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Effects of nitrogen on growth and carbohydrate formation in *Porphyridium cruentum*

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Running title: Nitrogen effects on growth and carbohydrates in red algae

Abstract

The microalgae *Porphyridium cruentum* (Rhodophyta) has industrial and pharmaceutical usages, especially for its polysaccharide production. This study aimed to investigate the influence of nitrogen levels as reflected by altered N:P ratios on the biomass and carbohydrate production and content. This was done in batch cultures with molar N:P ratios ranging from 1.6 to 50, using the Redfield ratio of 16:1 as a reference. Algal growth, estimated as final cell number, biomass concentration and maximum specific growth rate were negatively affected at low N:P ratios. The optimal N:P ratio for growth was identified at 35-50, with specific growth rates of 0.19 day^{-1} and maximum cell concentrations of $59 \cdot 10^8$ cells L^{-1} and 1.2 g dry weight of biomass L^{-1}. In addition, cell size varied, with larger cell diameters observed at higher N:P ratios. The cellular carbohydrate content increased under reduced nitrogen availability. However, because accumulation was moderate at the lowest N:P ratio, 0.4 g per g dry weight biomass compared to 0.24 at the Redfield ratio of 16:1, conditions for increased total carbohydrate formation were identified at the N:P ratios optimal for growth. Additionally, carbohydrates were largely accumulated in late exponential to stationary phase.

Keywords: Rhodophyta, red algae, Redfield ratio, nitrogen-to-phosphorous ratio, carbohydrate content, growth rate

1. Introduction

In a bio-based society, sustainable production processes should be based on renewable raw materials. Starch-based biomass of phototrophic organisms helps to fulfill this criterion. In this respect, studies on how culture conditions, such as nutrient availability, could enhance cellular carbohydrate content are important. The microalga *Porphyridium* sp. (Rhodophyta) is a potential source for several products, such as fatty acids and lipids [1, 2], pigments [3], and cell-wall polysaccharides [4]. These polysaccharides are sulphated and their structure gives rise to some unique properties that could lead to a broad range of industrial and pharmaceutical applications (patel et al., 2012). The polysaccharides surround the algal cell as an amorphous capsule. In the marine species *P. cruentum*, this capsule consists of glucose, galactose, xylose, and uronic acid as sugar monomers [5]. The outer part of the capsule can be partly excreted to the surroundings, *i.e.* exopolysaccharides, and give rise to increased viscosity of the medium [6]. Additionally, *P. cruentum* biomass contains starch [6], and cellular contents of carbohydrates of up to 57% have been reported [7]. Thus, the combined amount of carbohydrates in biomass and exopolysaccharides of this microalga could potentially provide the carbon source for fermentation processes, such as bioethanol production [8].

Culture conditions affect carbohydrate content in microalgae. Limited levels of macro-nutrients in the growth medium, such as nitrogen and phosphorous, have been shown to affect formation of carbohydrates in several algae species. For instance, nitrogen limitation increased carbohydrate formation in *Chlamydomonas mexicana* [9] and *Chlorella* spp. [10], and increased starch production in *Tetraselmis subcordiformis* [11]. Phosphorus limitation induced an increase of carbohydrate formation in *Ankistrodesmus falcatus* [12]. Thus, as a mechanism of nutrient depletion, increased accumulation of carbohydrates is expected in the

stationary growth phase, which has also been documented for, *e.g. Chlamydomonas mexicana* [9], *Tetraselmis gracilis* [13] and *Porphyridium aerugineum* [14]. Another effect of variable nitrogen concentration is that cell-wall polysaccharides become increasingly soluble in nitrogen deficient media, and the main part of these were found as exopolysaccharides [15]. Thus, to achieve optimal carbohydrate production, the nitrogen availability needs to be balanced to give a simultaneously high carbohydrate accumulation and a reasonable growth rate, and also to relate the concentration of nitrogen to the available phosphorus, *i.e.* the N:P ratio.

Nitrogen availability largely affects algae growth as indicated by the fact that nitrogen effluents intensify algal blooms [16]. Accordingly, in cultures of *Porphyridium* sp., high nitrogen concentrations were favorable for growth, whereas nitrogen deficiency limited growth [15, 17]. For example in *P. purpureum*, nitrogen limitation led to cessation of growth and significant reductions of chlorophyll and phycoerythrin contents (levy et al, 1990). In this respect, the Redfield ratio (C:N:P 106:16:1 molar composition) [18] has been proposed as a general optimal ratio for algal growth. However, recently the oversimplification of this ratio has been questioned, as optimal N:P ratios seem to be species specific, and in the range of 8.2-45.0 [19].

Algal cellular content may vary in response to light intensity and the type of metabolism, *i.e.* photosynthetic or respiratory and therefore also varies in response to light:dark cycles [20]. In *Porphyridium*, high light intensities stimulated starch and exopolysaccharide formation [6], whereas continuous light illumination enhanced exopolysaccharide formation only [21]. For cost-effective production, the algal cultures are preferably illuminated by natural sunlight, which provides illumination with light:dark cycles.

Given the potential to develop this species as a resource, an extended survey of the effects of N:P ratios is needed. Previous studies of *P. cruentum* and the effect of varying the N:P ratio

was limited to three ratios in the range of 5-20 [17]. The aim of this study was to compare a broader range of N:P ratios to determine the effect of nutrient availability on growth properties and carbohydrate content and production, and to identify the optimal molar N:P ratio for *P. cruentum* growth. This was done using batch cultures illuminated in dark/light cycles with N:P ratios ranging from 2-50, using the Redfield ratio as a reference.

2. Experimental procedures

2.1. Strain, medium and cultivation conditions

Porphyridium cruentum (strain GUMACC 25, UTEX 161) was obtained from Gothenburg University's Microalgae Algal Culture Collection, Sweden. The medium, f/2 [22], was prepared from filtered, autoclaved natural seawater with five times higher concentrations of sodium phosphate, trace metals, and vitamins compared to the original formula, to promote good algal growth and ensure other nutrients did not become limited. Altered N:P ratios (1.6-50) were achieved by changing the nitrate concentration using 0.29-9.1 mM of $NaNO_3$, while keeping the phosphate concentration constant at 0.18 mM, giving a range below (denoted as nitrogen limited) and above (denoted as nitrogen excess) the Redfield ratio of 16:1 mol N per mol P, used as the reference. The standard f/2 medium has an N:P ratio of 22. Cells were grown in batch cultures in culture flasks with ventilation caps (Sarstedt) at 23±1°C and 150 rpm agitation. The cultures were illuminated by fluorescent cool-white lamps (color code 840) at an irradiance of 98 µE m^{-2} s^{-1} (7000 lux) with an 18:6 hour light:dark cycle. Samples for total carbohydrates and cell concentration analyses were collected once a day during the dark phase. Two sets of experiments were conducted, one using cultures of 40 mL medium in 50 mL flasks, and the other using 150 mL medium in 250 mL flasks. This gave a volume to

illuminated surface area of 1.6 and 0.86 mL cm^{-2}, respectively. These experiments continued until the stationary phase was reached at days 14 and 12, respectively.

2.2. Analyses of cells and total carbohydrates

Cell concentration was estimated during cultivation using *in vivo* fluorescence of chlorophylls in 100 µL of cell suspension in black 96 well microtiter plates (FluoroNunc, Nunc Inc.). These were measured using a FLUOstar Omega plate reader (BMG LABTECH) held at a constant temperature of 25ºC, with optical filters for excitation at 400-480 nm and emission at 600-680 nm. Measurements on medium only were made in each run and used as background correction. Maximum specific growth rates were calculated using linear regression of the natural logarithm of the *in vivo* fluorescence data against time during the exponential growth phase [23].

Accurate cell numbers (cells L^{-1}) at the stationary phase were determined by counting cells in a Bürker chamber (C-Chip disposable hemocytometer DHC-B01, Digital Bio).

Biomass concentration (g L^{-1}) was obtained using the dry weight of cells collected from 5 mL of culture broth on a 0.45 µm hydrophilic polyethersulfonate filter (Sartorius Stedim Biotech, Germany). The filter was dried for 15 minutes in a microwave oven at 125 W, equilibrated in a desiccator overnight, and then weighed. An equivalent volume of media was filtered and the readings used for background correction [24].

Cell morphology was studied using an inverted microscope (Leica DMI4000B) with a CCD camera (Leica DFC360 FX) at 100× magnification (objective Leica HCX PL APO 100x NA 1.40). Average cell size for each N:P-ratio was estimated by determining the diameter of 90 cells from all replicates using the size tool of the accompanying software (Leica AF6000 E Software).

Total carbohydrates were analyzed using the phenol-sulfuric acid method [25] for the entire culture broth in triplicate. Glucose was used as the standard, and measurements on medium in

each run were used as background correction. The assay was performed in microtiter plates. Absorbance was measured at 488 nm in a FLUOstar Omega plate reader (BMG LABTECH).

2.3. Statistics

A two tailed student's t-test was used to test the null hypothesis that there was no difference between the different N:P ratios. Data was tested for significance against the reference (N:P ratio at 16). For deviant comparisons statements are given with the data. The average standard deviations were calculated from the mean of variances of replicates within each set of experiments.

3. Results

3.1. Batch growth of *P. cruentum* at different N:P ratios

The effects of altered nutrient levels on *P. cruentum* growth and accumulation of carbohydrates in biomass was studied in a first set of cultivations using media with a large range of molar N:P ratios. The growth curves displayed the expected characteristics, with a lag phase during the first one or two days, an exponential growth phase, a stationary phase when growth ceased, and finally a decay phase when cells were dying during the last few days (Figure 1A). The exponential growth phase ranged from the first to seventh day for cultures at N:P ratios of 16 and higher, whereas at nitrogen limitation the lag phase was longer (Figure 1A). The maximum cell concentration was lower at nitrogen limitation (N:P of 1.6 and 4.9) and higher at nitrogen excess (N:P of 35 and 50) compared to the reference condition of 16:1. Similarly, the total carbohydrate concentration was lower at nitrogen limitation and higher at nitrogen excess (Figure 1B). Cellular carbohydrate content slowly increased during the initial exponential growth phase until approaching the late exponential phase at day 4-5, when the content more rapidly increased (Figure 1C). Once the stationary phase was reached,

carbohydrate content remained constant until the end of the experiment. In the cultures at N:P ratios of 1.6 and 4.9 carbohydrate accumulation started later at day 6, at the beginning of the stationary phase (Figure 1C). However, all conditions reached a similar maximum carbohydrate content of approximately 26 µg $f.u.^{-1}$, except for the N:P ratio of 1.6, which was significantly lower at 15 µg $f.u.^{-1}$ ($p<0.01$, $df = 19$).

The low cell concentrations observed at the lowest nitrogen availability of 1.6 and 4.9 were associated with low maximum specific growth rates (Figure 2A). Correspondingly, nitrogen excess at N:P ratios of 35 and 50 promoted a maximum specific growth rate nearly two times faster compared to the specific growth rate of 0.12 day^{-1} at the Redfield ratio.

At the end of the experiment for the first set of cultures (day 14), cells at the two highest N:P ratios were significantly heavier, and at the two lowest N:P ratios were significantly lighter (Figure 2B). Furthermore, there was a non-significant tendency for the cell diameters to be larger at nitrogen excess for N:P ratios of 35 and 50, and smaller at nitrogen limitation for N:P ratios of 1.6 and 4.9 (Figure 2C).

3.2. Cellular properties of *P. cruentum* at the stationary phase after growth at different N:P ratios

In the second set of cultures using larger volumes, limited nitrogen availability at an N:P ratio of 1.6 significantly reduced cell density. By contrast, both cell number and biomass were significantly higher at nitrogen excess (N:P ratios of 35 and 50) compared to the reference of 16:1 (Table 1).

Total carbohydrate concentration followed a similar pattern to cell densities. However, only under nitrogen limiting conditions, at an N:P ratio of 4.9, a higher carbohydrate accumulation were strongly indicated at 0.32 g g^{-1} dry weight compared to the reference of 16:1 (Table 1). In spite of this, the carbohydrate content for the 1.6:1 ratio was even higher (0.40 g g^{-1} dry

weight) but yielded a non-significant comparison to the reference of 16:1, as the standard deviation was very large due to low cell densities. By contrast, no differences were found for the cellular carbohydrate content at the different N:P ratios (data not shown). Average content was 42±6 pg glc eq. $cell^{-1}$.

4. Discussion

Culturing conditions aiming at fast growth and high carbohydrate production for the red microalga *P. cruentum* were studied and described. For optimal production of a cellular component, both cellular abundance and total biomass formed are important for overall efficiency. In our study under nitrogen limited conditions, the largest amount of carbohydrates per cell was observed, but overall cell density was low. Thus, the total amount of carbohydrate per volume of culture is a better variable to examine when identifying optimal culturing conditions. The carbohydrate content of the cells only varied to a certain extent, and the increase in content was severely counteracted by the fact that less biomass was formed, yielding an overall lower formation at nitrogen limitation. Optimal production was thus identified during nitrogen excess.

The influence of nutrient limitation has been previously observed on accumulation of both carbohydrates and lipids [10, 26]. Nutrient limitation also imposes other physiological changes in algal cells. By limiting nitrogen, cell volume of the green alga, *Ankistrodesmus falcatus,* decreased, and displayed lower density. By contrast, limiting the availability of phosphorous resulted in the opposite effect [12]. Here, we observed effects on cell size in response to nutrient availability, with the smallest cell weight and diameters seen at reduced nitrogen availability, and the largest at increased availability. To some extent, this can be explained by altered macromolecular composition and also by the fact that nitrogen limitation

implies a decline in photosynthetic performance and remodeling of the photosynthetic machinery [27]. Additionally, nitrogen deprivation inhibits growth, only committed cells divide, and the progeny are smaller. Another explanation, possibly valid for *Porphyridum* spp., is that at conditions with small cells *i.e.* under nitrogen limitation, cell wall polysaccharides become increasingly soluble [17, 21]. The exertion of polysaccharides into the fluid could cause the cell size reduction and simultaneously increase the viscosity of the media, which was also observed during the ageing the culture. We did not analyse viscosity but we recommend it for future studies in order to increase the understanding of how nitrogen limitation alter the proportion of exopolysaccharide to intracellular carbohydrate content.

By contrast, phosphorus abatement at higher N:P ratios blocks the cell cycle, preventing the synthesis of nucleic acids and cell division. Consequently, cell size will be larger [28, 29]. Additionally, at higher nitrogen concentrations, the less soluble cell wall polysaccharides add to an increased cell size. At higher N:P ratios the increased cell size allows for greater accumulation of carbohydrates in the cell, which is also indicated by the similar cellular carbohydrate content of 42 pg glc eq. $cell^{-1}$ found for all N:P ratios.

Similar to other studies on *Porphyridium* strains [17, 21], we found that high nitrogen availability, as reflected by the high N:P ratios, was very beneficial for growth. However, increasing the level of nitrogen too much causes a limitation of other nutrients, such as phosphorous [29], trace metals and vitamins. Under such conditions, growth will be negatively affected. Other factors that might limit phototrophic based growth are reduced light availability due to shading, agglomeration, and poor gas exchange rates with limited CO_2 availability.

The effect of nutrient limitation occurs when cells enter the stationary phase and accumulation of carbohydrates is induced. This was observed here and manifested by a 10-15 fold increase

in the carbohydrate content, independent of the N:P ratio, when entering the stationary phase, as compared to the content for cells in the exponential growth phase (Figure 1C). However, as previously reported [15] an additional accumulation of carbohydrates during the stationary phase is expected. However, in our cultures only a small increase could be seen under some of the conditions (Figure 1C). In fact, the carbohydrates concentration in our study was lower than previous studies with the same species (ref) which might be either due to the limitation in carbon supply in our batch cultures without air bubbling or the presence of bacterial contamination in the culture which leads to consumption and further reduction of exopolysaccharides., For this purpose, usage of aerated axenic cultivation is desired for production of exopolysaccharide from *P. cruentum*.

The generalization of the canonical N:P ratio of 16, the Redfield ratio, has been debated in recent decades as not being a universally optimal ratio for all phytoplankton, but rather an average of species-specific N:P ratios [19]. Arrigo [30] classified phytoplankton as generalists, bloomers, and survivalists, which have cellular N:P ratios at the Redfield ratio, below 10 or above 30, respectively, and a consortium of these groups gives rise to the combined Redfield ratio. In respect to *P. cruentum*, the optimal growth and biomass production was observed in the range of N:P ratios at 35 to 50, far above the Redfield ratio. According to this classification, this alga could then be categorized in the group of survivalist microalgae, for which the cells contain large resource-acquisition machinery.

In summary, for an optimal carbohydrate formation, cellular accumulation and growth performance are important factors to consider. Our data suggest that the optimal N:P ratio for both growth and carbohydrate formation by *P. cruentum* is in the range of 35-50 mol N per mol P.

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Table including legend

Table 1. Cellular characteristics of *Porphyridium cruentum* at stationary phase (250 ml culture flasks). Data representative of the stationary phase are given - maximum number of cells (x10^8 cells L^{-1}), biomass concentration (g dry weight [dw] L^{-1}) and total carbohydrates (g glucose equivalents [glc eq.] L^{-1}) at different molar N:P ratios of 1.6, 4.9, and 11 (nitrogen limitation), 24, 35, and 50 (nitrogen excess), and the Redfield ratio at 16:1. Means for the triplicate cultures and all days during the duration of the stationary phase (day 5-10) are shown with ± standard deviation.

N:P ratio (N-mol P-mol^{-1})	Number of cells (x10^8 cells L^{-1})	Biomass concentration (g dw L^{-1})	Total carbohydrate concentration (g glc eq. L^{-1})	Carbohydrate content (g glc eq. g dw cells^{-1})
1.6	2.8±0.8***	0.04±0.01***	0.02±0.004***	0.40±0.14
4.9	32.7±5.5	0.47±0.10	0.15±0.02	0.32±0.03*
11	33.7±3.8	0.48±0.02	0.15±0.02	0.29±0.03
16	29.9±3.9	0.51±0.06	0.13±0.01	0.24±0.03
24	32.2±2.8	0.54±0.03	0.13±0.01	0.25±0.02
35	59.4±3.3***	1.18±0.08***	0.22±0.01***	0.20±0.02
50	58.7±1.7***	1.22±0.15*	0.23±0.01***	0.20±0.03

Data significant (df = 10) at *** $p<0.01$ and with strong indications at *$p<0.1$ compared to N:P ratio of 16.

Figure legends

Figure 1. *Porphyridium cruentum* cells in batch cultures (50 mL flasks) at different molar N:P ratios comparing nitrogen limitation at 1.6 (●), 4.9 (▲), and 11 (■); nitrogen excess at 24 (○), 35 (Δ), and 50 (□); and the Redfield ratio of 16:1 (X). A. Cell concentration estimated by *in vivo* fluorescence (f.u. = fluorescence units) on a logarithmic scale. B. Total carbohydrates (g L^{-1} broth). C. Carbohydrate content (µg glucose equivalents [glc eq.] per fluorescence units [f.u.]); representative data are shown (N:P of 11, 24, 35 and 50 are similar to the reference). Means for the triplicate cultivations at each time point are shown with ± standard deviation as error bars.

Figure 2. Cellular properties of *Porphyridium cruentum* cells in batch culture (50 mL flasks) at different molar N:P ratios, comparing nitrogen limitation at 1.6, 4.9, and 11; nitrogen excess at 24, 35, and 50; and Redfield ratio at 16:1. Means for the triplicate cultures are shown. A. Maximum specific growth rate ($days^{-1}$) at exponential phase calculated from *in vivo* fluorescence data. Error bars show the average standard deviation (9.44%) estimated using all data, with 19 degrees of freedom. B. Cellular dry weight (µg $f.u.^{-1}$) at the end of the experiment (f.u. = fluorescence units of *in vivo* fluorescence). Error bars show the average standard deviation (8.05%) estimated using all data giving 19 degrees of freedom. C. Cell diameter (µm) at the end of the experiment. The means of 90 cells from the triplicate cultures are shown, with standard deviation as error bars. Micrographs showing representative cells from N:P ratios 4.9, 16 and 35 are included with a scale bar representing 2 µm. Data significant at ***$p<0.01$ and **$p<0.05$ and with strong indications at *$p<0.1$ compared to N:P ratio of 16.

Figure 1

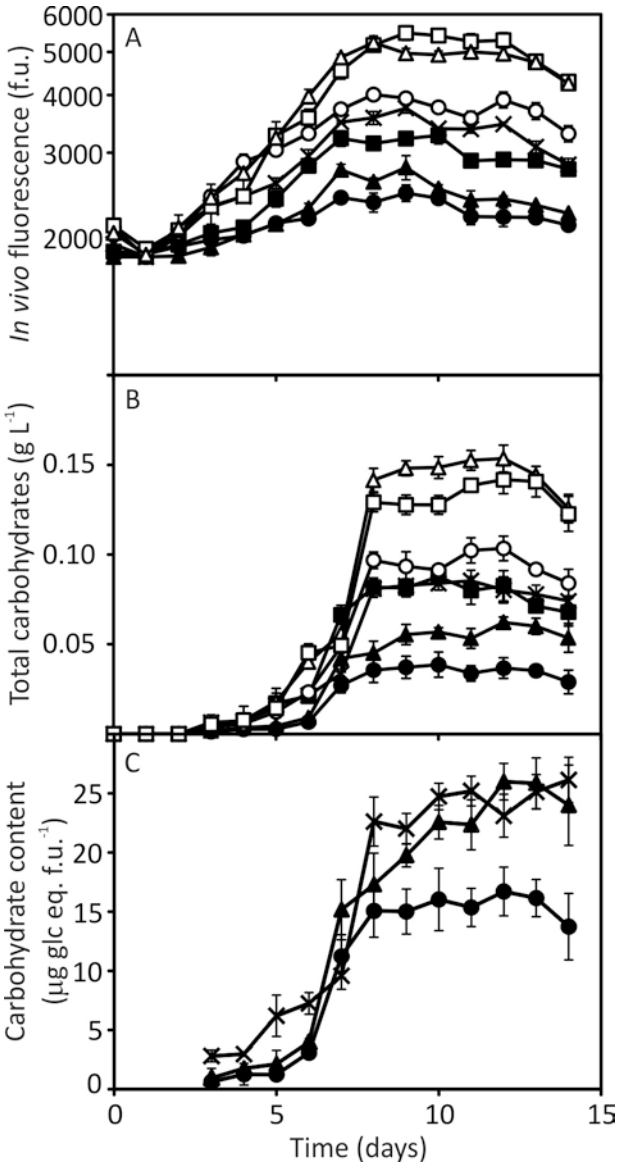
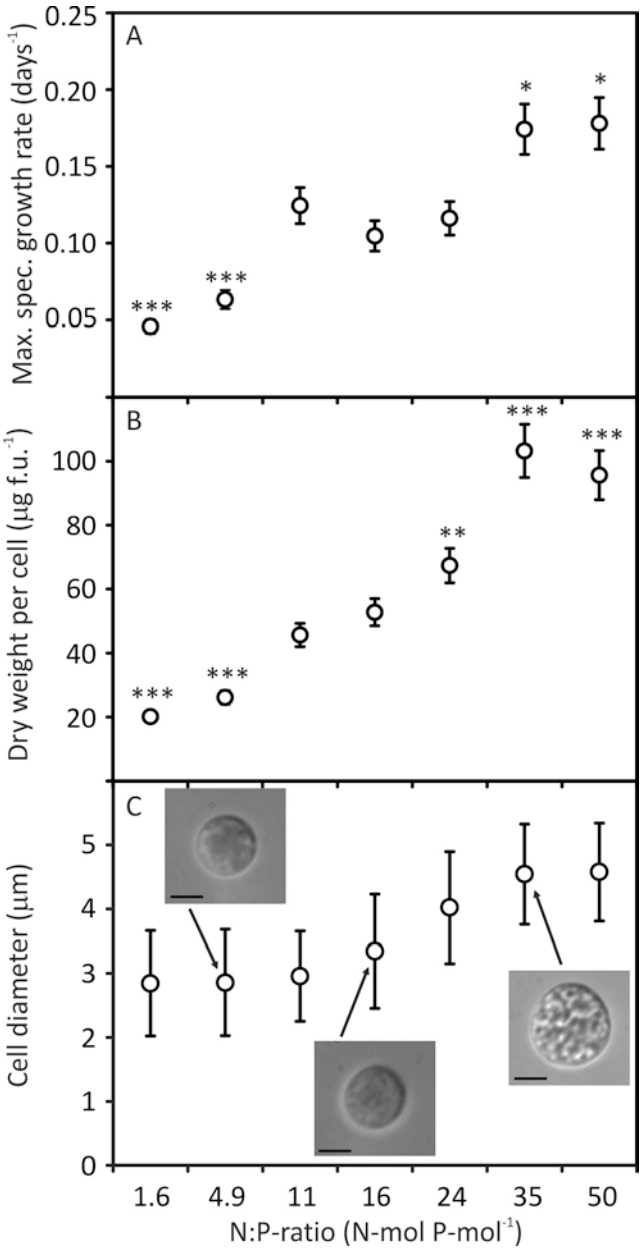

A
B
C
In vivo fluorescence (f.u.)
Total carbohydrates (g L-1)
Carbohydrate content (µg glc eq. f.u.-1)
Time (days)
6000
5000
4000
3000
2000
0.15
0.10
0.05
25
20
15
10
5
0
0
5
10
15

Figure 2


A
Max. spec. growth rate (days-1)
0.25
0.20
0.15
0.10
0.05
***
***
*
*
B
Dry weight per cell (µg f.u.-1)
100
80
60
40
20
***
***
**
***
***
C
Cell diameter (µm)
5
4
3
2
1
0
1.6
4.9
11
16
24
35
50
N:P-ratio (N-mol P-mol-1)