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# Quantifying Cyanothece growth under DIC limitation



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# ABSTRACT

The photoautotrophic, unicellular N<sub>2</sub>-fixer, *Cyanothece*, is a model organism that has been widely used to study photosynthesis regulation, the structure of photosystems, and the temporal segregation of carbon (C) and nitrogen (N) fixation in light and dark phases of the diel cycle. Here, we present a simple quantitative model and experimental data that together, suggest external dissolved inorganic carbon (DIC) concentration as a major limiting factor for *Cyanothece* growth, due to its high C-storage requirement. Using experimental data from a parallel laboratory study as a basis, we show that after the onset of the light period, DIC was rapidly consumed by photosynthesis, leading to a sharp drop in the rate of photosynthesis and C accumulation. In N<sub>2</sub>-fixing cultures, high rates of photosynthesis in the morning enabled rapid conversion of DIC to intracellular C storage, hastening DIC consumption to levels that limited further uptake. The N<sub>2</sub>-fixing condition allows only a small fraction of fixed C for cellular growth since a large fraction was reserved in storage to fuel night-time N<sub>2</sub> fixation. Our model provides a framework for resolving DIC limitation in aquatic ecosystem simulations, where DIC as a growth-limiting factor has rarely been considered, and importantly emphasizes the effect of intracellular C allocation on growth rate that varies depending on the growth environment.

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# 1. Introduction

By reducing atmospheric  $CO_2$  into bioavailable carbon (C), photosynthesis is the driving process of global ecosystem productivity

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and biogeochemical (nutrient) cycles. Phytoplanktonic organisms are responsible for most aquatic photosynthesis, and account for about half the primary production on earth [1]. A growing body of literature now reveals prokaryotic, nitrogen-fixing organisms as key players in the dynamics of phytoplanktonic communities and the world ocean's primary production. In particular, by their phototrophic capacity and their ability to fix molecular nitrogen (N<sub>2</sub>), unicellular N<sub>2</sub>-fixing cyanobacteria (UCYN) directly or indi-

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rectly contribute to and support primary production [2–4], exerting a direct coupling of the biogeochemical cycles of N and C [5,6].

One of the most intensively studied organismal models of unicellular cyanobacteria is Cyanothece sp. ATCC 51142 (hereafter *Cyanothece*), which also has a capability to fix dinitrogen  $(N_2)$  [7] to survive when bioavailable N, such as NH<sup>+</sup><sub>4</sub> or NO<sup>-</sup><sub>3</sub>, is inaccessible. As in other photo-autotrophic, unicellular N<sub>2</sub>-fixing cyanobacteria (UCYN-B and -C), N<sub>2</sub> fixation in Cyanothece is temporally segregated from carbon fixation [8–10], an evolution enabling protection of the  $O_2$ -sensitive, nitrogenase enzyme responsible for  $N_2$ fixation [11]. Recent studies show that N<sub>2</sub> fixation by UCYN-B is facilitated by the inactivation of PSII [12,13], which may apply to *Cyanothece*. There are cases with in-complete temporal segregation depending on the light periodicity and cellular energy requirements, but the largest part of N<sub>2</sub> fixation tends to occur at night [9.14]. The temporal separation of photosynthesis and  $N_2$  fixation imposes these strains to rely on fixed carbon stored within cells as polysaccharides and on their subsequent respiration, which support the energy costs of N<sub>2</sub> fixation. *Cyanothece* is not an obligate N<sub>2</sub>-fixer and grows well in the presence of bioavailable N, making it a relevant biological model of photo-autotrophic UCYN to investigate the cellular requirements imposed by N<sub>2</sub> fixation on the cellular carbon metabolism, in comparison to nitrate-supported growth. The cellular growth of Cyanothece and its resulting population dynamics thus closely depend on the metabolic pathways of photosynthesis, respiration,  $NO_3^-$  acquisition, and/or  $N_2$  fixation. Similar to other phytoplankton, the growth of autotrophic cyanobacteria is limited by the availability of macronutrients (nitrogen and phosphorus), trace metals (iron, copper) [15,16], light, and temperature [17]. However, the effect of CO<sub>2</sub> on their growth has only been started to be investigated intensively [10].

The effects of increasing  $CO_2$  on primary production are widely debated in the literature and motivated by the growing concern of ocean acidification [18–22]. Low DIC concentrations are likely to transiently occur [23] in areas with dense phytoplanktonic communities like the coastal regions, where *Cyanothece* are naturally present. Additionally, such low concentrations pose a potential, permanent risk in dense laboratory or industrial cultures and

photo-bioreactors running without  $CO_2$  enrichment in the air supply. In the natural environment, we expect  $CO_2$  limitation to be altered following the increasing temperatures the world ocean is facing globally, but how dissolved inorganic carbon (DIC: the sum of  $CO_2$ ,  $HCO_3^-$  and  $CO_3^{2-}$ ) affects the growth of *Cyanothece* has not been analyzed in detail. Given the tight links between C and N metabolisms, what causes the growth difference between N<sub>2</sub>-fixing and NO<sub>3</sub><sup>-</sup> assimilating conditions under DIC limitation [10]?

Here, we implement a simple, yet mechanistic model of Cyanothece (Cell Flux Model of Cyanothece: CFM-Cyano) and quantitatively simulate the growth of this model organism, focusing on the control that DIC exerts on carbon fixation and on the subsequent C metabolism (Fig. 1: see Methods). This coarse-grained approach has an advantage in predicting concentrations of each metabolite pool [24,25]. The flexibility and simplicity of CFM-Cvano allows the model to be adapted to different contexts (e.g., different datasets) and has provided intuitive overviews of cellular metabolism in unicellular N<sub>2</sub>-fixers [25–28]. The present modeling work builds upon an experimental study of DIC limitation in the UCYN Cyanothece ATCC 51142 grown in turbidostats, both under a non-limiting nitrate supply and under obligate N<sub>2</sub>-fixation [10]. This experimental approach addressed the additional energetic burden that cells face when growing with N<sub>2</sub> fixation compared to a  $NO_3^-$ -based growth. They also revealed how DIC limitation exerts a more severe control on N<sub>2</sub>-based growth compared with NO<sub>3</sub>-supplied cultures. In this study, we provide a simple, mechanistic and quantitative representation of DIC limitation. Model results illustrate that resultant growth rates differ significantly between these metabolic modes, in relation to the intracellular allocation of fixed C.

# 2. Methods

# 2.1. Key equations

The applied mechanistic model, CFM-Cyano, is based on a simplified metabolic flux network based on mass balances (Fig. 1) sim-



**Fig. 1.** Schematics of the cell flux model of *Cyanothece* (CFM-Cyano) in (A) N<sub>2</sub>-fixing cells and in (B)  $NO_3^-$  assimilating cells. The green boxes represent the cell. Ovals and rectangular boxes represent inorganic and organic molecules, respectively. Orange color represents C-dominant molecules and fluxes; while pink and red color represent N-dominant molecules and fluxes, respectively. *P*<sub>Pho</sub>, C fixation rate; *F*<sub>Cost</sub>, biosynthesis cost, which covers the electron and energy costs for biosynthesis, N<sub>2</sub> fixation and  $NO_3^-$  assimilation, thus differs between these two cases. See the definition in the main text below [eq. (7)].

ilar to previous CFMs [24,29,30] and earlier modeling on marine N<sub>2</sub> fixers [31–33]. Most of these studies are reviewed in a recent publication [6]. CFM-Cyano simulated two metabolic scenarios: 1. N<sub>2</sub>-fixing (diazotrophic) and 2. NO<sub>3</sub> assimilating. Under the N<sub>2</sub>-fixing condition, N<sub>2</sub> fixation accounted for the total N source, whereas under NO<sub>3</sub> assimilating condition, NO<sub>3</sub> was the total N source. Parameter units and values are listed in Supplementary Material (Table S1, S2). In the CFM-Cyano model, we considered C as the main "currency" of cellular growth, and computed the rates of photosynthesis, C storage production, and growth (biosynthesis) for each time step. The developed model was calibrated to reproduce the experimental data (Fig. 2, Fig. 3 and Fig. 6).

Cellular C is fixed by photosynthesis, whose rate depends on external DIC concentration, following Monod kinetics [34]:

$$F_{Pho} = F_{Pho}^{max} \left( \frac{[DIC]}{[DIC] + K_{DIC}} \right) \tag{1}$$

where  $F_{Pho}$  is the rate of photosynthesis,  $F_{Pho}^{max}$  is the maximum rate of photosynthesis, [*DIC*] is DIC concentration in the culture, and  $K_{DIC}$  is the half saturation constant of DIC uptake.  $F_{Pho}$  was assumed zero during the night. While the intracellular CO<sub>2</sub> concentration is the one that directly affects the rate of photosynthesis, the data for intracellular CO<sub>2</sub> are not available and here we consider external DIC as a proxy for intracellular CO<sub>2</sub>. This implicitly assumes a linear relationship between internal and external pools of DIC. More complex relationships could arise from the presence of a carbon concentrating mechanism, and could be easily be incorporated in the model if substantiated by more direct evidence.

Once we determined the rate of photosynthesis, we then computed the net rate of C storage production,  $F_{Csto}$ , based on the difference between maximum C storage capacity,  $C_{Sto}^{max}$ , and the current level of C storage,  $C_{Sto}$ , into starch-like molecules [35]:

$$F_{Csto} = min \left\{ F_{Csto}^{max} \left( \frac{C_{Sto}^{max} - C_{Sto}}{C_{Sto}^{max}} \right), F_{Pho} \right\}$$
(2)

where the rate is proportional to  $F_{Csto}^{max}$ , a maximum rate of C storage production. We adapted this formation from the Cell Flux Model of *Crocosphaera* (CFM-Croco) [30]. Since the storage production should not exceed the rate of photosynthesis,  $F_{Csto}$  was capped by  $F_{Pho}$ . Based on the mass balance, the rest of fixed C is used for growth. Thus, under N<sub>2</sub> fixing case:

$$\mu = \frac{F_{Pho} - F_{Csto}}{1 + E} \tag{3}$$

where  $\mu$  is the net growth rate, and *E* is a constant factor for respiration for providing energy for biosynthesis [25,26,29]. In reality, it is possible that stored C is used for the growth. Thus, the term *F*<sub>Csto</sub> instead represented the net C storage production: the difference between gross C storage production and the loss for the growth. Under NO<sub>3</sub><sup>-</sup> assimilating case:

$$\mu = \frac{F_{Pho} - F_{Csto}(1+E)}{1+E - C_{Sto}E}$$
(4)

This formula counts the cost for  $NO_3^-$  assimilation, to keep the cellular C:N constant as suggested by experimental data (see the section "3.4. *Cellular C:N and N assimilation*"). The derivations for [eq. (3)] and [eq. (4)] are in Supplementary text.



**Fig. 2.** Relations between C-based growth rate and DIC (dissolved inorganic carbon) concentrations during the light period. (A) and (B) C-based growth rate ( $\mu_C$ ) for N<sub>2</sub>-fixing and NO<sub>3</sub> assimilating cells, respectively. (C) and (D) DIC concentrations for N<sub>2</sub>-fixing and NO<sub>3</sub> assimilating cells, respectively. Blue curves are the results of model calculations, while red circles represent experimental data, deduced from growth rates determined by changes in OD<sub>720</sub>. Error bars represent standard deviation. The constancy of the DIC after h7 during the light period is supported by the observed constant pH [10].

In this study, we simulated two types of *Cyanothece* cells: N<sub>2</sub>-fixing and non-N<sub>2</sub>-fixing (Fig. 1). We provided different *E* values for the different N sources. Specifically, we followed the previously developed method, which computed *E* based on the mass, electron and energy balance [36]. Under NO<sub>3</sub><sup>-</sup> added case, NO<sub>3</sub><sup>-</sup> concentrations were abundant in the cultures (NO<sub>3</sub><sup>-</sup> culture; 16.16–22.67 mM), allowing us to focus on the C limitation. When NO<sub>3</sub><sup>-</sup> is not added, we assumed that there is sufficient N storage accumulated during the night to support biosynthesis. Since the data showed a decrease in biomass during the night, we allowed net cell growth only during the light periods ( $\mu$  = 0 at night), although we were aware that cell division may occur also in the dark. We considered any excretion of carbohydrates as a part of carbon storage.

#### 2.2. Time variations and model solutions

We then applied these four equations [eq. (1)]-[eq. (4)] to equations for the time variation in the experimental system of turbidostat cultures [10]. Here, the time variation of the non-C-stroage biomass concentration *X* increase based on the net growth rate [24]:

$$\frac{dX}{dt} = \mu X \tag{5}$$

here, the loss term was not included since we compared the model results to the cumulative optical density. We use the following equation for the time dependence of cellular C storage per non-C-storage biomass  $C_{Sto}$ :

$$\frac{dC_{Sto}}{dt} = F_{Csto} - \mu C_{Sto} - F_{Csto}^{N2fix}$$
(6)

where  $C_{Sto}$  increases with C storage production,  $F_{Csto}$ , but decreases with cell growth ( $\mu C_{Sto}$ ), as  $C_{Sto}$  is converted to new cells during the light period. Also, during the dark period under N<sub>2</sub>-fixing conditions,  $C_{Sto}$  decreases with N<sub>2</sub> fixation  $F_{Csto}^{N2fix}$ , which requires high consumption of C storage for intracellular O<sub>2</sub> management and ATP generation [26,29,30,33]. Under the NO<sub>3</sub><sup>-</sup> based condition,  $F_{Csto}^{N2fix}$  is zero. Finally, the time dependence of culture DIC is represented as follows:

$$\frac{d[DIC]}{dt} = F_{DIC}^{Gas} - k_{DIC}^{Cell} \left( F_{Pho} - F_{Cost} - F_{Csto}^{N2fix} \right)$$
(7)

which is determined by the rate of gas exchange  $F_{DIC}^{Gas}$  and the cellular DIC uptake (the second term). Here,  $F_{DIC}^{Gas}$ , is proportional to the DIC disequilibrium with a rate coefficient  $k_{DIC}^{Gas}$ :  $F_{DIC}^{Gas} = ([DIC]_{Eq} - [DIC])k_{DIC}^{Gas}$ .  $[DIC]_{Eq}$  is the equilibrium concentration of DIC in the environment,  $k_{DIC}^{Gas}$  is the gas exchange constant, and  $k_{DIC}^{Cell}$  is a constant factor for cellular DIC consumption, as a balance between photosynthesis,  $F_{Pho}$ , respiratory C cost,  $F_{Cost} (= \mu E$  for N<sub>2</sub>-based case, and  $= E(F_{Csto} - \mu C_{sto} + \mu)$  for NO<sub>3</sub>-based case: see Supplementary text), and C consumption for N<sub>2</sub> fixation during the dark period,  $F_{Csto}^{N2fix}$ .

We solved [eq. (5)]-[eq. (7)] with a finite difference method with  $F_{Pho}$ ,  $F_{Csto}$  and  $\mu$  computed for each time step from [eq. (1)]-[eq. (4)] with light:dark periods of 14 h:10 h, following the turbidostat experiment described in the companion paper [10]. We note that whereas a more detailed representation of C chemistry could be resolved [37], we chose to represent DIC as a pool for compatibility with the available data. Also, this way enabled us to keep our model simple with regard to extracellular carbonate chemistry and focus on a more detailed representation of intracellular carbon allocation over time. We assumed that influences of DIC speciation are relatively small compared to the large overall changes in DIC concentrations observed over the diel cycle. Once we obtained the solutions for the time series, we computed cellular C content:

$$[C_{Cell}] = X(1 + C_{Sto}) \tag{8}$$

the relative value of which was compared with the values for optical density (OD<sub>720</sub>). We also computed the C-based growth rate  $\mu_{C}$ :

$$\mu_{\rm C} = \frac{\mu + F_{\rm Csto}}{1 + C_{\rm Sto}} \tag{9}$$

 $\mu_c$  is formulated based on the net carbon assimilation rate normalized by the cellular C.  $\mu_c$  was compared with the growth rate obtained from photobioreactor data, based on the change in the cumulative OD<sub>720</sub> [10] (Fig. 3).

# 2.3. Obtaining N related values for $N_2$ fixing case during the light period

During the light period under the N<sub>2</sub>-fixing condition, the rate of N<sub>2</sub> fixation is small and the predicted integrated rate of biosynthesis is relatively small compared to that of C storage accumulation (Fig. 5). Thus, we approximate the cellular C:N, assuming a constant  $N_{Cell}$ , the cellular N content per non-C-storage biomass C:

$$C: N = \frac{1 + C_{Sto}}{N_{Cell}} \tag{10}$$

2.4. Obtaining N related values for  $NO_3^-$  added case during the light period

During the light period, the data showed largely constant cellular C:N (see below). Thus, we assumed constant cellular C:N. This allowed the computation of  $N_{Cell}$  with the following equations:

$$N_{Cell} = \frac{1 + C_{Sto}}{C : N} \tag{11}$$

Also using C : N, assuming all the N source is NO<sub>3</sub>, we could compute the NO<sub>3</sub> uptake rate  $V_{NO3}$ :

$$V_{\rm NO3} = \frac{\mu_{\rm C}}{C:N} \tag{12}$$

#### 2.5. Laboratory measurements

We tested model solutions and constrained its unknown using time-dependent observations of the variation of intracellular C and N content, obtained during GAP 10th International meeting [10,38]. Transmission electron microscopic (TEM) samples were processed as described in [38].

#### 3. Results and discussion

#### 3.1. C assimilation rate and DIC

The overall trend captures the data for both  $\mu_C$  (C assimilation rate) and DIC concentrations (Fig. 2). Under the N<sub>2</sub>-fixing condition, the model predicted a sharp decrease in  $\mu_C$  within ~2 h (Fig. 2A), as DIC became depleted (Fig. 2C). In between these phases, experimental data showed a minimum, virtually zero growth after about 3 h in the light (h3), which was not captured by the model (Fig. 2A, B). This drop in  $\mu_C$  may indicate a lag phase [39–41] during which cells acclimate to a changed environment with low DIC by upregulating the activity of their CO<sub>2</sub> concentration mechanisms, such as expression and synthesis of CO<sub>2</sub> uptake systems and HCO<sub>3</sub><sup>-</sup> transporters [42–48]. This observation highlights that DIC may become a limiting factor for growth even when  $CO_2$  is supplied by air bubbling. In natural systems, severe DIC draw-down, comparable to our experimental set-up, may develop in freshwater systems with dense cyanobacterial blooms with predicted steady-state DIC concentrations of 130 to 230 µmol L<sup>-1</sup> [37], in coastal regions [23], or within highly productive microenvironments such as cyanobacterial colonies in brackish water [49].

Under growth with NO<sub>3</sub><sup>-</sup>, the initial growth rate was much lower than with N<sub>2</sub>-fixation. However, it remained relatively high after h2 until h6-h7 compared to N<sub>2</sub>-fixing culture (Fig. 2B). This concurred with a relatively high DIC level during this period (Fig. 2D). Experimental data for NO<sub>3</sub><sup>-</sup> assimilating cells also exhibited a significant drop in  $\mu_c$ , not seen in the model curve, likely due to the energy demand of acclimation (e.g., introduction of carbon concentration mechanism) as suggested above. The major difference between the two growth regimes (N<sub>2</sub> vs. NO<sub>3</sub><sup>-</sup>) is the initial rate of photosynthesis, which is highlighted by a higher  $F_{Pho}^{max}$  for the N<sub>2</sub>-fixing condition. This difference can be explained by the energy and electron cost for NO<sub>3</sub><sup>-</sup> assimilation and intracellular C allocation (see 3.3. Fate of fixed C).

# 3.2. Carbon storage and cellular C concentration

Model simulations of  $C_{sto}$  and  $[C_{cell}]$  (Fig. 3) were comparable to cellular polysaccharide levels and OD<sub>720</sub>, respectively, from cultures. The data-model consistency (Fig. 3) suggests that most of the C storage is in the form of polysaccharides, while OD<sub>720</sub> is a proxy for total cellular C content rather than cell number. During

the dark period under N<sub>2</sub>-fixing conditions, OD<sub>720</sub> decreased drastically (Fig. 3C), reflecting the drop in polysaccharide content (Fig. 3A). At the beginning of the light period,  $C_{Sto}$  increased rapidly but the increase was moderated as the rate of photosynthesis decreased due to DIC limitation (Fig. 2C, 3A). The cellular level of  $C_{Sto}$  was higher for the N<sub>2</sub>-fixing condition than for the NO<sub>3</sub> supplementing treatment during the light period (Fig. 3A, B). However, the model predicts that  $C_{Sto}$  in both treatments reaches the similar level at the end of the dark period due to the high C requirement for N<sub>2</sub> fixation and O<sub>2</sub> management.

Interestingly, whilst the model closely predicted the  $OD_{720}$  and the total biomass C concentration, at the end of the dark period,  $C_{Sto}$  must return back to the initial value in the semi-steady state condition. This discrepancy may suggest that some of the C stored as polysaccharides is transformed to other molecules during the dark period. It is possible that a fraction of polysaccharides is used for synthesizing cyanophycin (N storing molecules with C:N of 2:1 [25]) or amino acids [38] or used to build structural elements such as the cell wall. In fact, protein synthesis from polysaccharides was observed during the night [38]. Such conversion must take place with negligible C consumption (i.e., small C storage loss to DIC) because the dark  $OD_{720}$  under  $NO_3^-$  availability is almost constant (Fig. 3D); high C loss would have appeared as in the N<sub>2</sub>-fixing situation (Fig. 3C).

Transmission electron microscopic (TEM) images taken at the beginning of the light period (thus, the end of the dark period) (Fig. 4, S1) showed more polysaccharide granules in  $N_2$ -fixing cells



**Fig. 3.** C storage and biomass C in  $N_2$ -fixing and  $NO_3^-$  assimilating cells. Blue curves are model results, while red circles and curves represent experimental data. The data for (A) and (B) are cellular polysaccharide content and those for (C) and (D) are  $OD_{720}$ . The data of  $OD_{720}$  are shown as a relative value to the initial state. The sudden change in the slope at h14 represent the onset of the dark period. Also,  $N_2$  fixation is assumed between h14 and h20, which also causes the changes in the slope. In (A) and (B) error bars represent standard deviation and dashed lines shows the expected effect of C storage conversion to close the diurnal cycle.

than in the NO<sub>3</sub><sup>-</sup> grown ones, in contrast to bulk measurements of carbohydrate, OD<sub>720</sub>, and the modelled  $C_{Sto}$  (Fig. 3). This additional difference suggests that C, represented by  $C_{Sto}$  and detected by the bulk analysis of carbohydrate content, includes C forms that are not visible as polysaccharide granules by TEM. The other forms of C could possibly be precursors of starches/carbohydrates of lower molecular weight [50]. Following this hypothesis, under NO<sub>3</sub><sup>-</sup>-based conditions, more of the C would be present in this lower molecular weight form in the morning, potentially indicating a faster turnover of C under these conditions. Conversely, in the middle of the light phase (h7, Fig. 4, S1), TEM images show an increased number of polysaccharide granules in NO<sub>3</sub><sup>-</sup> assimilating cells, while bulk analysis of carbohydrate and modelled  $C_{Sto}$  are higher in N<sub>2</sub> fixing cells, indicating that degradation or turnover of carbon may be higher in N<sub>2</sub> fixers at this time of day.

# 3.3. Fate of fixed C

The fate of fixed C is predicted to differ between the N<sub>2</sub>-fixing and NO<sub>3</sub><sup>-</sup> assimilating conditions (Fig. 5). Under N<sub>2</sub>-fixing condition, a significant fraction of C is initially channeled into C storage, leaving only a small fraction of newly fixed C for biosynthesis (cellular growth) (Fig. 5A). For non-N<sub>2</sub>-fixing cyanobacteria, it has been previously reported that biosynthesis is prioritized over C storage [38]. In contrast, our model suggests that N<sub>2</sub>-fixing unicellular cyanobacteria preferentially allocate fixed C to storage to support later N<sub>2</sub> fixation through respiration at night. Indeed, during the early half of the light period, the model predicted that within the N<sub>2</sub>-fixing cells virtually all newly fixed C is accumulated in storage, while new C is allocated to biosynthesis only after the C storage reaches a certain threshold level at around h9 (Fig. 3A and Fig. 5A).

Contrary to the N<sub>2</sub>-fixing condition, when NO<sub>3</sub><sup>-</sup> is available, biosynthesis starts soon after the onset of the light period and continues up to the end of the light period (Fig. 5B). This occurs because the maximum level of  $C_{Sto}$  is small and reaches its maximum much faster during the early light period (Fig. 3B), costing a significant amount of C. In the experiment, the total C fixation during the light period is similar between the two cases. However, given the high maximum rate of net C fixation under the N<sub>2</sub>-fixing conditions, if enough CO<sub>2</sub> were continuously added to the culture to prevent DIC limitation, the rate of C fixation in the N<sub>2</sub>-fixing case might exceed the NO<sub>3</sub> assimilating case (Fig. S2). However, this simulation does not consider limitation by the availability of fixed N, which, in reality, would likely become limited under the N<sub>2</sub>fixing case and constrain the rate of C fixation, since the N<sub>2</sub> fixation occurs mainly during the night.

### 3.4. Cellular C:N ratios and N assimilation

Based on the modeled C metabolisms and C:N data, we have simulated cellular C:N and cellular N per biomass C (without C storage) for both the N<sub>2</sub> fixing case and the NO<sub>3</sub><sup>-</sup> added case (Fig. 6). The data and the model revealed quantitative differences in daytime N metabolisms between these two cases. In the N<sub>2</sub> fixing case, C:N of the cell increases (Fig. 6A) due to the accumulation of C storage (Fig. 3A). The cellular level of N is largely constant since N<sub>2</sub> fixation does not occur (or is small) during the light period (Fig. 6B).

On the other hand, when  $NO_3^-$  is added, the cellular C:N is largely constant (Fig. 6C) since the  $NO_3^-$  uptake occurs simultaneously with the accumulation of C storage. Especially, during the early light period, the cellular N is enriched (Fig. 6D) due to  $NO_3^-$  uptake (Fig. 6E). The model shows that the  $NO_3^-$  uptake is about 200% larger during the early light period than the later light period, consistent with NanoSIMS results from the same experiment [38].

Based on the rate of NO<sub>3</sub><sup>-</sup> uptake and C fixation, we computed the ratio of electron use for these purposes (Fig. 6F). Despite the considerable rate of NO<sub>3</sub><sup>-</sup> uptake and high electron requirement for NO<sub>3</sub><sup>-</sup> reduction (8e<sup>-</sup>) relative to net C fixation (4e<sup>-</sup>) [36], the electron consumption for NO<sub>3</sub><sup>-</sup> is relatively small (~1/2.57) (Fig. 6F). Thus, the use of electrons for NO<sub>3</sub><sup>-</sup> reduction is not sufficient to explain the difference in the rate of photosynthesis between the N<sub>2</sub> fixing case and the NO<sub>3</sub><sup>-</sup> case during the light per-



**Fig. 4.** Transmission electron microscopic images of *Cyanothece* cells harvested at h0/h24, h2 and h7 in the light period. Top row –  $N_2$ -fixing conditions; Bottom row –  $N_{03}$  assimilating conditions, pc; polysaccharide (C storage), cy; cyanophycin (N storage), and cx; carboxysome. Black bars show 1  $\mu$ m. Additional images are available in Fig. S1.



Fig. 5. Fate of newly fixed C during the light period. (A) N<sub>2</sub>-fixing case. (B) NO<sub>3</sub> assimilating case. Green: biosynthesis cost. Red: C storage. Blue: C for non-C-storage biomass. Total value represents C fixation rates. The biosynthesis cost represents the sum of synthesis of non-C-storage biomass and the NO<sub>3</sub> assimilation.



**Fig. 6.** Cellular C:N ratio, N assimilation and electron allocation. (A) and (B) are under  $N_2$ -fixing condition and (C) - (F) are under  $NO_3^-$  added case. (A)(C) Cellular C:N. (B)(D) Cellular N per biomass C (which excludes C storage). (E)  $NO_3^-$  uptake rate. (F) The ratio of electron used for C fixation to that for  $NO_3^-$  reduction.

iod, since the maximum rate of photosynthesis is about 100% higher for N<sub>2</sub> fixing case (Fig. 2). The remaining difference can be explained by the energy cost (not electron cost) for NO<sub>3</sub> assimilation to biomass and the preferential allocation of C to C storage under the N<sub>2</sub>-fixing condition (Fig. 5).

#### 3.5. DIC and C-storage requirements co-limit fate of fixed C

Our model results highlight two major factors controlling cellular growth when the growth of *Cyanothece* is limited by inorganic C. Firstly, CO<sub>2</sub> (DIC) availability limits the rate of photosynthesis, and then, the storage requirement limits the portion of newly fixed C that is used for biosynthesis or growth (Fig. 7). Under N<sub>2</sub>-fixing conditions, the maximum rate of C fixation ( $F_{Pho}^{max}$ ) is higher. However, a large part of C is channeled into C storage, limiting the biosynthesis of new cells, thus limiting the growth rate. Secondly, despite the high maximum rate of photosynthesis in the N<sub>2</sub>-fixing condition, the photosynthesis rate rapidly decreases as it quickly depletes DIC. On the other hand, when NO<sub>3</sub><sup>-</sup> is available, a large part of fixed C is channeled directly into biosynthesis, thus resulting in higher growth (Fig. 7). The lower maximum rate of photosynthesis works favorably under DIC limitation since it keeps ambient DIC relatively high. However, if limitation by DIC becomes less severe, due to the high photosynthetic capacity, the cells under N<sub>2</sub>-fixing conditions might grow even faster, yielding a potential co-limitation of DIC and fixed N. This hypothesis needs to be tested with further experiments.

## 4. Conclusions

We have developed a simple, cellular model of *Cyanothece* (CFM-Cyano) focusing on DIC limitation. The model reproduced



**Fig. 7.** Summary of this study: differences in metabolisms between  $N_2$ -fixing and  $NO_3^-$  assimilating situations. (A)  $N_2$ -fixing case. (B)  $NO_3^-$  assimilating case. Under DIC limitation,  $N_2$ -fixing cells have a lower growth rate despite the higher net maximum photosynthesis rate due to high C storage requirement.

laboratory data both for N<sub>2</sub>-fixing and NO<sub>3</sub><sup>-</sup> available conditions demonstrating that, under N<sub>2</sub>-fixing conditions, C storage is prioritized during the early photoperiod to accumulate C in storage for N<sub>2</sub> fixation during the night, and later during the day, biosynthesis increases. This two-step growth limitation may apply to other photoautotrophic unicellular N<sub>2</sub>-fixers, such as *Crocosphaera watsonii*.

A recent study pinpointed the risk of significant biases brought by a lack of control of the DIC supply in cultures of *Cyanothece* [10]. Our study further emphasizes the potential for DIC limitation in laboratory studies, which may severely limit the growth rate of any photoautotrophs and may have been overlooked as a critical regulatory factor in previous studies. Our model is simple and efficient and can be incorporated into sophisticated ecological or physiological models to resolve intracellular carbon allocation, especially under conditions when DIC availability becomes limiting, such as dense cyanobacterial blooms or biotechnological mass cultures.

# 5. Model availability

CFM-Cyano is freely available from Zenodo at https://zenodo. org/record/3740245 (DOI: 10.5281/zenodo.3740245).

#### Author contributions

KI developed and run the model with suggestions from TM, ME, SR and OP. KI, TM and OP administered the project. TM, ME, SR, TZ, JČ, MV, GB, PC, EK, SS, DJS, OP contributed to obtaining data. KI wrote the original manuscript, which is revised by KI, TM, ME, SR, TZ, MV, GB, GA, PC, EK, SS, DJS, CD, OP.

### **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.csbj.2021.11.036.

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