Quantifying *Cyanothece* growth under DIC limitation

Keisuke Inomura a,e, Takako Masuda b, Meri Eichner b, Sophie Rabouille c, Tomáš Zavřel d, Jan Červený d, Marie Vancová e, Gábor Bernát b,f, Gabrielle Armin a, Pascal Claquin g, Eva Kotabová b, Susanne Stephan h, David J. Suggett i, Curtis Deutsch j, Ondřej Prášil b

a Graduate School of Oceanography, University of Rhode Island, Narragansett, Rhode Island, USA
b Institute of Microbiology, The Czech Academy of Sciences, Třebon, Czech Republic
c Sorbonne Université, CNRS, Laboratoire d’Océanographie Microbienne, LOMIC, F-66650 Banyuls-sur-Mer, France
d Department of Adaptive Biotechnologies, Global Change Research Institute, Czech Academy of Sciences, Brno, Czech Republic
*e* Laboratory of Electron Microscopy, Institute of Parasitology, Biology Centre of the Czech Academy of Sciences and Faculty of Science, University of South Bohemia, České Budějovice, Czech Republic
f Balaton Limnological Research Institute, Eötvös Loránd Research Network (ELKH), Tihany, Hungary
g Laboratoire de Biologie des Organismes et Ecosystèmes Aquatiques (BOREA), UMR 8067, Muséum National d’Histoire Naturelle, CNRS, IRD Sorbonne Université, Université de Caen Normandie, Normandie Université, Esplanade de la Paix, F-14032 Caen, France
h Department Experimental Limnology, Leibniz Institute of Freshwater Ecology and Inland Fisheries, Stechlin, Germany
i University of Technology Sydney, Climate Change Cluster, Faculty of Science, Ultimo, NSW 2007, Australia
j School of Oceanography, University of Washington, Seattle, WA, USA

**A R T I C L E   I N F O**

Article history:
Received 28 June 2021
Received in revised form 22 November 2021
Accepted 22 November 2021
Available online 29 November 2021

Keywords:
Cyanothece
DIC
CO₂
Nitrogen fixation
Nitrate
Diurnal cycle
Carbon
Carbon storage
Photosynthesis
Carbon allocation
Quantitative model
Mathematical model
Computer simulation
Cellular growth
Biomass
Growth limitation
Culture
Turbidostat

**A B S T R A C T**

The photoautotrophic, unicellular N₂-fixing, *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 N₂-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 N₂-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 N₂ 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.

© 2021 The Author(s). Published by Elsevier B.V. on behalf of Research Network of Computational and Structural Biotechnology. This is an open access article under the CC BY license (http://creativecommons.org/licenses/by/4.0/).

1. Introduction

By reducing atmospheric CO₂ into bioavailable carbon (C), photosynthesis is the driving process of global ecosystem productivity 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 (N₂), unicellular N₂-fixing cyanobacteria (UCYN) directly or indi-
directly 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 *Cyanothec* sp. ATCC 51142 (hereafter *Cyanothec*), which also has a capability to fix dinitrogen (N₂) [7] to survive when bioavailable N, such as NH₄⁺ or NO₃⁻, is inaccessible. As in other photo-autotrophic, unicellular N₂-fixing cyanobacteria (UCYN-B and -C), N₂ fixation in *Cyanothec* is temporally segregated from carbon fixation [8–10], an evolution enabling protection of the O₂-sensitive, nitrogenase enzyme responsible for N₂ fixation [11]. Recent studies show that N₂ fixation by UCYN-B is facilitated by the inactivation of PSII [12,13], which may apply to *Cyanothec*. There are cases with in-complete temporal segregation depending on the light periodicity and cellular energy requirements, but the largest part of N₂ fixation tends to occur at night [9,14]. The temporal separation of photosynthesis and N₂ 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 N₂ fixation. *Cyanothec* is not an obligate N₂-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 N₂ fixation on the cellular carbon metabolism, in comparison to nitrate-supported metabolism in unicellular N₂-fixers [25–28]. The present modeling approach has an advantage in predicting concentrations of each metabolite pool [24,25]. The flexibility and simplicity of CFM-Cyano allows the model to be adapted to different contexts (e.g., different datasets) and has provided intuitive overviews of cellular metabolism in unicellular N₂-fixers [25–28].

The effects of increasing CO₂ 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 *Cyanothec* are naturally present. Additionally, such low concentrations pose a potential, permanent risk in dense laboratory or industrial cultures and photo-bioreactors running without CO₂ enrichment in the air supply.

In the natural environment, we expect CO₂ limitation to be altered following the increasing temperatures the world ocean is facing globally, but how dissolved inorganic carbon (DIC; the sum of CO₂, HCO₃⁻ and CO₃²⁻) affects the growth of *Cyanothec* has not been analyzed in detail. Given the tight links between C and N metabolisms, what causes the growth difference between N₂-fixing and NO₃⁻ assimilating conditions under DIC limitation [10]?

Here, we implement a simple, yet mechanistic model of *Cyanothec* (Cell Flux Model of *Cyanothec*; 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-Cyano allows the model to be adapted to different contexts (e.g., different datasets) and has provided intuitive overviews of cellular metabolism in unicellular N₂-fixers [25–28].

**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-
ilar to previous CFMs [24,29,30] and earlier modeling on marine N 2 fixers [31–33]. Most of these studies are reviewed in a recent publication [6]. CFM-Cyano simulated two metabolic scenarios: 1. N 2-fixing (diazotrophic) and 2. NO 3 assimilating. Under the N 2-fixing condition, N 2 fixation accounted for the total N source, whereas under NO 3 assimilating condition, NO 3 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]:

\[ F_{\text{Pho}} = \frac{F_{\text{max,Pho}} \cdot [\text{DIC}]}{[\text{DIC}] + K_{\text{DIC}}} \]  

(1)

where \( F_{\text{Pho}} \) is the rate of photosynthesis, \( F_{\text{max,Pho}} \) is the maximum rate of photosynthesis, \([\text{DIC}]\) is DIC concentration in the culture, and \( K_{\text{DIC}} \) is the half saturation constant of DIC uptake. \( F_{\text{Pho}} \) was assumed zero during the night. While the intracellular CO 2 concentration is the one that directly affects the rate of photosynthesis, the data for intracellular CO 2 are not available and here we consider external DIC as a proxy for intracellular CO 2. 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, \( F_{\text{Csto}} \), based on the difference between maximum C storage capacity, \( C_{\text{max,Sto}} \), and the current level of C storage, \( C_{\text{Sto}} \), into starch-like molecules [35]:

\[ F_{\text{Csto}} = \min \left\{ \frac{F_{\text{max,Sto}} \cdot (C_{\text{max,Sto}} - C_{\text{Sto}})}{C_{\text{Sto}}}, F_{\text{Pho}} \right\} \]  

(2)

where the rate is proportional to \( F_{\text{max,Sto}} \), 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, \( F_{\text{Csto}} \) was capped by \( F_{\text{Pho}} \).

Based on the mass balance, the rest of fixed C is used for growth. Thus, under N 2 fixing case:

\[ \mu = \frac{F_{\text{Pho}} - F_{\text{Csto}}}{1 + E} \]  

(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 \( F_{\text{Csto}} \) instead represented the net C storage production: the difference between gross C storage production and the loss for the growth. Under NO 3 assimilating case:

\[ \mu = \frac{F_{\text{Pho}} - F_{\text{Csto}} (1 + E)}{1 + E - C_{\text{Sto}}E} \]  

(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 2-fixing and NO 3 assimilating cells, respectively. (C) and (D) DIC concentrations for N 2-fixing and NO 3 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 720. 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: N₂-fixing and non-N₂-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$_3$ added case, NO$_3$ concentrations were abundant in the cultures (NO$_3$ culture; 16.16–22.67 mM), allowing us to focus on the C limitation. When NO$_3$ 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 (μ = 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-storage biomass concentration $X$ increase based on the net growth rate [24]:

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

(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 $C_{Sto}$:

$$\frac{dC_{Sto}}{dt} = F_{Csto} - \mu C_{Sto} - F_{Qfix}$$

(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 N₂-fixing conditions, $C_{Sto}$ decreases with N₂ fixation $F_{O2fix}$, which requires high consumption of C storage for intracellular O₂ management and ATP generation [26,29,30,33]. Under the NO$_3$ based condition, $F_{O2fix}$ is zero. Finally, the time dependence of culture DIC is represented as follows:

$$\frac{dDIC}{dt} = F_{Gas} - k_{Gas}C_{Cell} - F_{sto} - F_{Qfix}$$

(7)

which is determined by the rate of gas exchange $F_{Gas}$ and the cellular DIC uptake (the second term). Here, $F_{Gas}$, is proportional to the DIC disequilibrium with a rate coefficient $k_{Gas}^C$:

$$F_{Gas} \propto (|DIC|_{eq} - |DIC|)k_{Gas}^C$$

$|DIC|_{eq}$ is the equilibrium concentration of DIC in the environment, $k_{Gas}^C$ is the gas exchange constant, and $k_{Gas}^C$ is a constant factor for cellular DIC consumption, as a balance between photosynthesis, $F_{sto}$, and respiratory C cost, $F_{sto}$ (= $\mu$ for N$_2$-based case, and $= E[F_{sto} - \mu C_{Cell} + \mu]$ for NO$_3$-based case; see Supplementary text). Also using $C : N$ for N$_2$ fixation during the dark period, $F_{sto}$. We solved eq. (5)-eq. (7) with a finite difference method with $F_{sto}$, $F_{sto}$, 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:

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

(8)

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

$$\mu_C = \frac{\mu + F_{Csto}}{1 + C_{Sto}}$$

(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$_{720}$ [10] (Fig. 3).

### 2.3. Obtaining N related values for N₂ fixing case during the light period

During the light period under the N₂-fixing condition, the rate of N₂ 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:

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

(10)

### 2.4. Obtaining N related values for NO₃ 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:

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

(11)

Also using $C : N$, assuming all the N source is NO$_3$, we could compute the NO$_3$ uptake rate $V_{NO3}$:

$$V_{NO3} = \frac{\mu_C}{C : N}$$

(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 N₂-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 CO₂ concentration mechanisms, such as expression and synthesis of CO₂ uptake systems and HCO$_3^−$ transporters [42–48]. This observation highlights that DIC may become a limiting factor for growth even when
CO₂ 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⁻¹ [37], in coastal regions [23], or within highly productive microenvironments such as cyanobacterial colonies in brackish water [49].

Under growth with NO₃⁻, the initial growth rate was much lower than with N₂-fixation. However, it remained relatively high after h₂ until h₆-h₇ compared to N₂-fixing culture (Fig. 2B). This concurred with a relatively high DIC level during this period (Fig. 2D). Experimental data for NO₃ assimilating cells also exhibited a significant drop in Nₑ, 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 (N₂ vs. NO₃) is the initial rate of photosynthesis, which is highlighted by a higher Fₘₐₓ for the N₂-fixing condition. This difference can be explained by the energy and electron cost for NO₃ assimilation and intracellular C allocation (see 3.3. Fate of fixed C).

3.2. Carbon storage and cellular C concentration

Model simulations of Cₘ₀ and [Cₑ₀] (Fig. 3) were comparable to cellular polysaccharide levels and OD₇₂₀, respectively, from cultures. The data-model consistency (Fig. 3) suggests that most of the C storage is in the form of polysaccharides, while OD₇₂₀ is a proxy for total cellular C content rather than cell number. During the dark period under N₂-fixing conditions, OD₇₂₀ decreased drastically (Fig. 3A), reflecting the drop in polysaccharide content (Fig. 3A). At the beginning of the light period, Cₘ₀ increased rapidly but the increase was moderated as the rate of photosynthesis decreased due to DIC limitation (Fig. 2C, 3A). The cellular level of Cₘ₀ was higher for the N₂-fixing condition than for the NO₃ supplementing treatment during the light period (Fig. 3A, B). However, the model predicts that Cₘ₀ in both treatments reaches the similar level at the end of the dark period due to the high C requirement for N₂ fixation and O₂ management.

Interestingly, whilst the model closely predicted the OD₇₂₀ and the total biomass C concentration, at the end of the dark period, Cₘ₀ 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 OD₇₂₀ under NO₃ availability is almost constant (Fig. 3D); high C loss would have appeared as in the N₂-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 N₂-fixing cells than in NO₃-assimilating cells.
than in the NO$_3$ grown ones, in contrast to bulk measurements of carbohydrate, OD$_{720}$ 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$_3$-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$_3$ assimilating cells, while bulk analysis of carbohydrate and modelled C$_{sto}$ are higher in N$_2$ fixing cells, indicating that degradation or turnover of carbon may be higher in N$_2$ fixers at this time of day.

### 3.3. Fate of fixed C

The fate of fixed C is predicted to differ between the N$_2$-fixing and NO$_3$ assimilating conditions (Fig. 5). Under N$_2$-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$_2$-fixing cyanobacteria, it has been previously reported that biosynthesis is prioritized over C storage [38]. In contrast, our model suggests that N$_2$-fixing unicellular cyanobacteria preferentially allocate fixed C to storage to support cellular growth (Fig. 5A). For non-N$_2$-fixing cyanobacteria, it has been previously reported that biosynthesis is prioritized over C storage [38]. In contrast, our model suggests that N$_2$-fixing unicellular cyanobacteria preferentially allocate fixed C to storage to support cellular growth (Fig. 5A). For non-N$_2$-fixing cyanobacteria, it has been previously reported that biosynthesis is prioritized over C storage [38]. In contrast, our model suggests that N$_2$-fixing unicellular cyanobacteria preferentially allocate fixed C to storage to support cellular growth (Fig. 5A). For non-N$_2$-fixing cyanobacteria, it has been previously reported that biosynthesis is prioritized over C storage [38]. In contrast, our model suggests that N$_2$-fixing unicellular cyanobacteria preferentially allocate fixed C to storage to support cellular growth (Fig. 5A). For non-N$_2$-fixing cyanobacteria, it has been previously reported that biosynthesis is prioritized over C storage [38]. In contrast, our model suggests that N$_2$-fixing unicellular cyanobacteria preferentially allocate fixed C to storage to support cellular growth (Fig. 5A). For non-N$_2$-fixing cyanobacteria, it has been previously reported that biosynthesis is prioritized over C storage [38]. In contrast, our model suggests that N$_2$-fixing unicellular cyanobacteria preferentially allocate fixed C to storage to support cellular growth (Fig. 5A). For non-N$_2$-fixing cyanobacteria, it has been previously reported that biosynthesis is prioritized over C storage [38]. In contrast, our model suggests that N$_2$-fixing unicellular cyanobacteria preferentially allocate fixed C to storage to support cellular growth (Fig. 5A). For non-N$_2$-fixing cyanobacteria, it has been previously reported that biosynthesis is prioritized over C storage [38]. In contrast, our model suggests that N$_2$-fixing unicellular cyanobacteria preferentially allocate fixed C to storage to support cellular growth (Fig. 5A). For non-N$_2$-fixing cyanobacteria, it has been previously reported that biosynthesis is prioritized over C storage [38]. In contrast, our model suggests that N$_2$-fixing unicellular cyanobacteria preferentially allocate fixed C to storage to support cellular growth (Fig. 5A). For non-N$_2$-fixing cyanobacteria, it has been previously reported that biosynthesis is prioritized over C storage [38]. In contrast, our model suggests that N$_2$-fixing unicellular cyanobacteria preferentially allocate fixed C to storage to support cellular growth (Fig. 5A). For non-N$_2$-fixing cyanobacteria, it has been previously reported that biosynthesis is prioritized over C storage [38]. In contrast, our model suggests that N$_2$-fixing unicellular cyanobacteria preferentially allocate fixed C to storage to support cellular growth (Fig. 5A). For non-N$_2$-fixing cyanobacteria, it has been previously reported that biosynthesis is prioritized over C storage [38]. In contrast, our model suggests that N$_2$-fixing unicellular cyanobacteria preferentially allocate fixed C to storage to support cellular growth (Fig. 5A). For non-N$_2$-fixing cyanobacteria, it has been previously reported that biosynthesis is prioritized over C storage [38]. In contrast, our model suggests that N$_2$-fixing unicellular cyanobacteria preferentially allocate fixed C to storage to support cellular growth (Fig. 5A). For non-N$_2$-fixing cyanobacteria, it has been previously reported that biosynthesis is prioritized over C storage [38]. In contrast, our model suggests that N$_2$-fixing unicellular cyanobacteria preferentially allocate fixed C to storage to support cellular growth (Fig. 5A). For non-N$_2$-fixing cyanobacteria, it has been previously reported that biosynthesis is prioritized over C storage [38]. In contrast, our model suggests that N$_2$-fixing unicellular cyanobacteria preferentially allocate fixed C to storage to support cellular growth (Fig. 5A). For non-N$_2$-fixing cyanobacteria, it has been previously reported that biosynthesis is prioritized over C storage [38]. In contrast, our model suggests that N$_2$-fixing unicellular cyanobacteria preferentially allocate fixed C to storage to support cellular growth (Fig. 5A). For non-N$_2$-fixing cyanobacteria, it has been previously reported that biosynthesis is prioritized over C storage [38]. In contrast, our model suggests that N$_2$-fixing unicellular cyanobacteria preferentially allocate fixed C to storage to support cellular growth (Fig. 5A). For non-N$_2$-fixing cyanobacteria, it has been previously reported that biosynthesis is prioritized over C storage [38]. In contrast, our model suggests that N$_2$-fixing unicellular cyanobacteria preferentially allocate fixed C to storage to support cellular growth (Fig. 5A). For non-N$_2$-fixing cyanobacteria, it has been previously reported that biosynthesis is prioritized over C storage [38]. In contrast, our model suggests that N$_2$-fixing unicellular cyanobacteria preferentially allocate fixed C to storage to support cellular growth (Fig. 5A). For non-N$_2$-fixing cyanobacteria, it has been previously reported that biosynthesis is prioritized over C storage [38]. In contrast, our model suggests that N$_2$-fixing unicellular cyanobacteria preferentially allocate fixed C to storage to support cellular growth (Fig. 5A). For non-N$_2$-fixing cyanobacteria, it has been previously reported that biosynthesis is prioritized over C storage [38]. In contrast, our model suggests that N$_2$-fixing unicellular cyanobacteria preferentially allocate fixed C to storage to support cellular growth (Fig. 5A). For non-N$_2$-fixing cyanobacteria, it has been previously reported that biosynthesis is prioritized over C storage [38]. In contrast, our model suggests that N$_2$-fixing unicellular cyanobacteria preferentially allocate fixed C to storage to support cellular growth (Fig. 5A). For non-N$_2$-fixing cyanobacteria, it has been previously reported that biosynthesis is prioritized over C storage [38]. In contrast, our model suggests that N$_2$-fixing unicellular cyanobacteria preferentially allocate fixed C to storage to support cellular growth (Fig. 5A). For non-N$_2$-fixing cyanobacteria, it has been previously reported that biosynthesis is prioritized over C storage [38]. In contrast, our model suggests that N$_2$-fixing unicellular cyanobacteria preferentially allocate fixed C to storage to support cellular growth (Fig. 5A). For non-N$_2$-fixing cyanobacteria, it has been previously reported that biosynthesis is prioritized over C storage [38]. In contrast, our model suggests that N$_2$-fixing unicellular cyanobacteria preferentially allocate fixed C to storage to support cellular growth (Fig. 5A). For non-N$_2$-fixing cyanobacteria, it has been previously reported that biosynthesis is prioritized over C storage [38]. In contrast, our model suggests that N$_2$-fixing unicellular cyanobacteria preferentially allocate fixed C to storage to support cellular growth (Fig. 5A). For non-N$_2$-fixing cyanobacteria, it has been previously reported that biosynthesis is prioritized over C storage [38]. In contrast, our model suggests that N$_2$-fixing unicellular cyanobacteria preferentially allocate fixed C to storage to support cellular growth (Fig. 5A). For non-N$_2$-fixing cyanobacteria, it has been previously reported that biosynthesis is prioritized over C storage [38]. In contrast, our model suggests that N$_2$-fixing unicellular cyanobacteria preferentially allocate fixed C to storage to support cellular growth (Fig. 5A). For non-N$_2$-fixing cyanobacteria, it has been previously reported that biosynthesis is prioritized over C storage [38]. In contrast, our model suggests that N$_2$-fixing unicellular cyanobacteria preferentially allocate fixed C to storage to support cellular growth (Fig. 5A). For non-N$_2$-fixing cyanobacteria, it has been previously reported that biosynthesis is prioritized over C storage [38]. In contrast, our model suggests that N$_2$-fixing unicellular cyanobacteria preferentially allocate fixed C to storage to support cellular growth (Fig. 5A). For non-N$_2$-fixing cyanobacteria, it has been previously reported that biosynthesis is prioritized over C storage [38]. In contrast, our model suggests that N$_2$-fixing unicellular cyanobacteria preferentially allocate fixed C to storage to support cellular growth (Fig. 5A). For non-N$_2$-fixing cyanobacteria, it has been previously reported that biosynthesis is prioritized over C storage [38]. In contrast, our model suggests that N$_2$-fixing unicellular cyanobacteria preferentially allocate fixed C to storage to support cellular growth (Fig. 5A). For non-N$_2$-fixing cyanobacteria, it has been previously reported that biosynthesis is prioritized over C storage [38]. In contrast, our model suggests that N$_2$-fixing unicellular cyanobacteria preferentially allocate fixed C to storage to support cellular growth (Fig. 5A). For non-N$_2$-fixing cyanobacteria, it has been previously reported that biosynthesis is prioritized over C storage [38]. In contrast, our model suggests that N$_2$-fixing unicellular cyanobacteria preferentially allocate fixed C to storage to support cellular growth (Fig. 5A). For non-N$_2$-fixing cyanobacteria, it has been previously reported that biosynthesis is prioritized over C storage [38]. In contrast, our model suggests that N$_2$-fixing unicellular cyanobacteria preferentially allocate fixed C to storage to support cellular growth (Fig. 5A)

![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 – NO$_3$ assimilating conditions. pc; polysaccharide (C storage), cy; cyanophycin (N storage), and cx; carboxysome. Black bars show 1 μm. Additional images are available in Fig. S1.](image-url)
iod, since the maximum rate of photosynthesis is about 100% higher for N₂ fixing case (Fig. 2). The remaining difference can be explained by the energy cost (not electron cost) for NO₃/C₃ assimilation to biomass and the preferential allocation of C to C storage under the N₂-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, CO₂ (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 N₂-fixing conditions, the maximum rate of C fixation (Fmax Pho) 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 N₂-fixing condition, the photosynthesis rate rapidly decreases as it quickly depletes DIC. On the other hand, when NO₃ 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 N₂-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
laboratory data both for N₂-fixing and NO₃ assimilating conditions demonstrating that, under N₂-fixing conditions, C storage is prioritized during the early photoperiod to accumulate C in storage for N₂ fixation during the night, and later during the day, biosynthesis increases. This two-step growth limitation may apply to other photoautotrophic unicellular N₂-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, JC, MV, GB, PC, UK, 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, UK, 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.

Acknowledgements

We thank Steven G. Ball for sharing useful insights about C storage in *Cyanothece*, José Bonomi-Barufi for contributing to data acquisition, Douglas A. Campbell for providing feedback, Martin Lukeš for his help in measurements and interpretation of data. This project was supported by the Simons Foundation (Simons Postdoctoral Fellowship in Marine Microbial Ecology, Award 544338, KI), the National Science Foundation under EPSCoR Cooperative Agreement (Award OIA-1655221, KI, GA), the Czech Research Foundation GAČR (Award 20–17627S, OP and Award 18–24397S, TZ, JC), MEYS CR (LM2018129 Czech-BioImaging, MV), the Ministry of Education, Youth and Sports of the Czech Republic (OP RDE grant number CZ.02.1.01/0.0/0.0/16–588 026/0008413, TZ, JC), and the National Research, Development and Innovation Office of Hungary, NKFIH, (Award K 128950 and NKFIH–471–3/2021, GB). We thank the generous support from these foundations.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.csbj.2021.11.036.

References

[1] Field CB, Behrenfeld MJ, Randerson JT, Falkowski P. Primary production of the biosphere: Integrating terrestrial and oceanic components. Science 1998;281(5374):237–40.
[2] Zehr JP, Waterbury JB, Turner PJ, Montoya JP, Omorgé E, Steward GF, et al. Unicellular cyanobacteria fix N₂ in the subtropical North Pacific Ocean. Nature 2001;412(6847):635–8.
[3] Montoya JP, Holl CM, Zehr JP, Villareal TA, Capone DG. High rates of N₂ fixation by unicellular diazotrophs in the oligotrophic Pacific Ocean. Nature 2004;430(7003):1027–31.
[4] Moisander PH, Beinart RA, Hewson I, White AE, Johnson KS, Carlson CA, et al. Unicellular cyanobacterial distributions broaden the oceanic N₂ fixation domain. Science 2010;327(5972):1512–4.
[5] Gruber N, Galloway JN. An Earth-system perspective of the global nitrogen cycle. Nature 2008;451(7176):293–6.
[6] Inomura K, Deutsch C, Masuda T, Prášil O, Follows MJ. Quantitative models of nitrogen-fixing organisms. Comput Struct Biotechnol J 2020;18:3905–24.
[7] Reddy R, Haskell JB, Sherman DM, Sherman LA. Unicellular, aerobic nitrogen-fixing cyanobacteria of the genus *Cyanothece*. J Bacteriol 1993;175(5):1284–92.
[8] Meunier PC, Colón-lópez MS, Sherman LA. Temporal changes in state transitions and photosystem organization in the unicellular, diazotrophic cyanobacterium *Cyanothece* sp. ATCC 5112. Plant Physiol 1997;115:991–1000.
[9] Rabouille S, Van de Waal DB, Matthijs HCF, Huisman J. Nitrogen fixation and respiratory electron transport in the cyanobacterium *Cyanothece* under different light/dark cycles. FEMS Microbiol Ecol 2014;87(3):630–8.
K. Inomura, T. Masuda, M. Eichner et al.

[10] Rabouille S, Campbell DA, Masuda T, Zavřel T, Bernat G, Polerecky L, et al. Electron and biomass dynamics of Cyanothece under interacting nitrogen and carbon limitations. Front Microbiol 2021;12:617802.

[11] Callion JR, Tansley Review No. 44 Reconciling the incompatibile: N₂ fixation and O₂. New Phytol 1992;122(4):571–609.

[12] Rabouille S, Claquin P. Photosystem-II shutdown evolved with nitrogen fixation in the unicellular diazotroph Crocosphaera watsonii. Environ Microbiol 2016;18(2):477–85.

[13] Masuda T, Bernat G, Bečková M, Kotabová E, Lawrenz L, Lukš M, et al. Diel regulation of photosynthetic activity in the oceanic unicellular diazotrophic cyanobacterium Crocosphaera watsonii WH8501. Environ Microbiol 2016;18(2):546–60.

[14] Dron A, Rabouille S, Claquin P, Talec A, Raimbault V, Scandiara A. Photoperiod length paces the temporal orchestration of cell cycle and carbon-nitrogen metabolism in Crocosphaera watsonii. Environ Microbiol 2013;15:3292–304.

[15] Moore CM, Mills MM, Arrigo KR, Berman-Frank I, Bopp L, Boyd PW, et al. Processes and patterns of oceanic nutrient limitation. Nat Geosci 2013;6(9):701–10.

[16] Huertas M, López-Maury L, Giner-Lamau J, Sánchez-Riego A, Florencio F. Metals in cyanobacteria: Analysis of the copper, nickel, cobalt and arsenic homeostasis mechanisms. Life 2014;4(4):865–86.

[17] Dechatwongse P, Srisamai S, Maitland G, Hellgardt K. Effects of light and temperature on the photosynthetic growth and photosynthesis in nitrogen-fixing cyanobacterium Cyanothoe sp. ATCC 51142. Algol Res 2014;5:103–11.

[18] Rebelsei U, Wolf-Gladrow DA, Smetacek V. Carbon dioxide limitation of marine phytoplankton growth rates. Nature 1993;361(6400):249–51.

[19] Gattuso JP, Magnan A, Billé R, Kotabova E, Lawrenz L, Lukš M, et al. Diel regulation of photosynthetic activity in the oceanic unicellular diazotrophic cyanobacterium Crocosphaera watsonii WH8501. Environ Microbiol 2016;18(2):477–85.

[20] Moore CM, Mills MM, Arrigo KR, Berman-Frank I, Bopp L, Boyd PW, et al. Processes and patterns of oceanic nutrient limitation. Nat Geosci 2013;6(9):701–10.

[21] Yang Y, Håkanson L, Gattuso JP. Data compilation on the biological response to ocean acidification: An update. Earth Syst Sci Data 2016;8(1):79–87.

[22] Gao K, Beardall J, Häder DP, Hall-Spencer JM, Gao G, Hutchins DA. Effects of ocean acidification on marine photosynthetic organisms under the concurrent influences of warming, UV radiation, and deoxygenation. Front Mar Sci 2019;6:1–18.

[23] Eichner M, Rost B, Kranz S. Diversity of ocean acidification effects on marine N₂ fixers. J Exp Mar Biol Ecol 2014;457:199–207.

[24] Evans W, Hales B, Strutton PC. Seasonal cycle of surface ocean pCO₂ on the Oregon shelf. J Geophys Res Oceans 2011;116:C05012.

[25] Inomura K, Masuda T, Gauglitz JM. Active nitrogen fixation by Crocosphaera expands their niche despite the presence of ammonium – A case study. Sci Rep 2019;9:15064.

[26] Inomura K, Omta AW, Talmy D, Bragg J, Deutsch C, Follows MJ. A Mechanistic model of macromolecular allocation, elemental stoichiometry, and growth rate in phytoplankton. Front Microbiol 2020;11.

[27] Inomura K, Bragg J, Riemann L, Follows MJ, Virolle M-J. Quantifying oxygen management and temperature and light dependencies of nitrogen fixation by Crocosphaera watsonii. mSphere 2019;4(6). https://doi.org/10.1128/mSphere.00531-19.

[28] Babu PR, Babu MA, Babu J, Babu S, Babu CA, Babu J. Modeling the dynamic regulation of nitrogen fixation in the cyanobacterium Trichodesmium sp. Appl Environ Microbiol 2006;72(5):3217–27.

[29] Agawi NR, Rabouille S, Veldhuis MJW, Servatius L, Hol S, van Overzee H, et al. Competition and facilitation between unicellular nitrogen-fixing cyanobacteria and non-nitrogen-fixing phytoplankton species. Limnol Oceanogr 2007;52(5):2233–48.

[30] Grimaud CM, Rabouille S, Dron A, Scandiara A, Bernard O. Modelling the dynamics of carbon – nitrogen metabolism in the unicellular diazotrophic cyanobacterium Crocosphaera watsonii WH8501, under variable light regimes. Ecol Model 2014;291:121–33.

[31] Monod J. The growth of bacterial cultures. Ann Rev Mar Sci 1949;3:371–94.

[32] Deschamps P, Colleoni C, Nakamura Y, Suzuki E, Putaux J-L, Rufeles A, et al. Metabolic symbiosis and the birth of the plant kingdom. Mol Biol Evol 2008;25(3):536–48.

[33] Rittmann BE, McCarty PL. Environmental Biotechnology: Principles and Applications. New York, NY: McGraw-Hill; 2001.

[34] Ji X, Verspagen JMH, van de Waal DB, Rost B, Huuskman J. Phenotypic plasticity of carbon fixation stimulates cyanobacterial blooms at elevated CO₂. Sci Adv 2020;6:eaae2926.

[35] Porecky L, Masuda T, Eichner M, Rabouille S, Vancová M, Kiemhov MVM, et al. Temporal patterns and intra- and inter-cellular variability in carbon and nitrogen assimilation by the unicellular cyanobacterium Cyanothoe sp. ATCC 51142. Front Microbiol 2021;12:620915.

[36] Swinnen IAM, Bernaerts K, Dens EJJ, Geeraerd AH, Van Impe JF. Predictive modelling of the microbial lag phase: A review. Int J Food Microbiol 2004;94:137–59.

[37] Mulder G, Mooij WM, Smolders AJP, Donk EV. Allelopathic inhibition of phytoplankton by exudates from Stratiotes aloides. Aquat Bot 2005;82(4):284–96.

[38] Rolfe MD, Rice CJ, Lucchini S, Pin C, Thompson A, Cameron ADS, et al. Lag phase is a distinct growth phase that prepares bacteria for exponential growth and involves transient metal accumulation. J Bacteriol 2012;194(3):686–701.

[39] Miller AG, Colman B. Active transport and accumulation of bicarbonate by a unicellular cyanobacterium. J Bacteriol 1980;143(3):1253–9.

[40] Miller AG, Espie GS, Canvin DT. Physiological aspects of CO₂ and HCO₃⁻ transport by cyanobacteria: a review. Can J Bot 1990;68(6):1291–302.

[41] Kaplan A, Badger MR, Berry JA. Photosynthesis and the intracellular inorganic carbon pool in the bluegreen alga Anabaena variabilis: Response to external CO₂ concentration. Plants 1980;149(3):215–26.

[42] Badger MR, Spalding MH (2000) CO₂ acquisition, concentration and fixation in cyanobacteria and algae. In: Leegood RC, Sharkey TD and von Caemmerer S (eds), Advances in Photosynthesis, Vol 9. Photosynthesis: Physiology and Metabolism, 9: 399–434.

[43] Price GD, Badger MR, Woodger FJ, Long BM. Advances in understanding the cyanobacterial CO₂-concentrating-mechanism (CCM): Functional components, Ci transporters, diversity, genetic regulation and prospects for engineering into plants. J Exp Bot 2008;59:1441–61.

[44] Ogawa T, Kaplan A. Inorganic carbon acquisition systems in cyanobacteria. Photosynth Res 2003;77:105–15.

[45] Eichner M, Thomas S, Kranz SA, Rost B. Cellular inorganic carbon fluxes in Trichodesmium: A combined approach using measurements and modelling. J Exp Bot 2015;66:749–59.

[46] Ploog H, Adam B, Musat N, Kalvelage T, Laviç, G, Wolf-Gladrow D, et al. Carbon, nitrogen and O₂ fluxes associated with the cyanobacterium Nodularia spumigena in the Baltic Sea. ISME J 2011;5:1549–58.

[47] Zavřel T, Čermášová P, Sinetova M, Červeny J. Determination of Storage (Starch/Glycogen) and Total Saccharides Content in Algae and Cyanobacteria by a Phenol-Sulfuric Acid Method. Bio-ProTOCOL. 2018;8:1–13.