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Insight into nitrogen and phosphorus coupling effects on mixotrophic *Chlorella vulgaris* growth under stably controlled nutrient conditions

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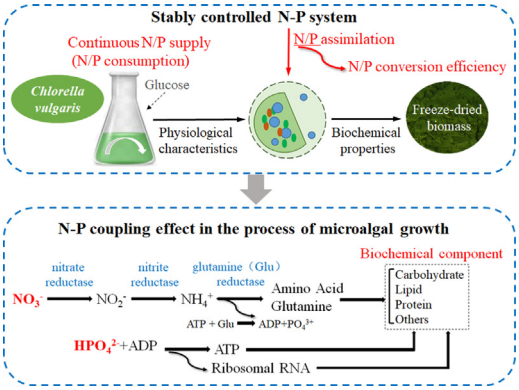
HIGHLIGHTS

- Mixotrophic *C. vulgaris* cultivation was conducted at stably controlled N-P levels.
- N-P coupling effect was significant on algal nutrient uptake, dry mass and pigments.
- Physiological and biochemical properties of algae varied greatly in lower N-P range
- Algal metabolic pathway was more strongly influenced by nitrogen than phosphorus.

GRAPHICAL ABSTRACT



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ABSTRACT

In water environment, nitrogen (N) and phosphorus (P) are biochemically dependent nutrients following the co-limitation concept for algae growth under mixotrophic mode. From a practical viewpoint, algae growth may not bring about significant change of the background nutrient concentration of an actual waterbody in contrast to a conventional batch system. In order to better understand the growth pattern of microalgae in aquatic environments, a series of experiments were conducted under stably controlled N-P levels for studying the N-P coupling effect on mixotrophic *Chlorella vulgaris* growth process, with attention paid to the physiological and biochemical characteristics. It was found that within the concentration range of $N = 1$–$8\ mg \cdot L^{-1}$ and $P = 0.1$–$1.0\ mg \cdot L^{-1}$, the variation of the N-P level slightly affected the specific growth rate, but significantly influenced nutrients uptake, biomass dry weight, chlorophyll contents of the grown *C. vulgaris*. The biochemical and elemental composition of the microalgae tended to be more sensitive to the N-P concentrations and ratios in the lower nutrient range (1–$2\ mg\ N \cdot L^{-1}$, 0.1–$0.4\ mg\ P \cdot L^{-1}$) in which the highest N and P conversion rates were gained as $90.18 \pm 1.23\%$ and $60.47 \pm 1.59\%$, respectively. The P assimilation and conversion efficiencies were much affected by both N and P supplies, while the P supply showed little influence on N assimilation and conversion efficiencies. It was also noticed that the N level greatly affected the metabolic pathway involving nutrient assimilation, carbohydrate fixation and monosaccharide profile, resulting in conversion of the dominant fraction of protein at $N \le 2\ mg \cdot L^{-1}$ into other biochemical compositions including lipids at $N \ge 3\ mg \cdot L^{-1}$. The fatty acid methyl esters (FAMEs) composition tended to differ with varied nutrient levels. These findings may deepen our understanding of algal growth in aquatic environment and provide perspective for eutrophication control.

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1. Introduction

Recently, nutrient and organic pollutants have been considered as a serious threat to aquatic ecosystems (Morgane et al., 2019). *Chlorella vulgaris* is the most commonly cultivated Chlorophyceae and can grow in mixotrophic modes in aquatic environments in the presence of organic carbon (Rincon et al., 2019). Total organic carbon (TOC) and nutrient levels remain relatively constant, as the large dilution capacity of lakes or rivers precludes significant temporal fluctuations (Guedes et al., 2014). Most current studies examining the effect of nutrients on microalgal photosynthetic activity, biomass production, physiological, biochemical ecological and genetic aspects are based on batch cultivation (Deng et al., 2019; Li et al., 2019; Zhang et al., 2020). However, during batch cultivation periods, the growth rate of microalgae has been observed to be downregulated by the reduction of substrates and eventually ceases as a consequence of substrate depletion (Lee et al., 2015). In addition, cellular physiology varied temporally during batch cultivation as cells aged and the bulk environment changed (Klok et al., 2013). Therefore, continuous systems are generally more representative of actual lakes or rivers compared with batch systems. Hagström et al. (2011) confirmed the superiority of continuous culture because of the dynamic equilibrium between nutrient influent and cell metabolism.

Although the continuous cultivation technique of photosynthetic microorganisms has been studied since the 1960s, the complexity of its operation and maintenance has limited its development (Fernandes et al., 2015). Different control and monitoring techniques have been explored during microalgal cultivation processes (Sharma and Stal, 2014). For example, Novoveská et al. (2016) conducted continuous experiments by diluting the culture medium once per day to the same optical density. Su et al. (2012) used a peristaltic pump under a constant dilution rate to maintain the continuous cultivation of *Thermosynechococcus* sp. Continuous control can also be achieved through supplementation with nutrients and maintenance of the culture medium volume at the initial level of enrichment following partial periodic harvesting (Ashokkumar et al., 2014). Stably controlled nutrient cultivation (hereafter "stable cultivation") is a simplified version of continuous cultivation that maintains constant N and P concentrations (Marchetti et al., 2012). To date, most modes of stable cultivation have been conducted for the aquaculture of single algal species to obtain desirable traits, such as lipids or proteins (Ashokkumar et al., 2014; Feng et al., 2014), whereas others have focused on microalgal-based wastewater treatment in photobioreactors (Daneshvar et al., 2019; Yang et al., 2016). To our knowledge, no studies have examined the N and P coupling effect on the supplies of nutrients in aquatic environments under stably controlled nutrient cultivation.

The presence of both nitrogen (N) and phosphorus (P) resources in aquatic ecosystems might have a synergistic effect on microalgal growth; however, the biochemical processes underlying their effects, as well as their interactions, are not fully understood (Hagström et al., 2011; Li et al., 2019). Microalgal species utilize nutrients in the cultivation medium followed by recycle them into biomass, which is then further transformed into energy and other raw chemical products (Mata et al., 2010). Generally, N-P co-limitation or the N: P ratio appears to have a greater influence on the biochemical composition of algae relative to N or P alone (Ross et al., 2018). The nutrient level also significantly affects the atomic carbon (C), N, P and nutrient uptake in microalgal strains (Gao et al., 2018a). Although many studies have examined microalgal growth rates and biomass production under various nutrient supplies (Gao et al., 2018b; Leite et al., 2019), there is still a lack of publicly available data on the impacts of varied N and P concentrations on microalgal physiological and biochemical characteristics in stable cultivation (Wu et al., 2017). Therefore, microalgal trait-based approaches may provide insight into the underlying mechanisms of the relationships between microalgal communities and environmental substrates and promote their further use in ecological studies.

Here, we aimed to explore the extent to which mixotrophic *C. vulgaris* can be influenced by co-limited nutrient concentrations at aquatic environment levels and identify a possible pathway for the transformation of vital nutrients present in biomass components and maintain their levels in equilibrium. Specifically, the growth parameters, nutrient availability, dry cell weight and the elemental and biochemical composition of microalgae at varied N-P levels were examined. This study provided more detailed insight into the co-limitation of nutrients on microalgal growth from biochemical, physiological and molecular perspectives, improving our knowledge of several aspects of aquatic environments.

2. Material and methods

2.1. Microalgal cultivation

2.1.1. Microalgal strains and cultivation conditions

The microalgae breeds were inoculated and acclimated on BG11 medium plates containing agar and glucose and were cultured at 25 °C for 7–10 days. Single colonies were then extracted and transferred carefully and individually to new plates. Isolated *C. vulgaris* were cultured in 1000-mL laboratory flasks maintaining 500-mL stoke cultures. Acclimation, initial inoculation and cultivation conditions of microalgae were based on methods reported by our previous study (Huang et al., 2019). The autoclaved BG11 medium mixed with a concentrated glucose solution (through a sterile 0.22-μm filter) and varied N and P levels were used for growing microalgae. The chemical oxygen demand in the medium was maintained at 40 $mg \cdot L^{-1}$ with glucose as the organic carbon source. The TOC contents and the range of nutrient levels were determined according to the surface water environmental quality (GB 3838-2002) and the discharge standard of pollutants for municipal wastewater treatment plants (GB 18918-2002). Twenty-five combinations of N-P concentrations were prepared, and the periodic nutrient concentrations of N ($NaNO_3$) and P (K_2HPO_4) were 1.0, 2.0, 3.0, 6.0 and 8.0 mg $N \cdot L^{-1}$ and 0.1, 0.2, 0.4, 0.6 and 1.0 mg $P \cdot L^{-1}$.

2.1.2. Stably controlled nutrient system

The schematic diagram of the stably controlled nutrient system (abbreviated as "stable system") process is shown in Fig. S1. The operational process of the stable system was as follows. The 1-L sterilized Erlenmeyer flask was used as a bioreactor. The stable technique was implemented through partial periodic harvesting (every 6 h/day, dilution rate: 4 d^{-1}). NO_3-N, PO_4^{3-}-P and TOC measurements and supplementary calculations were conducted during the 6 h period. After measurements and calculations, the medium was topped off to the initial volume of 500 mL in the Erlenmeyer flask and supplemented with $NaNO_3$, K_2HPO_4 and glucose to reach the original set concentrations. To allow better gas exchange, mixing was performed under constant rotational speed in a light incubator, the light intensity was maintained at 45–50 μmol photon $\cdot m^{-2} \cdot s^{-1}$ with continuous illumination of 12 h/12 h (light/dark) photoperiod.

2.2. Microalgal analytical methods

2.2.1. Microalgal growth

Microalgal cell density and optical density were used as growth indicators and were measured daily using a cellometer and UV spectrophotometer. The standard analytical procedures described in our previous study were used to calculate the specific growth rate (Huang et al., 2019).

2.2.2. Carbon, nitrogen and phosphorus analysis

Samples were taken periodically to evaluate the replenishment of TOC, N and P as well as nutrient consumption and conversion efficiency. Ten milliliters of the microalgal culture sample was centrifuged (10 min, 6000 rpm) and filtered by 0.45-μm glass fiber filters to determine TOC

and nutrient (NO_3^- and PO_4^{3-}) contents. TOC and soluble N and P concentrations were determined using a method described by our previous studies (Huang et al., 2019).

2.2.3. Microalgal biomass dry weight and elemental analysis

At the end of the microalgal growth period, cultures were collected by centrifugation for analysis of elemental composition and dry cell weight (DCW). The remaining medium with an initially set concentration and volume was collected and washed with deionized water three times in a centrifuge tube (6000 rpm, 10 min). The precipitation without the supernatant was then dried with a freeze dryer for weighing (Iasimone et al., 2018). The atomic C, N and sulfur (S) fraction in the freeze-dried microalgal biomass (%) were measured by a tin capsule, while the atomic hydrogen (H) and oxygen (O) contents (1–2 mg) were determined by a silver capsule for elemental analysis (vario PYRO cube, Elementar Company, Germany) (Liu et al., 2016). Sulfanilamide and benzoic acid were used as calibration standards. The concentration of elemental P was determined using the standard methods with modifications described in our previous studies. Briefly, the freeze-dried biomass was pretreated by diluting with deionized water and treated in the Hash digestion instrument (Huang et al., 2019).

2.2.4. Microalgal biochemical composition

The most abundant microalgal pigment in *C. vulgaris* is chlorophyll (chlorophyll-a + chlorophyll-b), while *C. vulgaris* also contains a variety of carotenoids (xanthophylls + carotenes). Photosynthetic pigments were measured by a method described in our previous studies by using different equations to calculate pigment concentrations (Huang et al., 2019).

Carbohydrate content was compared with glucose as the standard using the anthrone method (Raunkjær et al., 1994). Briefly, 1 mL of filtered microalgal culture was acidified by adding 5 mL of anthrone-sulfate solution under an ice bath. The acidified solution was then hydrolyzed at 100 °C for 10 min and then transferred to ice water for cooling. A spectrophotometer was used to determine the absorbance of the samples at 625 nm. In order to determine the detailed monosaccharide composition, five lyophilized biomass samples at N-P concentrations of 1-1, 2-1, 3-1, 6-1 and 8-1 $mg \cdot L^{-1}$ under stable cultivation were selected as research targets. Samples (20 mg) were hydrolyzed by trifluoroacetic acid (TFA) and derivatized by 1-phenyl-3-methyl-5-pyrazolone (PMP)-methanol solution. The chromatographic analysis of the derivatized sugars (both standard sugars and microalgae samples) was conducted on an HPLC system (LC-2010AHT, Shimadzu Corp., Kyoto, Japan) to determine D-mannose (Man), L-rhamnose (Rha), D-glucose (Glu), D-galactose (Gal), D-xylose and L-arabic (Xyl/Ara) in microalgae biomass (Ortiz-Tena et al., 2016). Supplementary S1 provide more details on the specific experimental operation.

Proteins were collected by mixing 5 mL of ethanol (80%) into a 20-mL centrifuged microalgal culture without the supernatant and then were left to sit for 3 h at 65 °C; this process was repeated after centrifugation (6000 rpm, 15 min). After precipitation, the solution was centrifuged (9000 rpm, 5 min), and the supernatant was removed. Two milliliters of 2 $mol \cdot L^{-1}$ NaOH was then mixed with the residue, followed by transfer to a 100 °C water bath for 10 min. The pH of the sample was maintained at 7.1 ± 0.1 using 2 $mol \cdot L^{-1}$ HCl. After further centrifugation, the protein in the supernatant was determined using the method of Bradford (1976). The varying concentrations of bovine serum albumin were utilized as the calibration samples.

Lipid determination was conducted by slightly modifying previously used methods (Paranjape et al., 2016). Nile red solution (100/1 in acetone) was added in the samples and cultivated in the dark for 15 min at 25 °C. The fluorescence of the culture was obtained using a Hitachi F-4500 fluorescence spectrophotometer. Triglycerides are widely used to create the standard calibration curve. The fatty acid methyl esters (FAMEs) content and composition of the abovementioned five lyophilized biomasses (20 mg) were also measured by using transesterification reagent (see Supplementary S2). 20 μL nonadecanoic acid (1 $mg \cdot mL^{-1}$ in N-heptane) were added as internal standard. The content and composition of FAMEs were analyzed with gas chromatography–mass spectrometry (GC–MS, Agilent 7890A/5975C, Santa Clara, USA).

2.3. Model presentation and calculation procedures

The modified Monod and Droop models are basic microalgal growth models that were used to describe the dynamic relationships between algae growth, nutrient uptake and nutrient contents (Bougaran et al., 2010). The effect of the dilution rate on microalgal growth kinetics during continuous cultivation can be observed in the formulas. All measurements were made on culture samples during the steady state. The extended Droop model equations can be written as follows [Eq. (1)]:

$$\begin{aligned} N_t &= DN_{in} - r_N(S, q_N, q_P)X - DN \\ P_t &= DP_{in} - r_P(S, q_N, q_P)X - DP \\ q_{Nt} &= r_N(S, q_N, q_P) - \ddot{E}(q_N, q_P)q_N \\ q_{Pt} &= r_P(S, q_N, q_P) - \ddot{E}(q_N, q_P)q_P \\ X_t &= \ddot{E}(q_N, q_P)X - DX \end{aligned} \quad (1)$$

where N_t and P_t denote the concentrations of dissolved inorganic N and P ($mg \cdot L^{-1} \cdot d^{-1}$), respectively; q_{Nt} and q_{Pt} are the internal N and P cell quota ($mg \cdot g^{-1}$ DW$\cdot d^{-1}$), respectively; and X_t is the microalgal biomass concentration ($mg \cdot L^{-1} \cdot d^{-1}$). D is the dilution rate during cultivation (d^{-1}); N_{in} and P_{in} are the concentrations of N and P in the initial medium ($mg \cdot L^{-1}$), respectively; and N and P are the concentrations of N and P in the culture medium ($mg \cdot L^{-1}$), respectively. X is the biomass weight in the culture medium ($mg \cdot L^{-1}$). The functions $r_N(S,q_N,q_P)$, $r_P(S,q_N,q_P)$ and $\mu(q_N,q_P)$ represent inorganic N uptake ($mg \cdot g^{-1}$ DW$\cdot d^{-1}$), P uptake ($mg \cdot g^{-1}$ DW$\cdot d^{-1}$) and the specific growth rate (d^{-1}), respectively. q_N and q_P represent the N and P cell quota in the microalgal biomass ($mg \cdot g^{-1}$ DW), respectively.

The calculation of nutrient uptake rate (r) and specific growth rate (μ) is based on the Michaelis-Menten model [Eq. (2)] (Bougaran et al., 2010) and modified Monod functions proposed by Huang et al. (2019).

$$r_{N,P} = r_{max}\frac{C_{N,P}}{C_{N,P} + R_{N,P}} \quad (2)$$

where $R_{N,P}$ is the half-saturation constant for the N or P substrate uptake; r_{max} and $r_{N,P}$ are the highest nutrient uptake rate and N or P uptake rate ($mg \cdot g^{-1}$ DW$\cdot day^{-1}$), respectively; and $C_{N,P}$ is the N or P content ($mg \cdot L^{-1}$) in the substrate.

Added N or P was monitored and recorded during partial periodic harvesting so that the nutrient levels were maintained at their original concentrations. Consumed N and P in microalgae under the stable system was calculated by the total amount of N or P addition through Eq. (3):

$$\text{Consumed N (P)} = (\text{N or P})_i + (\text{N or P})_a - (\text{N or P})_m \quad (3)$$

where $(\text{N or P})_i$ is the initial N or P in the medium before inoculation (mg), $(\text{N or P})_a$ is the N or P added during the cultivation period (mg), and $(\text{N or P})_m$ is the N or P in the medium at the end of cultivation under the steady state (mg).

The assimilated N and P in microalgae under the stable system was calculated using Eq. (4):

$$\text{Assimilated N (P)} = \text{DCW} \times \text{N (P)}\% - \text{N (P)}_i \quad (4)$$

where DCW is the dry cell weight of microalgae (mg), N(P)% is the elemental composition of microalgal biomass and $N(P)_i$ is the initial N or P (mg) in the inoculated microalgae.

The N and P conversion efficiency ($E_{N,P}$) of microalgae under the stable system was calculated based on Eq. (5):

$$E_{N\ or\ P} = \frac{\text{Assimilated N or P}}{\text{Consumed N or P}} \times 100\% \quad (5)$$

2.4. Data analysis

Carbohydrate, protein and lipid content of the biomass is portrayed as % DCW. Data were analyzed and processed by SPSS 21.0 software for Windows. Each of the experiment was conducted three times by taking the average of all parallel data samples (mean ± SD, $n = 3$). The statistical differences in the microalgal growth rates, DCW, pigments, biochemical and elemental composition under supply of varied nutrient levels during stable cultivation were calculated by paired *t*-test and analysis of variance, a *P*-value less than 0.05 was confirmed as significant (Fegade et al., 2013). The data calculation, analysis and plotting were carried out with Microsoft excel and Origin 8.5 software. The error bars in all figures represent the standard deviation, columns with different letters represent significant differences ($P < 0.05$) between varied N-P groups. First optimization software was applied for kinetic model evaluation and growth parameters calculation.

3. Results and discussion

3.1. C. vulgaris growth at varied nutrient levels

3.1.1. Specific growth rate

A robust linear correlation fit was obtained between cell density and optical density measurements at 680 nm under stable cultivation (Fig. 1). Several studies have used calibration curves for measuring microalgal cell density by correlating the number of cells with optical values (Pahija and Hui, 2019). This equation allows measurements of cell density and cell numbers to be taken by anyone with common equipment (optical microscope and spectrophotometer). The microalgal specific growth rate under varying nutrient levels in stable cultivation is shown in Table 1 (Fig. S2). The growth rate reached up to 0.40–0.51 d^{-1} under stable cultivation, which was much higher than that for mixotrophic batch cultivation under the same N-P levels (Huang et al., 2019). The downregulation of nutrient contents during batch cultivation limited the growth of microalgae (Lee et al., 2015). However, under stable cultivation, the assimilation of organic carbon and the uptake of nutrients accelerated the growth of microalgae. Previous studies have revealed that continuous cultivation permits growth and photosynthetic rates to be maintained near their maximum values (Fernandes et al., 2015). The increase in the specific growth rate also stemmed from the high dilution rate (Fouilland et al., 2014). The growth rates in this study significantly ($P < 0.05$) increased as N and P concentrations increased when N was 1, 2–3 or 6–8 mg·L^{-1} and P was 0.1–0.2 or 0.4–1 mg·L^{-1} (Table 1), and within those nutrient concentrations, the increase is not obvious. For example, the change of the specific growth rate on mixotrophic *C. vulgaris* under stably controlled N-P levels at 1–0.2 mg·L^{-1} or 2–0.1 mg·L^{-1} and 3–0.1 mg·L^{-1} was not significant ($P > 0.05$). Novoveská et al. (2016) found that nutrient level was one of the most important factors affecting the growth rate in a semi-continuous culture.

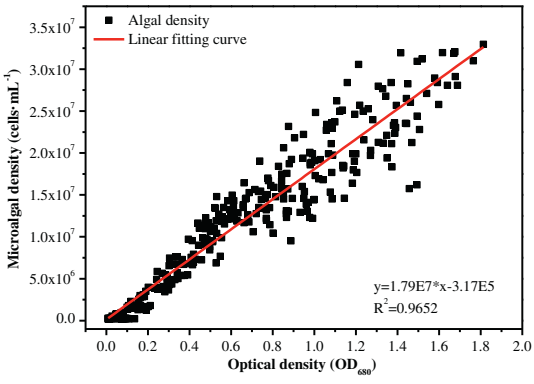

Fig. 1. Relation between OD_{680} and microalgal cell density of *Chlorella vulgaris* at varied N-P levels.

3.1.2. Growth kinetics

Microalgal growth kinetic parameters at varied nutrient levels under stable cultivation are shown in Table 2. Eq. (2) and modified Monod functions were derived from the growth calculations, nutrient determinations and model fitting. Biomass N or P per cell was calculated to determine Eq. (1). These parameters were expected, as this model could accurately represent the capability of microalgae to consume nutrients in the stable cultivation mode. The specific growth rate and nutrient uptake rate were influenced by the coupling effect of the N and P supply. $K_{N(P)}$ was significantly lower than $R_{N(P)}$, showing that $S_{N(P)}$ had a more obvious effect on nutrient uptake than on microalgal growth. Indeed, Cho et al. (2016) confirmed that the most persistent limiting resource for microalgae under continuous cultivation was light intensity rather than the contents of nutrients.

3.2. N and P uptake by C. vulgaris at varied nutrient levels

3.2.1. N and P consumption

Nutrient levels can greatly influence microalgal nutrient uptake, as biomass consumption and assimilation are the main contributors to N and P conversion (Cai et al., 2013). The addition of nitrate (mg NO_3^--N) and phosphorus (mg PO_4^{3-}-P) in the growth medium was monitored during the entire cultivation period, and N and P conversion in the *C. vulgaris* biomass was calculated. Fig. 2a, b shows the values of consumed and assimilated N and P. The amount of consumed N calculated by Eq. (3) was slightly affected by the P levels in the substrate and was significantly ($P < 0.05$) increased when the N levels increased from 1 to 6 mg N·L^{-1}. The highest average value of 24.32 ± 2.83 mg of N consumption was at $N = 6$ mg·L^{-1}. The amount of consumed P by *C. vulgaris* under stable cultivation was significantly ($P < 0.05$) elevated when the P supply was increased (0.1–0.4 mg·L^{-1}). A constant nutrient supply of 0.4–1 mg P·L^{-1} and 3–8 mg N·L^{-1} (nine groups of N-P levels) resulted in the highest P consumption value of 3.43 ± 0.11 mg. The average intracellular N:P ratio in this study decreased from 46.77 to 6.47 when the N and P supplies were elevated. The highest levels of N and P consumption were observed at N = 6 mg·L^{-1} and $P =$ 0.4–1 mg·L^{-1} with an average N:P ratio of 7.58. These results are consistent with previous studies revealing that the N:P ratio has a strong influence on nutrient consumption; the optimal N:P ratio for *C. vulgaris* was 7.2:1 (Cai et al., 2013; Xin et al., 2010). Indeed, in our study, the intracellular (elemental) N:P ratio, rather than the ratio of supplied nutrients, strongly affected the consumption of N and P.

3.2.2. N and P assimilation and conversion efficiency

Higher nutrient consumption does not result in higher degrees of N and P assimilation. The mechanism of nitrogen consumption has been proposed to not only include direct uptake and assimilation by microalgal cells but also the removal of ammonia during the photosynthetic activity of abiotic processes, which might explain the variation observed in nitrogen consumption and conversion (Iasimone et al., 2018). Microalgae can assimilate high levels of phosphate that accumulate as polyphosphate granules in insoluble form, whereas P adsorption and chemical precipitation provide another means of P consumption (Shriwastav and Bose, 2015). Luxury N and P uptake resulted in high N and P assimilation when N ranged from 1 to 2 mg·L^{-1} with elevated P from 0.1 to 1 mg·L^{-1}. The conversion rate of N and P under stable

Table 1
Specific growth rate of mixotrophic *Chlorella vulgaris* at varied N-P levels.

N levels	Specific growth rate (d^{-1})				
	P = 0.1 mg·L^{-1}	P = 0.2 mg·L^{-1}	P = 0.4 mg·L^{-1}	P = 0.6 mg·L^{-1}	P = 1.0 mg·L^{-1}
1 mg·L^{-1}	0.40 ± 0.003	0.41 ± 0.002	0.46 ± 0.007	0.47 ± 0.005	0.47 ± 0.003
2 mg·L^{-1}	0.45 ± 0.008	0.46 ± 0.003	0.48 ± 0.003	0.48 ± 0.009	0.48 ± 0.002
3 mg·L^{-1}	0.45 ± 0.005	0.46 ± 0.009	0.48 ± 0.002	0.49 ± 0.007	0.49 ± 0.006
6 mg·L^{-1}	0.46 ± 0.004	0.47 ± 0.002	0.49 ± 0.006	0.50 ± 0.006	0.51 ± 0.004
8 mg·L^{-1}	0.46 ± 0.008	0.48 ± 0.000	0.50 ± 0.005	0.51 ± 0.005	0.51 ± 0.002

Table 2
Kinetic parameters related to *Chlorella vulgaris* growth and nutrient uptake at varied N-P levels.

Parameter	$r_{N,P} = r_{max}\frac{C_{N,P}}{C_{N,P}+R_{N,P}}$			$\mu = \mu_{max}\frac{C_N}{K_N+C_N}\frac{C_P}{K_P+C_P}$	
	$r_{max,\,N}$	$r_{max,\,P}$	$R_{N(P)}$	μ_{max}	$K_{N(P)}$
Value	0.15	0.02	0.88(0.06)	0.52	0.12 (0.013)
R^2	0.9170	0.9201	–	0.9308	

cultivation was evaluated to verify whether the N and P consumption from the substrate matched their assimilation in the *C. vulgaris* biomass. Higher nutrient conversion efficiency of stable cultivation provides a feasible means for consuming nutrients in aquatic environments. The N and P conversion efficiency of *C. vulgaris* under stable cultivation at fixed N or P concentrations was calculated using Eq. (5). The conversion efficiency of N by microalgae was limited by N concentration and was P-independent, whereas the P conversion efficiency was limited by both N and P concentrations (Fig. 2c, d). Overall, a relatively higher N and P conversion efficiency was obtained with 1–2 mg N·L^{-1} when the addition of P was elevated in the stable system. The average N conversion efficiency was 91.24 ± 7.49% and 61.92 ± 14.77%, and the average P conversion rate was 40.22 ± 9.90% and 61.98 ± 12.65% at 1 and 2 mg N·L^{-1} at various P levels. The variable conversion efficiencies demonstrated the effect of varied nutrient supplies on the capacity for nutrient transformation and accumulation. Because the model species and the stable system used here are reliable, this same phenomenon is likely broadly applicable to other large-scale systems. Therefore, control of the N concentration within 2 mg·L^{-1} in the aquatic environment can optimize nutrient conversion into intracellular components.

3.3. *C. vulgaris* biomass characteristics at varied nutrient levels

3.3.1. Biomass dry weight

Both N and P under stable cultivation influenced the production of microalgal biomass (Fig. 3a), and N concentration had a larger impact.

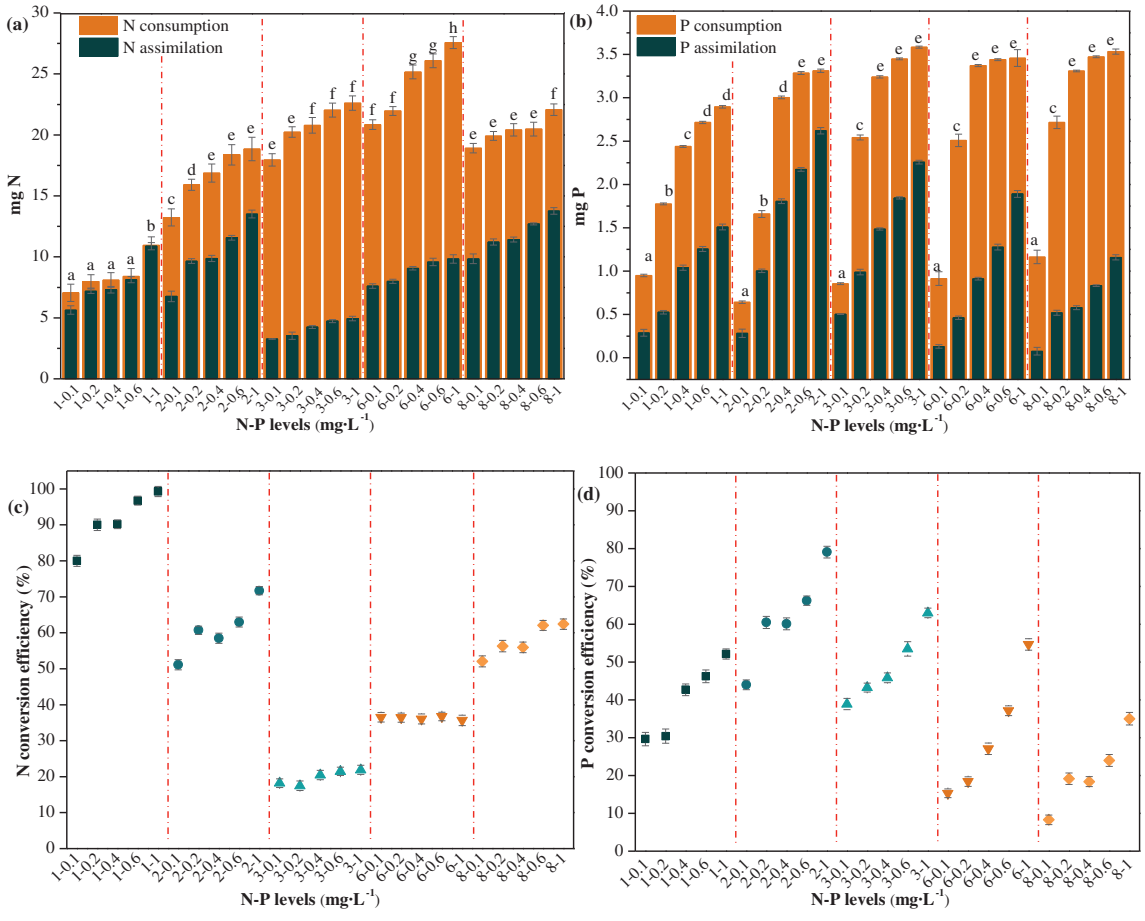

Fig. 2. Nitrogen and phosphorus (a, b) consumption, assimilation and (c, d) conversion efficiency of *Chlorella vulgaris* at varied N-P levels.

Biomass accumulation occurs when nutrients are sufficient in the cultivation medium (Xin et al., 2010). The change in DCW (1.71–2.23 fold) of different N levels (1–8 mg·L^{-1}) at fixed P concentrations was more variable than the change in DCW (1.13–1.63 fold) of varied P levels (0.1–1 mg·L^{-1}) at fixed N levels. This pattern may stem from the luxury uptake of nutrients at these N-P levels described in Section 3.2.2. Fundamentally, NO^{3-} passively or actively enters across the cell membrane, reduces to NH^{4+} and amino acids and is further assimilated into macromolecules, such as proteins, enzymes, nucleic acids or chlorophyll, to increase microalgal cell weight (Ross et al., 2018). Kube et al. (2018) found that N contributed to 1–10% of the DCW for producing microalgal biomass and converting energy. P is mostly used for the biosynthesis of rRNA and phospholipids, which are critical for the genetic and energy cycle for cell division and growth (Huang et al., 2019). The high DCW indicated that the continuously supplied TOC, N and P were absorbed and transformed into storage components to elevate biomass dry weight.

DCW comparisons between batch and stable cultivation under various nutrient supplies are shown in Fig. 3b. The maximum DCW in this study was 4.86–13.67-fold higher than that of the batch cultivation when compared pairwise for each nutrient combination. These data were consistent with previous studies reporting higher productivity in continuous cultivation when compared with batch cultivation using optimal cultivation parameters (Salati et al., 2017). Cho et al. (2016) presented two standard parabolic equations to explain the dilution rate as a function of both biomass and lipid productivity. They found that biomass production cumulatively increased by 10 times when the dilution rate was 0.75 d^{-1}. The most plausible explanation for this pattern is that mixotrophic cultivation of microalgae under a stable system can accelerate growth characteristics, shorten the growth period and diminish DCW loss during the dark hours through pure respiration to enhance microalgal biomass production (Park et al., 2012).

3.3.2. Photosynthetic pigments

A positive correlation between chlorophyll pigments and N-P availability was observed at the end of stable cultivation (Fig. 4). Consistent results were obtained in the changes of chl-*a*, chl-*b* and carotenoid, respectively. In contrast to the results obtained for DCW, P had a larger effect on pigment synthesis than N. The change in pigments (1.69–2.11 fold) at different P levels (0.1–1 mg·L^{-1}) at fixed N concentrations was more variable than the change in pigments (1.43–1.69 fold) at varied N levels (1–8 mg·L^{-1}) at fixed P concentrations. Beuckels et al. (2015) indicated that P is essential for ATP synthesis and the bioenergy cycle in microalgal biomass. The decrease in actual photochemical efficiency and the inactivation of the PSII system was closely related to the fall in P content (Jiao et al., 2017). The higher concentration of pigments in *C. vulgaris* might be related to the higher concentration of

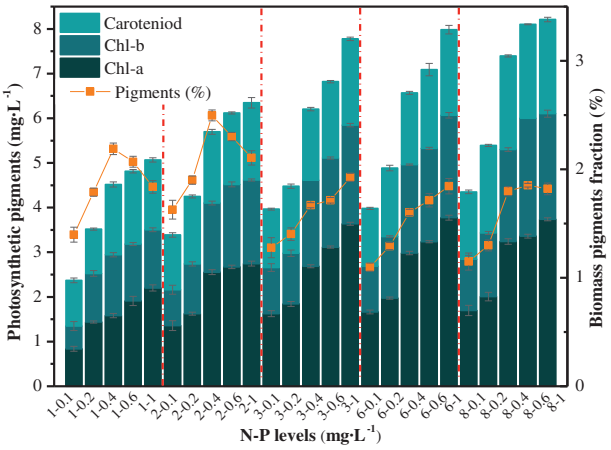

Fig. 4. Microalgal chlorophyll (Chl-*a* and Chl-*b*) and carotenoid concentrations and pigments fraction (% of dry weight) in *Chlorella vulgaris* at varied N-P levels.

microalgal cells in the culture medium. According to Daneshvar et al. (2019), chlorophyll can be considered an intracellular nutrient reservoir; thus, the availability of N and P leads to the healthy growth of microalgae and the accumulation of chlorophyll. Chl-*a*, Chl-*b* and carotenoid concentrations in this study elevated during the cultivation period from day 1 to day 11; concomitant increases in pigments reflected adequate carbon and nutrient supplies (Jiao et al., 2017). The content of pigments in DCW increased as N contents increased from 1 to 2 mg·L^{-1} in all P-constant groups and decreased with the N supply from 3 to 8 mg·L^{-1}. When N was fixed at 1 and 2 mg·L^{-1}, biomass pigments increased and reached their maximum values of 2.18% and 2.49% when the supplied P content ranged from 0.1 to 0.4 mg·L^{-1} and decreased with elevated P contents from 0.4 to 1.0 mg·L^{-1}. This could be explained by the fact that the increase in DCW was more rapid than the increase in biomass pigments (%) (Su et al., 2012). When N was fixed at 3–8 mg·L^{-1}, biomass pigments increased as the supply of P increased. Thus, the luxury consumption of N and P was used for chlorophyll accumulation in *C. vulgaris* at 1–2 mg N·L^{-1} and 0.1–0.4 mg P·L^{-1}. Trends in the reduction of pigments with N also demonstrated the more important function of N in cell weight gain rather than in the increase in pigments. Chlorophyll concentrations in *C. vulgaris* under the stable system were significantly higher (1–5.85, 1.12–3.80 and 2.73–28.95-fold differences in Chl-*a*, Chl-*b* and carotenoids, respectively) than values under the batch mode obtained by our previous studies (Huang et al., 2019).

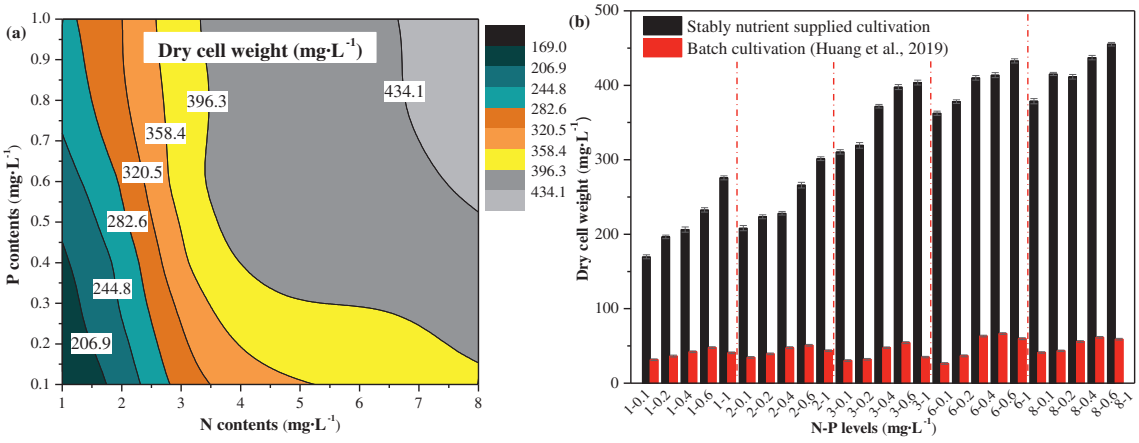

Fig. 3. (a) Dry cell weight of grown *Chlorella vulgaris* at varied N-P levels and (b) Comparison of dry cell weight between Huang et al., 2019 (batch cultivation with initial N-P levels) and this study (cultivation at stable N-P levels).

3.3.3. Biochemical composition

Microalgae are rich in carbohydrates, proteins (primary metabolites), lipids, pigments and other components (dietary fiber, ash, moisture and other insoluble matters) and also contain considerable amounts of secondary metabolites and minerals (Cai et al., 2013). Understanding the biochemical composition and transformation of components in microalgal cells is essential for studying their biomass characteristics. Different components in *C. vulgaris* can contribute to the processes associated with cell metabolism (Li et al., 2018; Ross et al., 2018). Microalgal carbohydrate and protein components (% of dry weight) showed significant differences between varied levels of N and were slightly influenced by P levels (Fig. 5a). The carbohydrate content significantly ($P < 0.05$) decreased with increased N contents in all P-constant groups, whereas the values decreased significantly ($P < 0.05$) with increased P under low P levels and changed little when P was higher than 0.4 mg·L^{-1} in N-constant groups. The total protein content significantly ($P < 0.05$) increased when the N supply increased from 1 to 2 mg·L^{-1} and decreased to its minimum value when $N = 3$ mg·L^{-1}. Total protein content then increased as N supply increased from 3 to 8 mg·L^{-1} but never exceeded the values observed when N ranged between 1 and 2 mg·L^{-1}. Thus, the emergence of a large protein gap between 1 and 2 mg N·L^{-1} and 3, 6 and 8 mg N·L^{-1} suggests that during stable cultivation of *C. vulgaris*, when N was less than or equal to 2 mg·L^{-1} and P was less than or equal to 0.4 mg·L^{-1}, microalgae experienced an inadequate supply of nutrients and may have over-absorbed and transformed C, N and P to promote the accumulation of carbohydrates and proteins. The synthesis of biomass lipids was significantly influenced by the addition of both N and P under stable cultivation (Fig. 5a). Microalgal lipids were significantly ($P < 0.05$) increased with elevated P addition in N-constant groups. The higher protein composition at 1–2 mg N·L^{-1} limited carbohydrate production; however, the elevated production of lipids was accompanied by a more pronounced reduction in proteins in 3, 6 and 8 mg N·L^{-1} groups than in the 1 and 2 mg N·L^{-1} groups. The acceleration in organic carbon assimilation under higher nutrient concentrations may also inhibit protein synthesis (Lai et al., 2019). Carbohydrates significantly ($P < 0.05$) decreased and were increasingly converted to lipids under 3–8 mg N·L^{-1} when P was higher than or equal to 0.4 mg·L^{-1} under stable cultivation. The chemical composition of the biomass developed in microalgae ultimately depends on nutrient availability and the supplied nutrient concentrations (Rani et al., 2020). Gao et al. (2018a) also observed a re-allocation of cellular C from carbohydrates to lipids with a modest N supply; an increased P supply accompanied by a form of energy input contributed to this transformation process.

As shown in Fig. 5b, under a fixed P content of 1 mg·L^{-1}, the carbohydrate contents in *C. vulgaris* (% of dry weight) decreased gradually from 51.80% to 20.79% when N was increased from 1 mg·L^{-1} to 8 mg·L^{-1}. The most significant decrease was seen in the content of glucose (Glu) which was 45.02% at the N-P level of 1–1 mg·L^{-1}, and gradually decreased to 28.98%, 22.92%, 18.22% and 15.22% when the N-P levels varied to 2–1 mg·L^{-1}, 3–1 mg·L^{-1}, 6–1 mg·L^{-1} and 8–1 mg·L^{-1}, respectively. Although the contents of other saccharides, such as 0.17% (Man), 2.92% (Rha), 3.17% (Gal) and 0.52% (Xyl/Ara) also varied to certain extents with elevated N concentration, they were always the less dominant monosaccharides than Glu at varied N-P levels. The distribution of the contents of the five saccharides is similar to that reported by Ortiz-Tena et al. (2016). The significant decrease of carbohydrate content, especially glucose ($P < 0.05$) with increasing N level revealed the structural characteristics and cellular component in microalgal biomass (Mayers et al., 2018), suggesting that the synthesis of carbohydrates in *C. vulgaris* might be nitrogen-dependent.

Fig. 5c shows variation of fatty acid methyl esters (FAMEs) composition (% of total FAMEs) and their contents (% of dry weight) under the fixed P content of 1 mg·L^{-1} and varied N content from 1 mg·L^{-1} to 8 mg·L^{-1}. The approximate tendency was an increase of the FAMEs content from 2.56% at the N-P level of 1–1 mg·L^{-1} to 3.50% at the N-P level of 1–8 mg·L^{-1}. Linoleic acid (C18:2), oleic acid (C18:1) and palmitic acid (C16:0) were found to be the main components of FAMEs in *C. vulgaris* biomass, similar to the finding of Park et al. (2014).

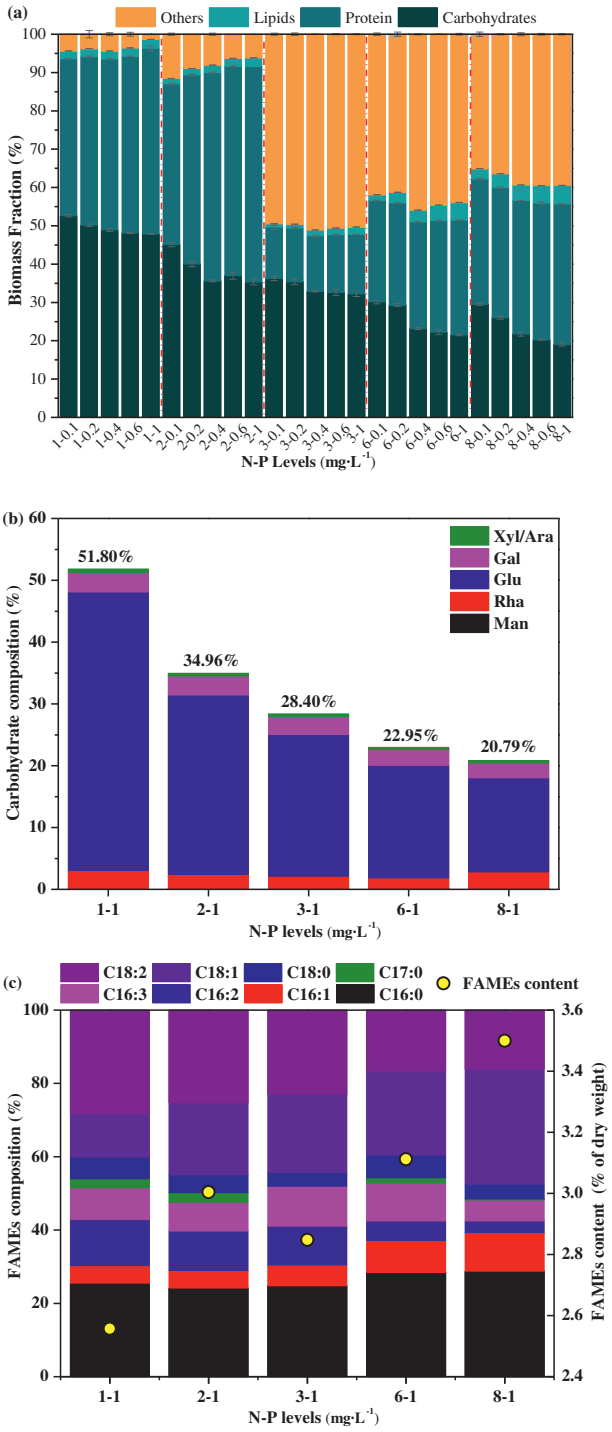

Fig. 5. (a) Biochemical composition, (b) Carbohydrate amounts and (c) Fatty acid methyl esters (FAMEs) composition and content (% of dry weight) of *Chlorella vulgaris* at varied N-P levels.

3.3.4. Elemental composition

The elemental composition (e.g., C, N, P, H, O, S and others trace elements) observed at the end of stable cultivation at varied N-P levels is shown in Fig. 6. Interestingly enough, the elemental composition of microalgae in each of the N-P combinations exhibited the same pattern

as that observed under the fixed supplies of P and N. When the N supply remained constant under elevated P concentrations from 0.1 to 1 mg·L^{-1}, the biomass C and N changed little ($P > 0.05$) among all five of the groups, while the biomass P significantly increased ($P < 0.05$), especially when P was less than or equal to 0.4 mg·L^{-1}. Conversely, under a constant supply of P with N concentrations (1–8 mg N·L^{-1}), C, N and P concentrations all significantly increased and the biomass O significantly declined when the supply of N ranged from 1 to 2 mg·L^{-1}. These observations stemmed from the essential participation of nitrogen in the biomass structure proposed in Section 3.3.3. The biomass P decreased when N levels increased from 2 to 8 mg N·L^{-1}, showing that biomass P was also affected by the N supply (Fig. 6). Both biomass C and N (%) were at their minimum and O% at its maximum, when N was 3 mg·L^{-1}. This downward trend was ascribed to the microalgal carbohydrates and protein degradation, which provided the C and N required to maintain normal metabolic functions (Azadeh et al., 2017). When the N supply elevated from 3 to 8 mg·L^{-1}, the values of C and N (%) significantly ($P < 0.05$) increased, and the values of O (%) decreased. Thus, biomass P (%) was influenced by both the N and P supply under stable cultivation, while the biomass C (%), N (%) and O (%) were not related to the P supply and were primarily affected by the N supply ($P < 0.05$). Biomass H and S (%) did not significantly vary among the N-P groups. Our findings are similar to those previously obtained for both *Chlorella and Scenedesmus* species under batch cultivation by Beuckels et al. (2015), who explained these patterns by the different functions of N and P in microalgal intercellular anabolism and catabolism. The maximum biomasses C, N and P were observed at 2 mg N·L^{-1} under various P supplies. The stoichiometric formula and molecular weight of microalgae under 1–8 mg N·L^{-1} and 0.1–1.0 mg P·L^{-1} were calculated by their elemental compositions. The common formula of *C. vulgaris* under stable cultivation was: $C_{107.16-123.18}H_{214.32-245.20}O_{60.77-82.88}N_{4.74-19.94}P_{0.04-1.50}S_{0.27-1.34}$. The molecular weights of stably cultivated *C. vulgaris* under varied N-P levels were all lower than the Redfield formula weight. Notably, C:N ratios ranged from 5.04 to 19.71, and C:P ratios ranged from 1.91 to 130.34, demonstrating the stoichiometric plasticity of stably cultivated *C. vulgaris* under aquatic nutrient levels in this study (1–8 mg N·L^{-1} and 0.1–1.0 P mg·L^{-1}).

3.4. Coupling effect of N and P in the process of microalgal growth

Based on the results presented above, N and P thus play a major role in cell metabolism, as they are jointly involved in biochemical cycles (Bougaran et al., 2010). First, the nutrient consumption, biomass dry weight and photosynthetic pigment accumulation under stable cultivation were significantly influenced by the N and P coupling effect, especially when nutrient supplies were low (1–2 mg N·L^{-1} or 0.1–0.4 P mg·L^{-1}). This pattern can be explained by the ability of microalgae to assimilate excess C, N and P under a relatively inadequate supply of nutrients (Cai et al., 2013). Increasing N and P contents positively influenced the specific growth rates of microalgae given that the adequate energy produced by both chemicals (organic carbon) and light (photosynthesis) was utilized for nutrient assimilation and transformation. Second, the biomass of P, P assimilation and conversion efficiency not only depended on the supply of P but also on the supply of N, while the biomass of N, N assimilation and conversion efficiency were only slightly affected by the P supply under the stable system. The energetic and genetic features of P in cellular processes could explain these observations. 1) Transportation of nitrate passes across the plasma membrane, and then reduced via nitrate reductase and nitrite reductase. Nitrite is then reduced to ammonium followed by the incorporation of ammonium into amino acids. This process can be accomplished without the involvement of phosphorus and result in the production of PO_4^{3-} for the energetic process. However, supplied HPO_4^{2-} acts as an intermediate in energy cycle of microalgae and is incorporated as an essential component during synthesis (N involvement) by phosphorylation (Beuckels et al., 2015; Cai et al., 2013). 2) Proteins (mainly composed of cellular C and N) play major roles in most microalgal species for microalgal growth, repair and maintenance and also act as cellular motors, chemical messengers, cellular activity regulators and cellular stress receptors (Safi et al., 2014). The synthesis of protein will significantly influence the production of metabolic enzymes, ribosomes and further affect the amount of rRNA, which were all closely related to cellular P concentrations (Kube et al., 2018).

The physiological effects discussed above motivated biochemical characteristics, thus, the coupling effect of N and P on cellular components should be thoroughly explored. The cellular mechanism was characterized into two parts: the assembly and acquisition machinery (Christensen and Tilgner, 2004). Whereas the former corresponds to rRNA that contains N and P, the latter consists of proteins, carbohydrates and lipids that contain N but little or no P. The contents of microalgal carbohydrates and proteins are solely governed by N supply. Carbohydrates are primarily produced through NADPH and ATP in the dark reaction of the Calvin cycle during photosynthesis (Ross et al., 2018). The stable system with a constant nutrient supply was considered a non-nutrient starved culture with high protein content and low lipid and

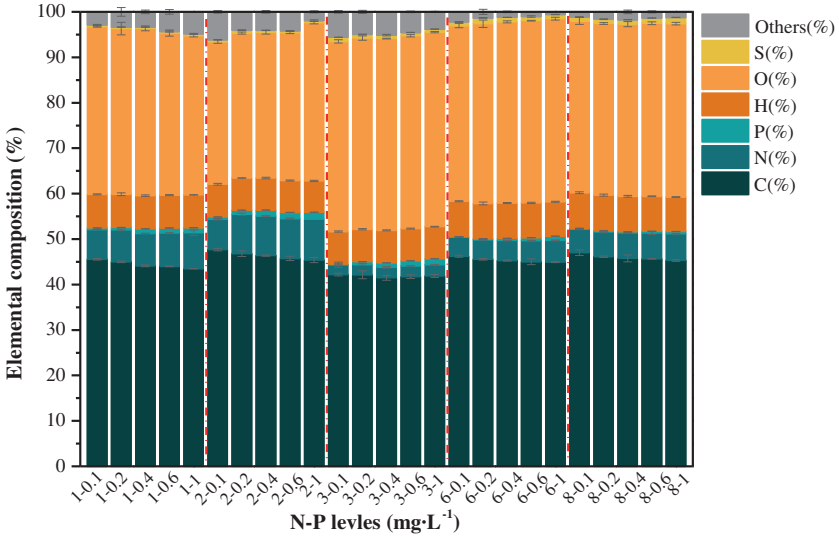

Fig. 6. Elemental analysis (% of dry weight) of *Chlorella vulgaris* at varied N-P levels.

carbohydrate contents (Mayers et al., 2017). With increasing N supply, saccharides decrease while proteins increase in the *C. vulgaris* biomass. Such phenomena are due to the metabolic pathway of microalgae that when N is sufficient in the culture, the storage of polysaccharides will shift to the storage of proteins, lipids and other components in the microalgal biomass (Li et al., 2019; Ross et al., 2018). The higher concentrations of proteins and carbohydrates under 1–2 mg $N \cdot L^{-1}$ and 0.1–0.4 mg $P \cdot L^{-1}$ are probably due to the luxury consumption of N and P for cell metabolism (Rhee, 1980). The synthesis of lipids, as well as the FAMEs accumulation, in microalgal biomass is significantly related to both the supply of N and P. The metabolic pathways of carbon assimilation in *C. vulgaris* involve the conversion of fixed carbon into protein when $N \leq 2$ mg $\cdot L^{-1}$ and its further conversion to lipids and pigments when $N \geq 3$ mg $\cdot L^{-1}$.

4. Conclusions

Mixotrophic microalgal cultivation under stably controlled N-P levels can better capture the characteristics of algal growth in actual water environment. In this study attention was mainly paid to the N-P coupling effect on nutrients uptake, dry cell weight, chlorophyll contents, biochemical and elemental composition of the grown microalgae. An important finding was the highest N and P conversion obtained at the lower nutrient levels of 1–2 mg $N \cdot L^{-1}$ and 0.1–0.4 mg $P \cdot L^{-1}$, indicating that a background nutrients concentration in this range would be sufficient for luxury nutrients uptake to enhance algal growth rather than higher N-P levels. For *Chlorella vulgaris*, the intracellular (elemental) N:P ratio was found to be averaged as 7.58:1 which might be a nutrient ratio beneficial to fast algal growth. The N supply under stable cultivation greatly influenced the carbohydrate contents in the microalgal biomass. It was further found that the microalgal metabolic pathway would be primarily governed by nitrogen, resulting in the conversion of the dominant fraction of protein at $N \leq 2$ mg $\cdot L^{-1}$ into other biochemical compositions including lipids at $N \geq 3$ mg $\cdot L^{-1}$. The characteristic distribution of FAMEs in the microalgal biomass was also revealed and those with carbon chains of C16 and C18 were found to be the dominant contents. These findings may assist a better understanding of the characteristics of algal growth in aquatic environment and a perspective of eutrophication control.

CRediT authorship contribution statement

Yue Huang: Investigation, Data curation, Formal analysis, Writing - original draft. **Chenghao Lou:** Methodology, Software. **Li Luo:** Conceptualization, Visualization. **Xiaochang C. Wang:** Supervision, Writing - review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2020.141747.

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