutrient deficient medium, and its physiological activities could not normally proceed. The algae protein content and other inclusions decreased, and cell division slowed down or was blocked, which resulted in a low growth rate (Li et al. 2011). The 1000 mg/L TN group showed a similar result, likely due to the serious imbalance of N:P and high nitrogen concentration (serious phosphorus limitation). In such a nurturing environment, algal cell division was strongly inhibited, which led to low biomass, and low protein content.

From Fig. 3b, when the TP concentration was in the range of 0.5–4.5 mg/L, the algal protein content constantly rose with the increase of TP concentration ($P < 0.05$). The protein content was the highest when the TP concentration was 4.5 mg/L, and reached to 12.88 mg/L. When the TP concentration was more than 4.5 mg/L, the protein content decline with the increasing concentration of TP. Previous studies have shown that P is the main component of the nucleic acids, proteins, and phospholipids of microalgae cells, which was also involved in the growth of the algae in the process of metabolism, playing an important role in the growth of microalgae (Li 2003). When the algae grew in a low phosphorus environment, the algae could not get enough phosphorus nutrition which led to less biomass and protein synthesis. Therefore, the protein content of the phosphorus limited group was the lowest among all the experimental groups.

Combining Fig. 3 and Table 2, the relationship between the protein content of each group, the size of the biomass, and growth trends were positively correlated. The two plots showed that the rapid increase period of the protein content (from the beginning of the 9th day) lagged behind the biomass accelerated growth period (from the beginning of the 5th day). This was because the algae absorbed the medium nutrition and grew rapidly. After the nutrient was consumed completely, protein was able to be synthesized.

From Fig. 3c, the “high nitrogen and phosphorus (960:60) group” appeared at the lowest level. The final protein content of *C. reinhardtii* was 12.34 mg/L. The protein of the other groups greatly increased on the 7th day, and then they increased gradually to the respective “stationary phase” at different times. The protein content of the low nitrogen and phosphorus (12:0.75) group tended to be stable after the 11th day, thus, its final protein content was 13.78 mg/L, which was just higher than that of the 960:60 group. The protein level of other groups kept changing till to the 15th day. The 72:4.5 group was not always at the top of the curves; it was roughly higher than the other groups from the 9th day, and the final protein content was 20.01 mg/L, 23.28% higher than the control group. Moreover, this work showed that when the N: P ratio was fixed at an appropriate value, changing the concentration of N and P could also affect the protein synthesis of the microalgae. When the ratio of the concentration of nitrogen

and phosphorus was in correct range, increasing the concentration of N and P could promote a protein synthesis. However, when the concentration exceeded the optimum, an increase would inhibit the growth of algae and restrict the synthesis of protein.

Effects of different N and P on the ultrastructure in *C. reinhardtii*

When the concentration of TN was 72 mg/L, both the chloroplast and mitochondrial showed good structure (Fig. 4c). Compared with control group (Fig. 4a, Fig. 4b), algal cells of this group had larger chloroplasts and clearer thylakoids. The amount of well-formed mitochondria grew in the space between the plasma membrane and the chloroplast. It was inferred that enriching the N concentration appropriately might promote the structure growth of the chloroplast and the mitochondria, which contributed to the growth of *C. reinhardtii*. The observed results of using electron microscopy further confirmed the conclusion that the ultrastructure of *C. reinhardtii* was consistent with its growth characteristics under different N conditions. However, when the concentration of TN increased to 1000 mg/L (Fig. 4d), the organelle ultrastructure in *C. reinhardtii* was obviously damaged: internal shrinkage caused deformation of chloroplast membrane, while the thylakoid lamellae were clear and did not show layer differentiation; the mitochondria became swollen, with cavitation and rupture of the membranes, and some of the mitochondria were even partially disintegrated and dissolved; the cytoderm and cytomembrane showed plasmolysis; the cytoplasmic stretched into the protein pith (electron dense region in the center), and the starch sheath of the protein core periphery was broken, which showed extremely high concentration of TN could directly inhibit the respiratory function of the mitochondria and photosynthesis of the chloroplast. *C. reinhardtii* adapted to the stress of the high concentration of nitrogen by increasing the volume of mitochondria. Thus, the mitochondrial structure was damaged more seriously, and the electron transport system and oxidative phosphorylation circulating in cells were inhibited. The respiratory function of the cells was weakened, and energy conversion was destroyed, which affected the growth of *C. reinhardtii*. Therefore, it can be inferred that mitochondria are more sensitive to a high concentration of N than the chloroplasts. With a high N concentration, plasmolysis appeared as *C. reinhardtii*'s adaptive response. As a result, the algae, which evolved in the process of long-term adaptation of the high concentration of N, form a protective inhibition to resist, or weaken the damage of the organelles caused by high concentration of N deep inside the cell.

In the 4.5 mg/L TP environment, the *C. reinhardtii*'s cells grew well (Fig. 4e). Compared with control group, cells in this group had better chloroplast which accounted for most of the

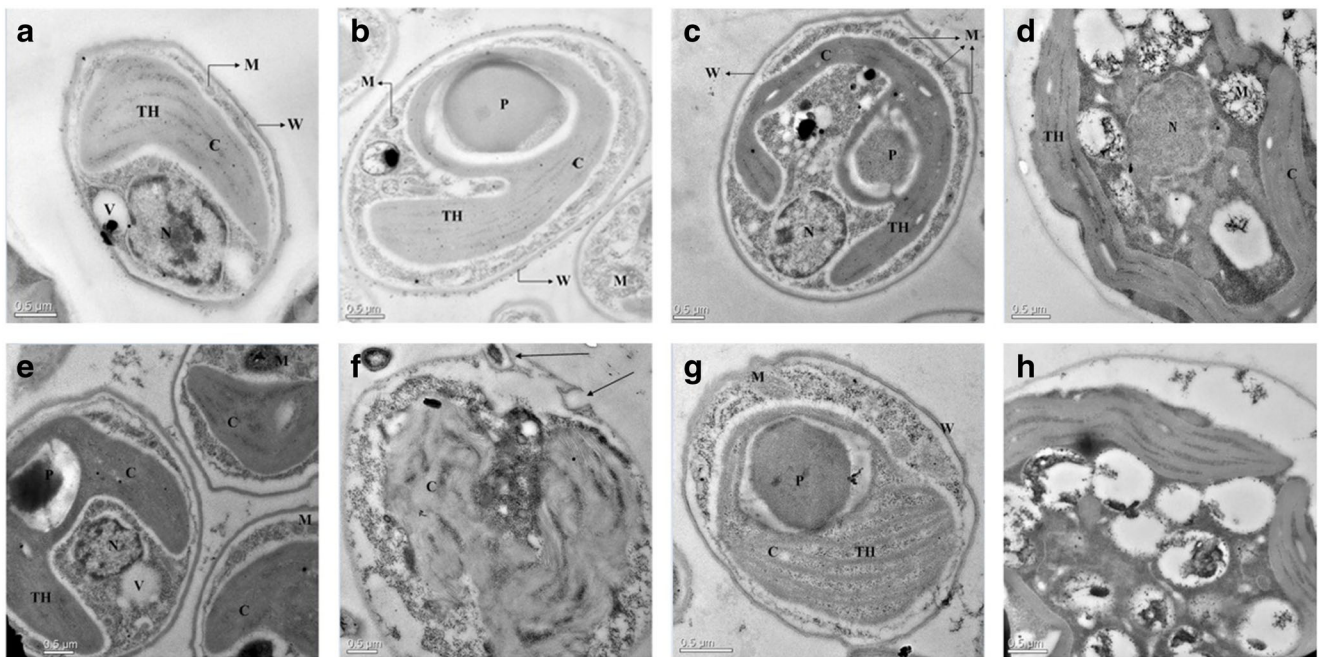


Fig. 4 The ultrastructure of *C. reinhardtii* under different conditions: **a, b** are the control groups (14 mg/L TN, 1.55 mg/L TP); **c, d** are the enriching N groups (keeping TP at 1.55 mg/L) and the TN concentrations are 72 and 1000 mg/L, respectively; **e, f** are the enriching P groups (keeping TN at 14 mg/L) and the TP concentrations are 4.5 and 0.5 mg/L, respectively;

g, h are the enriching N and P groups (keeping the N:P ratio at 16:1) and the ratios are 72:4.5 and 960:60, respectively. C, chloroplast; M, mitochondrion; N, nucleus (nuclear); P, pyrenoid; TH, thylakoid; V, vacuole; W, cell wall

volume of cells, with the chloroplasts consisting of a large number of thylakoids which were clear and neat. This showed that well-grown chloroplasts could be a good basic for the photosynthesis of *C. reinhardtii*. The situation of mitochondria was similar to the control group, and it could be inferred that chloroplasts were more sensitive to TP than mitochondria. Figure 4f showed that the cells were damaged, and the majority of the organelles were deformed. Only chloroplasts could be identified clearly. The chloroplast membrane became fuzzy, while the thylakoid was disordered, blurred, and even dissolved. This confirmed that when other groups were in the logarithmic growth phase, the line of the experimental group (TP 0.5 mg/L) moved into the growth inflection point, or entered the death period. This also explained that, in the growth curve in Fig. 1b, the OD₇₅₀ of this group entered the stable growth period earlier than other groups.

When the N: P ratio was 72:4.5, *C. reinhardtii* was in the best condition (Fig. 4g). Compared with control group, the chloroplast's volume was larger, and occupied about 75% of the cell. The thylakoids' layer was clear and neatly arranged; the chloroplast protein with a larger nucleus was surrounded by starch sheath; mitochondria was larger than that of the control group, which reflected the structural integrity with inner and outer membrane, but the amount was small; some cells had slightly large vacuoles. It could be inferred that the photosynthesis and respiration of the algae cells in this group were better than that in the control group. It confirmed that, when the

concentration of N: P was 72:4.5, *C. reinhardtii* grew better and its energy metabolism was more vigorous. However, with high N and P concentration stress, organelles of the algal cell were severely affected, and then further caused the growth inhibition, which also reflected in the growth trend curve of the *C. reinhardtii*. When the N: P ratio was 960:60 (Fig. 4h), the organelle of *C. reinhardtii* was obviously affected: (1) The algae cells were larger than the other groups. (2) The vacuoles singularly became large and the quantity increased, but the content decreased and even dissolved. (3) A part of the chloroplast shrunk or was deformed seriously, and the thylakoid layer was disordered. (4) The plasmolysis phenomenon appeared. (5) In an environment with high concentration of nitrogen and phosphorus, the cells seemed to be dying: The cell internal structure appeared chaotic, and organelles were severely damaged, even cell structure could not be distinguished.

Conclusion

Under the experimental conditions used, these conclusions could be drawn as follows:

1. *C. reinhardtii* has certain tolerability to N and P stress, but if the concentration of N and P was too much higher (1000 mg/L TN) or lower (0.5 mg/L TP) than the optimum concentration, that could be adverse to the microalgae

growth. Besides, *C. reinhardtii* was more sensitive to high-N and low-P stress, especially the low-P stress.

- 24–72 mg/L TN and 4.5–13.5 mg/L TP were the best concentration ranges for *C. reinhardtii*. In such good conditions, the final biomass and protein content of *C. reinhardtii* reached the highest production, the ultra-structure of cells were in the best condition, and the better removal efficiency could be achieved. Thus, this study could provide the basis for the practical use of *C. reinhardtii* in wastewater treatment especially in the advanced stage of wastewater treatment.
- The chloroplasts of *C. reinhardtii* were more sensitive to P stress than mitochondria, while mitochondria were more sensitive to high-N stress than chloroplasts.

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