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The effect of light on the cellular stoichiometry of *Chlorella* sp. in different growth phases: implications of nutrient drawdown in batch experiments

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Abstract Identification of nutrient status is essential to *Chlorella* sp. cultivation for biodiesel production. Critical cellular elemental ratio is a commonly used parameter to assess nutrient status, but the definition of critical cellular elemental ratio is inconsistent, and the influence of light and growth phase on this ratio is still unclear. In this study, *Chlorella* sp. was batch cultured under different conditions of light and nutrient supply ratio. Nitrate and phosphate consumption in the medium and particulate cellular carbon (C), nitrogen (N), and phosphorus (P) contents were measured. We first obtained the critical nutrient supply ratio to assess nutrient limitation and then utilized this ratio to deduce the critical cellular elemental ratio. The critical nutrient supply ratio was significantly affected by light intensity, and was approximately 45, 15, and between 30 to 45 under light intensities of 50, 100, and 200 μmol photons m^{-2} s^{-1}, respectively. Critical cellular C/N/P ratios of *Chlorella* sp. at three light intensities of 50, 100, and 200 μmol photons m^{-2} s^{-1} in exponential growth phase were C$_{159.91-237.30}$N$_{15.90-22.52}$P, C$_{104.02-167.39}$N$_{11.71-17.35}$P, and C$_{144.30-243.66}$N$_{12.84-19.84}$P, and in stationary growth phase the ratios were C$_{201.47-406.14}$N$_{16.55-32.67}$P, C$_{125.05-298.44}$N$_{10.12-24.24}$P, and C$_{168.65-342.42}$N$_{14.47-29.18}$P,

respectively. These results supported the hypothesis that the critical cellular elemental ratio, which is used to predict nutrient status, varies and depends on light intensity and growth phase.

Keywords *Chlorella* sp. · Critical nutrient supply ratio · Critical cellular elemental ratio · Light intensity · Growth phase

Introduction

Chlorella has a high nutritional value because of its high protein content (>55 % dry weight) and is consumed as an alternative food supplement (Safi et al. 2014). This microalga can also be used in medicine (Agyei and Danquah 2011) due to its immunity-modulating and anti-cancer properties (Safi et al. 2014). Most importantly, *Chlorella* is capable, as an alternative to having a higher protein content, of accumulating significant amounts of lipid suitable for biodiesel production (Zheng et al. 2011). Because of the oil crisis, global climatic change and obvious advantages, such as a high lipid percentage and production capacity, no competition with arable lands, or conflict with food production (Singh et al. 2011) and reduction in greenhouse emissions (Wang et al. 2008), *Chlorella* (as one of the potential microalgae species) cultivation for biodiesel production has received a great deal of attention (Lan et al. 2015).

Special nutrient-limited cultivation conditions can strongly impact the physiology of microalgae and thus influence the content of cellular lipids (White et al. 2011). For example, under nitrate-limited condition, lipid is accumulated in *Chlorella* sp. cells (Rodolfi et al. 2009). However, the final lipid productivity is influenced by both biomass and lipid content, requiring culture conditions for generating cells with high growth rates and lipid contents. Nutrient-limited

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conditions that enhance lipid accumulation can also decrease the growth rate and therefore may not improve overall lipid productivity (Griffiths et al. 2014). One strategy to address this problem is to first cultivate microalgae in a medium without limitation or colimitation for maximizing biomass production and then cultivate it under nutrient-limited condition for high lipid content (White et al. 2011). Therefore, identification of the nutrient status is important in *Chlorella* sp. cultivation for biodiesel production.

Measurable parameters for assessing nutrient status are cell growth rate, population density, and maximum quantum efficiency of PSII photochemistry (F_v/F_m). Although these indices can provide useful information regarding whether microalgal growth is nutrient limited, they cannot offer further insight into which nutrient is limiting or whether a certain nutrient is present in excess; this is important, since excessive nutrient supply is not economical. Nutrient status can also be evaluated based on nutrient supply parameters (concentration, supply ratio, and absorption kinetics) and cellular composition parameters (cell macromolecular composition, elemental ratio, and enzyme activity, such as alkaline phosphatase activity), as well as nutrient enrichment experiments (Beardall et al. 2001a, b).

The critical cellular elemental ratio is a commonly used parameter to assess nutrient status. It is assumed that there is a distinct transition point between different nutrient limitations, such as the Redfield ratio nitrogen (N)/phosphorus (P) = 16:1 (Redfield 1960); when N/P >16, phosphate is the limiting nutrient; when N/P <16, nitrate is the limiting nutrient; and when N/P = 16, nitrate and phosphate are the colimiting nutrients (Geider and La Roche 2002). However, the definition of critical cellular elemental ratio is inconsistent. It was originally defined as sharp transition point between nitrate and phosphate limitations predicted by the Droop model (Droop 1968; Geider and La Roche 2002); it is also used to define the ratio of the minimum cell nutrient quota (Rhee 1978; Wynne and Rhee 1986). Although the minimum cell nutrient quota ratio is convenient to measure, it is of less value for assessing the switchover point from nitrate to phosphate limited when cells are actively growing. Goldman et al. (1979) found that the cellular elemental ratio of rapidly growing microalgae converged to a single value regardless of the nutrient conditions, and the authors defined this value as the critical cellular elemental ratio for an investigated species. The critical cellular elemental ratio, renamed as the structural stoichiometry by Klausmeier et al. (2004a), is the cellular elemental ratio that maximizes growth rate or minimizes the requirement of the limiting nutrient under special conditions. In addition to the inconsistent definition of critical cellular elemental ratio, this ratio is strongly influenced by light intensity (Wynne and Rhee 1986; Hall et al. 2007), because light-harvesting pigment–protein complexes and components of the photosynthetic electron transfer chains account for a large but variable fraction of the cellular mass (Geider et al. 1996).

Under low light conditions, the cell invests heavily in light harvesting and other photosynthetic components to optimize energy capture; conversely, under high light conditions, intracellular quantities of light-harvesting components are decreased, minimizing photo-inhibitory damage (Larkum and Howe 1997). Finally, light intensity may differently affect microalgal cellular composition in different growth phases (exponential growth and stationary growth phases) due to acclimation and then influence the critical cellular elemental ratio (Terry et al. 1985; Klausmeier et al. 2004a). Because of the inconsistent definition of critical cellular elemental ratio and the strong influence of light intensity and growth phase on this ratio, we cannot directly know critical cellular elemental ratio by measurement of particulate cellular elemental contents during microalgal cultivation process.

Nutrient concentration in the medium is a typical parameter measured during microalgal cultivation, giving the nutrient drawdown patterns and drawdown ratios, which may provide an effective and accessible approach for the definition and deduction of critical cellular elemental ratio. In the past few decades, two types of nutrient drawdown patterns have been proposed (see Fig. 1). When the microalgal stoichiometry is assumed to be fixed (Tilman 1982), the cells will consume two nutrients at a fixed ratio until the concentration of one nutrient is less than R* (the critical nutrient concentration or zero net growth isocline), but the other may remain plentiful. In contrast, the flexible stoichiometry pattern posits that microalgae can consume both nutrients to subdetection levels. In this scenario, the temporal dynamics of nutrient drawdown occur in two phases. Nutrients presented at high concentration are initially consumed at a fixed ratio in the first phase, followed by consumption of one residual nutrient in the second phase (Klausmeier et al. 2004b). In both nutrient drawdown patterns, when microalgae could consume two nutrients with a fixed ratio to the lower limit of detection, nutrient supply ratio is the critical one (Fig. 1) and can be used to determine the limiting nutrient (Klausmeier et al. 2004b). In other words, if one nutrient supply ratio is the critical one, the nutrient drawdown ratio should equal the nutrient supply ratio and there is no detectable residual nutrient in the medium. Critical nutrient supply ratio and critical cellular elemental ratio are different parameters used to assess nutrient status. At any specific growth condition, evaluation results about nutrient status should be the same with either of those two parameters. If the critical nutrient supply ratio is measured, then the critical cellular elemental ratio can also be estimated from measurements of the particulate cellular elemental comments. In this study, we defined the critical cellular elemental ratio as the cellular carbon (C)/N/P of *Chlorella* sp. grown at the critical nutrient supply ratio; the relationship between critical cellular elemental ratio and critical nutrient supply ratio is shown in Fig. 2.

The aims of this study are to identify an effective and accessible approach for obtaining the microalgal critical cellular
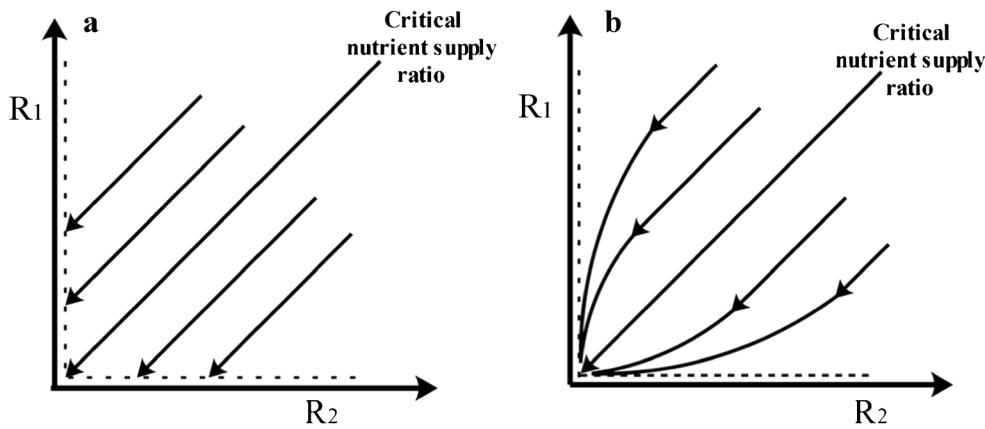
Fig. 1 Nutrient drawdown patterns for a range of nutrient supply ratios, where R_1 and R_2 are nutrient concentrations and the *dashed line* is R^* (the critical nutrient concentration or zero net growth isocline). **a** Nutrient drawdown pattern in fixed microalgal stoichiometry (*left figure*). **b** Nutrient drawdown pattern in flexible microalgal stoichiometry (*right figure*). The *longest arrow* represents critical nutrient supply ratio; if the nutrient supply ratio is greater than this ratio, microalgal growth is R_2 limited; if the nutrient supply ratio is equal to this ratio, microalgal growth is R_1 and R_2 colimited; otherwise, microalgal growth is R_1 limited

elemental ratio and then evaluate the influences from light intensity and growth phase on this ratio. To this end, *Chlorella* sp. was batch cultured under different nutrient conditions and light intensities and the intracellular elemental content and nutrient consumption process were monitored. We first obtain the critical nutrient supply ratio to assess nutrient limitation and then utilize this ratio to deduce the critical cellular elemental ratio and then evaluate the influence of the growth phase and light intensity on the critical cellular elemental ratio.

Materials and methods

Microalgae cultivation

Chlorella sp. (FACHB-1298) was obtained from the Freshwater Algae Culture Collection at the Institute of Hydrobiology, Chinese Academy of Sciences, which is known as the FACHB collection (http://algae.ihb.ac.cn/). The cells were grown in modified BG-11 medium (Allen 1968) with nitrate and phosphate as the sole sources of inorganic N and P, and ferric ammonium citrate in BG-11 was replaced by ferric citrate with same concentration of Fe. In all formulations, the nitrate concentration was adjusted to 100 μmol L^{-1}, and the phosphate concentration was

Fig. 2 Relationship between critical cellular elemental ratio and critical nutrient supply ratio

varied to yield five nutrient supply ratios of 5:1, 15:1, 30:1, 45:1, and 60:1 (mol ratio). The pH was adjusted to 7.1.

Chlorella sp. cells under exponential phase were transferred to the culture medium with no nutrient addition for 48 h at three light intensities, 50 μmol photons m^{-2} s^{-1} (light-50), 100 μmol photons m^{-2} s^{-1} (light-100), and 200 μmol photons m^{-2} s^{-1} (light-200), for light acclimation. Light intensity was measured with LI-190SA Light Meter (Li-Cor Inc., USA). Cells were centrifuged (15 min, 4500 rpm), and after discarding the supernatant, the *Chlorella* sp. cells were resuspended in sodium bicarbonate (15 mg L^{-1}). The suspension was centrifuged to remove any nutrients from the surface of the cells, and then, *Chlorella* sp. cells were transferred to 1.5-L modified BG-11 medium in 3-L glass flasks, yielding an initial algal concentration of 1×10^5 cells mL^{-1}. The culture was grown at 25 °C under a 12:12 light/dark cycle with the above three light intensities. The culture was shaken three to four times each day to mix uniformly and to promote the dissolution of carbon dioxide into the culture medium. Culture and experimental samples were taken in duplicate. The samples for nutrient analysis were taken in the middle of the light phase to avoid diurnal effects (Leonardos and Geider 2004) on days 1, 2, 3, 4, 5, 6, 7, 9, and 11 at light-50 and days 1, 2, 3, 4, 5, 6, 7, and 9 at light-100 and light-200.

Nutrient analysis

The concentrations of dissolved nitrate (NO_3^--N) and phosphate (PO_4^{3-}) were determined as follows. Soluble forms of the those compounds were retrieved by filtering samples through a glass fiber (Whatman GF/F, UK) pretreated at 450 °C for 4 h in a muffle furnace. All nutrient parameters were analyzed by a UV-vis spectrophotometric method,
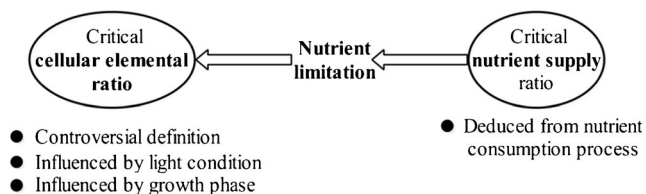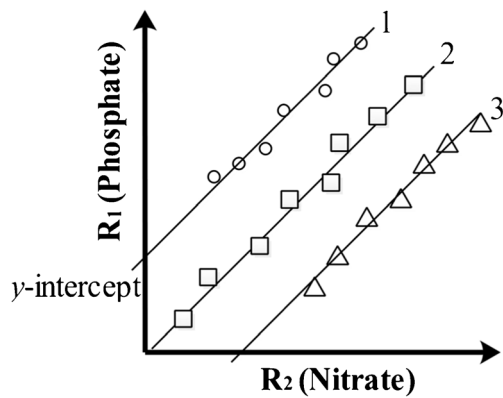
Fig. 3 Nutrient drawdown ratio calculated by linear regression of the phosphate (R_1) and nitrate (R_2) concentrations in the medium during the cultivation process. *1*: nutrient drawdown ratio <nutrient supply ratio, *y*-intercept >0, and microalgal growth is R_2 limited; *2*: nutrient drawdown ratio = nutrient supply ratio, *y*-intercept = 0, microalgal growth is R_1 and R_2 limited, and this nutrient supply ratio is the critical nutrient supply ratio; *3*: nutrient drawdown ratio > nutrient supply ratio, *y*-intercept <0, and microalgal growth is R_1 limited. *Y*-intercept increases with degree of nutrient R_2 limitation

following standard methods in Monitoring and Analyzing Methods for Water and Wastewater (CEPA 2002).

Cellular C, N, and P content analysis

Particulate samples were collected by glass fiber filters (Whatman GF/F, diameter = 47 mm) that had been precombusted at 450 °C for 4 h. For particulate phosphorus, the samples were transferred to 50-mL colorimetric cylinders with screw caps, and 25 mL of deionized water and 4 mL potassium peroxydisulfate (5 %) were added. The cylinders were tightly capped and autoclaved at 120 °C for 30 min. The particulate phosphorus was prepared in the same way (CEPA 2002) as phosphate, the samples were filtered prior to analysis to eliminate debris (Pujopay and Raimbault 1994). Particulate carbon and nitrogen were analyzed using a Euro Vector EA3000 CHNS/O Elemental Analyzer (Euro Vector Instrument & Software, Italy) with the Callidus 5.1 software. The parameters were as follows: run time = 250 s, front

furnace temperature = 980 °C, GC oven temperature = 140 °C, sample delay = 10 s, and oxidation time = 8.7 s. The samples were dried at 60 °C for 24 h and preserved at −20 °C until analysis. During the analysis, all microalgae samples were scraped from the glass fiber filter and then enclosed in tinfoil. Scraping represents a loss of biomass as it is impossible to remove all algal cells from a GF filter, but this does not matter as the resulting data are used only to determine the elemental ratios, not the elemental content per cell. The glass fiber filters (Whatman GF/F; diameter = 25 mm) for elemental analysis were also preheated at 450 °C for 4 h. The instrument was calibrated using sulfanilamide ($C_6H_8O_2N_2S$).

Critical nutrient supply ratio and critical cellular elemental ratio

In batch experiments, concentrations of extracellular phosphate and nitrate decrease with time, and the nutrient drawdown ratio can be calculated by linear regression of the nitrate and phosphate concentrations in the medium during the cultivation process. The slope of the linear regression is the nutrient drawdown ratio, and the *y*-intercept of linear regression is the residual nutrient (see Fig. 3). When the linear regression equation yields a *y*-intercept of zero, as well as slope = nutrient supply ratio, this nutrient supply ratio is the critical one (see nutrient supply ratio 2 in Fig. 3). There is only one critical nutrient supply ratio among all possible nutrient supply ratios under a specific light intensity, but we cannot experimentally test all possible nutrient supply ratios. Therefore, we can only estimate the range of the critical nutrient supply ratio, and the *y*-intercepts of those nutrient supply ratios closest to 0, i.e., the lower limit of the range with negative *y*-intercept and upper limit of the range with positive *y*-intercept, such as nutrient supply ratios 1 and 3 in Fig. 3.

The critical cellular elemental ratio is the C/N/P ratio in *Chlorella* sp. cells grown under critical nutrient supply ratio, and the range of the critical cellular elemental ratio is the range of cell elemental ratio under nitrate and phosphate limitations.
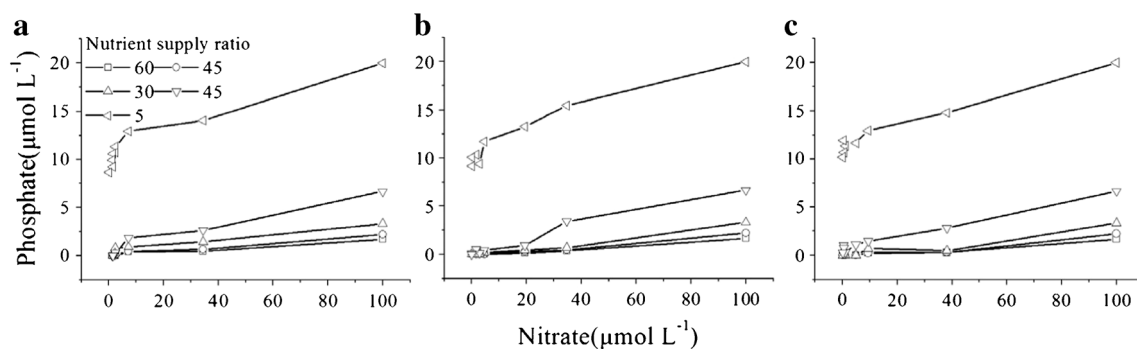
Fig. 4 Phosphate versus nitrate drawdown curves for *Chlorella* sp. grown under three light intensities (**a** light-50, **b** light-100, **c** light-200). Concentrations of phosphate and nitrate decreased with culture time; their values are the highest in the first day and gradually declined to the lowest at the end of the experiment

Table 1 Critical nutrient supply ratios of *Chlorella* sp. grown under three light intensities

Light intensity (μmol photons m^{-2} s^{-1})	Critical nutrient supply ratio	
50	30–45	Approaches 45
100	5–15	Approaches 15
200	30–45	Midway between 30 and 45

Nutrient limitation is evaluated by the possible range of critical nutrient supply ratios.

All figures were constructed using OriginPro 8, and statistical analyses were performed using IBM SPSS Statistics 19.

Results

Nutrient consumption

Our data support the second nutrient drawdown pattern (Fig. 1). In the first phase, the nutrients of nitrate and phosphate were consumed at a fixed ratio until one decreased to subdetection levels. Then, the other nutrient was consumed to subdetection levels in the second phase (Fig. 4). At a nutrient supply ratio of 5, *Chlorella* sp. first consumed all nitrate while phosphate remained plentiful at the end of the experiment. In the other nutrient treatments (nutrient supply ratios of 294 10, 15, 30, 45, and 60) *Chlorella* sp. first consumed all the phosphate, followed by all the nitrate.

Implications for nutrient limitation based on nutrient drawdown ratio

The critical nutrient supply ratio (Table 1) under light-50, light-100, and light-200, as determined from the *y*-intercepts of the linearly regressed nutrient drawdown curves, is listed in Table 2. Under high and low intensities (light-200 and light-50), the critical nutrient supply ratio ranged from 30 to 45. Under medium light intensity (light-100), the critical nutrient supply ratio ranged from 5 to 15.

The response of cellular stoichiometry in different growth phases

In the first growth phase, *Chlorella* sp. cellular C/P and N/P increased with increasing nutrient supply ratio up to nutrient supply ratio = 45; however, they decreased when the nutrient supply ratio = 60 under light-50 and light-100. *Chlorella* sp. cellular C/P and N/P increased with increasing nutrient supply ratio up to nutrient supply ratio = 60 under light-200. However, the *Chlorella* sp. cellular C/N was not influenced by the nutrient supply ratio. In addition, intracellular N/P and C/P ratios were much lower under nitrate limitation than phosphate limitation (Fig. 5).

The critical cellular C/N/P ratios under light-50, light-100, and light-200 were approximately $C_{227}N_{21}P_1$, $C_{115}N_{13}P_1$, and $C_{196}N_{17}P_1$, respectively, and the average critical cellular C/N/P ratio was $C_{179}N_{17}P_1$ in the first phase. However, a more useful quantity would appear to be the range of the critical cellular elemental ratio. Between the upper and lower thresholds of the cellular N/P ratio, cell growth is N and P colimited; cell growth is nitrogen limited when the cellular N/P ratio falls below the lower threshold, whereas cellular N/P ratios exceeding the upper threshold indicate phosphate limitation (Hecky et al. 1993; Falkowski 1997; Tyrrell 1999). Similarly, growth is nitrogen and phosphate limited if the cellular C/N and C/P ratios exceed their upper thresholds, respectively. The critical cellular C/N ranges were 10.53–11.16, 8.92–9.19, and 11.79–12.66 (average 10.41–11.00); the critical cellular N/P ranges were 15.90–22.52, 11.71–17.35, and 12.84–19.84 (average 13.48–19.90), and the respective critical cellular C/P ranges were 159.91–237.30,

Table 2 Summary of phosphate versus nitrate drawdown ratio for *Chlorella* sp. grown under different nutrient supply ratios and three light intensities (μmol photons m^{-2} s^{-1})

Nutrient supply ratio	60			45			30			15			5		
Initial phosphate	PO$_4^{3-}$-P = 1.67			PO$_4^{3-}$-P = 2.22			PO$_4^{3-}$-P = 3.33			PO$_4^{3-}$-P = 6.67			PO$_4^{3-}$-P= 20		
	Slope	Intercept	*R*	Slope	Intercept	*R*	Slope	Intercept	*R*	Slope	Intercept	*R*	Slope	Intercept	*R*
Light intensity 50	52.70	12.90	0.98	44.68	1.04	1.00	38.44	−28.27	1.00	15.60	−7.96	0.96	11.77	−135.55	0.98
n	8			10			8			8			8		
Light intensity 100	47.47	21.95	0.99	39.37	13.84	0.99	28.69	5.90	0.99	13.97	0.36	0.95	11.53	−134.70	0.98
n	8			8			10			12			8		
Light intensity 200	49.29	17.74	1.00	42.48	5.44	0.98	29.85	−4.48	0.96	15.23	−6.07	0.98	11.89	−138.67	0.99
n	8			8			8			10			8		

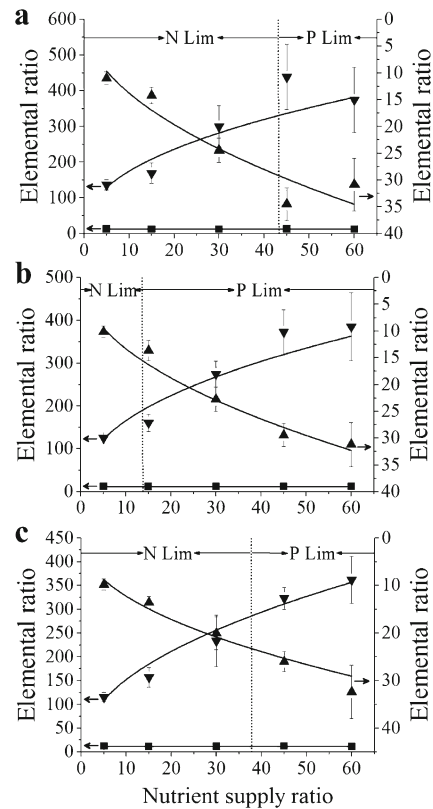*n* number of independent replicates, *R* Pearson's correlation coefficient of linear regression
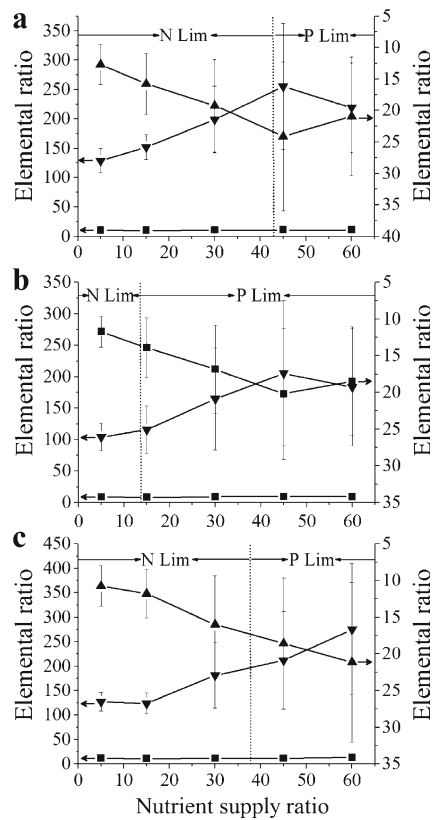
Fig. 5 The *Chlorella* sp. cellular elemental ratio in the first growth phase (light-50 (**a**), n = 6 ; light-100 (**b**), n = 6 ; light-200 (**c**), n = 6), *triangles* N/P ratio in cells; *inverted triangles* C/P ratio in cells; *squares* C/N ratio in cells. *Error bars* represent the standard errors, *arrows* denote the corresponding *y* axes, and *N Lim* and *P Lim* denote nitrate and phosphate limitations, respectively

Fig. 6 The *Chlorella* sp. cellular elemental ratio during the second growth phase under different light intensities (light-50 (**a**), n = 12; light-100 (**b**), n = 10; light-200 (**c**), n = 10). *Triangles* N/P ratio in cells; *inverted triangles* C/P ratio in cells; *squares* C/N ratio in cells. *Error bars* represent the standard errors, *arrows* denote the corresponding *y* axes, and *N Lim* and *P Lim* denote nitrate and phosphate limitations in the medium, respectively

104.02–167.39, and 144.30–243.66 (average 136.21–216.12) at light-50, light-100, and light-200, respectively (Table 3).

In the second growth phase, intracellular N/P and C/P ratios increased as the nutrient supply ratio increased. More specifically, intracellular N/P and C/P ratios increased from 9 to 32 and from 115 to 385, respectively, while the C/N ratio remained almost constant. In addition, the intracellular N/P and C/P ratios were much lower under nitrate limitation than phosphate limitation (Fig. 6).

In the second growth phase, the critical cellular C/N ranges were 12.31–12.36, 12.25–12.44, and 11.69–11.88 (average 12.08–12.23); the critical cellular N/P ranges were 16.55–32.67, 10.12–24.24, and 14.47–29.18 (average 13.71–28.70), and the critical cellular C/P ranges were 201.47–406.14, 125.05–298.44, and 168.65–342.42 (average 165.12–349.00) at light-50, light-100, and light-200, respectively. In addition, the critical cellular N/P and C/P ratios were much higher in the second growth phase than in the first growth phase (Table 4).

Table 3 Summary of critical *Chlorella* sp. cellular elemental ratio under different light intensities in the first growth phase

Light intensity (μmol photons m^{-2} s^{-1})			50	100	200
First growth phase	P Lim	C/N	11.16 (1.62, n = 12)	9.19 (1.65, n = 24)	12.66 (1.82, n = 12)
	N Lim		10.53 (1.96, n = 18)	8.92 (1.63, n = 6)	11.79 (2.45, n = 18)
	P Lim	N/P	22.52 (10.35, n = 12)	17.35 (6.79, n = 24)	19.84 (9.60, n = 12)
	N Lim		15.90 (5.79, n = 18)	11.71 (2.07, n = 6)	12.84 (4.88, n = 18)
	P Lim	C/P	237.30 (90.79, n = 12)	167.39 (87.73, n = 24)	243.66 (117.75, n = 12)
	N Lim		159.91 (46.02, n = 18)	104.02 (21.91, n = 6)	144.30 (47.93, n = 18)

Numbers in parentheses = SD

n number of independent replicates, *N Lim* nitrate limitation, *P Lim* phosphate limitation

Table 4 Summary of critical *Chlorella* sp. cellular elemental ratio under different light intensities in the second growth phase

Light intensity (μmol photons m^{-2} s^{-1})			50	100	200
Second growth phase	P Lim	C/N	12.36 (1.92, *n* = 24)	12.25 (1.34, *n* = 40)	11.88 (1.25, *n* = 20)
	N Lim		12.31 (2.37, *n* = 36)	12.44 (0.99, *n* = 10)	11.69 (1.37, *n* = 30)
	P Lim	N/P	32.67 (4.43, *n* = 24)	24.24 (7.46, *n* = 40)	29.18 (5.25, *n* = 20)
	N Lim		16.55 (6.06, *n* = 36)	10.12 (1.08, *n* = 10)	14.47 (4.80, *n* = 30)
	P Lim	C/P	406.14 (95.13, *n* = 24)	298.44 (103.73, *n* = 40)	342.42 (42.44, *n* = 20)
	N Lim		201.47 (80.48, *n* = 36)	125.05 (5.96, *n* = 10)	168.65 (59.58, *n* = 30)

Numbers in parentheses = SD

n number of independent replicates, *N Lim* nitrate limitation, *P Lim* phosphate limitation

Discussion

Nutrient drawdown ratio In contrast with the same nutrient drawdown ratios under different nutrient supply ratios in the second nutrient drawdown pattern, nutrient drawdown ratio increased with increasing nutrient supply ratio in our study (Table 2). The increased nutrient drawdown ratio may result from different nutrient requirements of *Chlorella* sp. for synthesizing various cellular machineries (Klausmeier et al. 2004a; Arrigo 2005), For example, light and nutrient uptake machinery requires more N than P, and growth machinery (such as ribosomal and RNA) requires more P than N. Nutrient condition, which is the nutrient supply ratio, is the main factor that affects cellular machinery composition; therefore, various cellular machinery compositions influence cellular nutrient requirement and finally resulted in different nutrient drawdown ratios. The increased nutrient drawdown ratio may also result from luxury consumption of phosphate with increased phosphate concentration as the nutrient supply ratio decreases. Finally, insufficient data for statistical analysis may result in increased nutrient drawdown ratio; if we increase the initial nutrient concentration or decrease the initial biomass density, the difference in nutrient drawdown ratio with a different nutrient supply ratio may decrease, because many studies have shown that microalgae require equal amounts of both nutrients at different nutrient supply ratios when nutrient concentrations are high.

Critical nutrient supply ratio in different light intensities Compared with medium light intensity, *Chlorella* sp. required more nitrate under high and low intensities, so light intensity had a large effect on the critical nutrient supply ratio in our research. Because light-harvesting pigment–protein complexes and components of the photosynthetic electron transfer chain account for a large but variable fraction of the cell mass and cellular N (Geider et al. 1996), microalgae allocate most of the nitrate taken up for photosynthetic purposes. At low light intensity, microalgae allocate large quantities of nitrate to photosynthetic light-harvesting apoproteins, enabling the capture of sufficient energy to meet cell growth and maintenance requirements. At high light intensity, much of the nitrate is allocated to the synthesis of proteins that protect the photosynthetic system from photo-oxidative damage and photoinhibition (Larkum and Howe 1997). Medium light intensity is suitable for microalgal growth without photo-inhibition or photo-insufficiency and, then, a relatively lower critical nutrient supply ratio.

Critical cellular elemental ratios in different light intensities and growth phases The critical cellular N/P ratios obtained in our research are consistent with those of other freshwater and marine chlorophytes. Hillebrand and Sommer (1999) reported a critical cellular N/P ratio of 17 in benthic microalgae, while Klausmeier et al. (2004a) showed that the ranges of critical cellular N/P in chlorophytes were 5 to 21. Furthermore, the average critical cellular N/P in our study is close to the Redfield ratio (Redfield 1960), indicating that the Redfield ratio of 16 is not a universal optimum but, instead, represents the average of cellular N/P ratios under different growth conditions. Arrigo and colleagues (Arrigo 2005) also established this point. The critical C/P ratio varied from 136.21:1 to 216.12:1 in the first growth phase and 165.12:1–349.00:1 in the second growth phase; this is much higher than the Redfield ratio, suggesting that *Chlorella* sp. has low phosphate cost in carbon fixation.

Wynne and Rhee (1986) investigated how the critical cellular elemental ratio in algae changed with various light intensities. They reported that light intensities can strongly influence algal critical cellular elemental ratio, and this agrees well with our results. Wynne and Rhee then attributed the various critical cellular elemental ratios to the modulation of cell N content in different light conditions. In our study, the critical cellular N/P at light-50 and light-200 is much higher than at light-100, which reflects a high N content in *Chlorella* sp. cells at light-50 and light-200. At light-50, more nutrients (N) are devoted to the photosynthesis module, increasing the cells' ability to absorb light energy and convert it to chemical energy, thereby increasing the critical cellular N/P ratio.

Conversely, at light-200, microalgae are exposed to excess energy and more nutrients (N) are devoted to the photo-protection module, which resulted in a high critical cellular N/P ratio under light-200 (Larkum and Howe 1997).

In our research, the critical cellular N/P ratio varied from 13.48:1 to 19.90:1 in the first phase and from 13.71:1 to 28.70:1 in the second phase, and the critical cellular C/P ratio varied from 136.21:1 to 216.12:1 in the first phase and from 165.12:1 to 349.00:1 in the second phase. These results support the inference that the critical cellular elemental ratios are significantly affected by cell growth phase. Critical cellular N/P and C/P values in the second phase are much higher than those in the first growth phase. Furthermore, our results are consistent with the model prediction by Klausmeier et al. (2004a), whose theoretical analysis indicated that critical cellular N/P varied with growth phase and a lower critical cellular N/P in exponential growth phase compared with stationary growth phase. Because of relatively sufficient nutrient in the medium during the first growth phase, the state of *Chlorella* sp. during the first growth phase in our study is close to exponential growth phases, and the state of *Chlorella* sp. during the second phase in our study is close to stationary, when one nutrient in the medium is completely consumed and there is no significant growth.

In conclusion, the critical nutrient supply ratio (nitrate/phosphate) was used to estimate critical cellular elemental ratio under different conditions of light intensity and growth phase. Light intensity was an important factor influencing the critical nutrient supply ratios of *Chlorella* sp., which were approximately 45, 15, and between 30 to 45 under light intensities of 50, 100, and 200 μmol photons m^{-2} s^{-1}, respectively. Critical cellular C/N/P ratios of *Chlorella* sp. in the exponential growth phases were $C_{159.91-237.30}N_{15.90-22.52}P$, $C_{104.02-167.39}N_{11.71-17.35}P$, and $C_{144.30-243.66}N_{12.84-19.84}P$, and these ratios in the stationary growth phase were $C_{201.47-406.14}N_{16.55-32.67}P$, $C_{125.05-298.44}N_{10.12-24.24}P$, and $C_{168.65-342.42}N_{14.47-29.18}P$ at light intensity of 50, 100, and 200 μmol photons m^{-2} s^{-1}, respectively. These results support the hypothesis that the critical cellular elemental ratio, which is used to predict nutrient status, varies and depends on light intensity and growth phase.

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