Temporal Patterns and Intra- and Inter-Cellular Variability in Carbon and Nitrogen Assimilation by the Unicellular Cyanobacterium *Cyanothece* sp. ATCC 51142

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Unicellular nitrogen fixing cyanobacteria (UCYN) are abundant members of phytoplankton communities in a wide range of marine environments, including those with rapidly changing nitrogen (N) concentrations. We hypothesized that differences in N availability (N2 vs. combined N) would cause UCYN to shift strategies of intracellular N and C allocation. We used transmission electron microscopy and nanoscale secondary ion mass spectrometry imaging to track assimilation and intracellular allocation of 13C-labeled CO2 and 15N-labeled N2 or NO3 at different periods across a diel cycle in *Cyanothece* sp. ATCC 51142. We present new ideas on interpreting these imaging data, including the influences of pre-incubation cellular C and N contents and turnover rates of inclusion bodies. Within cultures growing diazotrophically, distinct subpopulations were detected that fixed N2 at night or in the morning. Additional significant within-population heterogeneity was likely caused by differences in the relative amounts of N assimilated into cyanophycin from sources external and internal to the cells. Whether growing on N2 or NO3, cells prioritized cyanophycin synthesis when N assimilation rates were highest. N assimilation in cells growing on NO3 switched from cyanophycin synthesis to protein synthesis, suggesting that once a cyanophycin quota is met, it is bypassed in favor
of protein synthesis. Growth on NO₃ also revealed that at night, there is a very low level of CO₂ assimilation into polysaccharides simultaneous with their catabolism for protein synthesis. This study revealed multiple, detailed mechanisms underlying C and N management in Cyanothece that facilitate its success in dynamic aquatic environments.

**Keywords:** Crocosphaera subtropica (former Cyanothece sp. ATCC 51142), Cyanothece, photosynthesis, carbon fixation, nitrogen fixation, nanoSIMS, TEM

**INTRODUCTION**

Nitrogen fixing microorganisms (diazotrophs) are critical suppliers of bioavailable forms of nitrogen (N, e.g., ammonium) in natural ecosystems. In the surface ocean where primary suppliers of bioavailable forms of nitrogen (N, e.g., ammonium) Nitrogen fixing microorganisms (diazotrophs) are critical int...
photosons m\(^{-2}\) s\(^{-1}\) with a 14 h:10 h light:dark cycle (14L:10D), with L0 at 07:30 and D0 at 21:30. The initial and final hours of each light cycle were set to follow a sinusoidal increase or decrease in light intensity, respectively. TriPLICATE cultures were grown in ASP2 medium (Provasoli et al., 1957; Van Baalen, 1962) either supplemented with 17 mM NO\(_3\) ("NO\(_3\) culture") or prepared with no inorganic N added ("N\(_2\) culture"). Cultures were bubbled with ambient air (400 mL min\(^{-1}\)) and maintained under turbidostat mode at OD\(_{680}\) of \(\sim 0.5\). Another set of triplicate cultures were grown in \(\sim 300\) mL glass tubes in ASP2 medium without NO\(_3\) amendment at 28°C under 300 \(\mu\)mol photons m\(^{-2}\) s\(^{-1}\) (14L:10D, same light regime as above) under semi-continuous, fed-batch mode ("SC-N\(_2\) culture"). The SC-N\(_2\) cultures were cultivated and maintained to ensure a "back-up" option in case the turbidostat (N\(_2\) culture) failed during the experiment. Because both culture conditions were stable throughout the experiment, we opportunistically sampled both cultures. The SC-N\(_2\) culture exhibited a wider range of phenotypic variability compared to the N\(_2\) culture that may be akin to some more dynamic natural environments. Therefore, we chose to include results for both diazotrophic cultures. All cultures maintained constant population sizes for >21 days prior to sampling. Culture and cell characteristics for each treatment are given in Table 1. An analysis of photosynthetic electron transport and the energetic costs of N and C acquisition in the same cultures studied here are given in Rabouille et al. (In Revision).

Cell density and size distributions were determined using a Multisizer 4 Coulter Particle Counter (Beckman Coulter Inc., Brea, CA, United States). Particulate organic carbon (POC) and nitrogen (PON) were analyzed using an elemental analyzer (PerkinElmer PE2400, PerkinElmer Inc., Waltham, MA, United States) following sample collection (10 mL), centrifugation (28°C, 8,000 rpm), and drying at 60°C. Chl \(a\) and polysaccharide contents were determined following the previously reported protocols (Zavrel et al., 2015a,b, 2018). Cyanophycin cell content was quantified by Sakaguchi reaction (Messineo, 1966), after sample concentration (30 mL) by centrifugation (28°C, 8,000 rpm, 7 min).

**Stable Isotope Probing Experiments**

For stable isotope probing experiments, samples were collected from one culture replicate from each condition. Incubations were performed by sub-sampling cultures into 6 mL gas-tight vials and amending the ASP2 medium with NaH\(_{13}\)CO\(_3\) (all cultures) and either \(^{15}\)N\(_2\) (N\(_2\) and SC-N\(_2\) cultures) or Na\(^{15}\)NO\(_3\) (NO\(_3\) culture). Vials were incubated under light and temperature conditions that were equivalent to culture conditions. Incubation durations were 2 h in the morning, 2.5 h during the day, and 10 h during the night, with shorter incubations in early night (3 h) and late night (5 h). Isotope labeling was calculated from the known amounts of label added to the incubation medium and measured initial concentrations of unlabeled substrate in the bioreactors (Supplementary Table 1). The \(^{15}\)N\(_2\) enriched stock was prepared by injecting 10 mL of \(^{15}\)N\(_2\) gas into 43 mL of the ASP2 medium, followed by an equilibration for >24 h. The \(^{15}\)N-N\(_2\) atom fraction in the incubation medium was calculated assuming that \(^{15}\)N\(_2\) was fully equilibrated with the stock solution. Since this may lead to an underestimation of N\(_2\) fixation rates (Mohr et al., 2010), we refrain from comparisons of N assimilation rates between N treatments (N\(_2\) vs. NO\(_3\)). However, comparisons over time and among cells within each treatment are not affected since any potential underestimation would be similar in all \(^{15}\)N\(_2\) incubations. NO\(_3\) concentration in the incubation medium was estimated by averaging NO\(_3\) concentrations measured in the bioreactor on the respective day of the experiment. Dissolved inorganic carbon (DIC) concentration in the incubation medium was estimated by measuring DIC concentrations in the bioreactor at three time points during the day and interpolating them to the starting time points of our stable isotope incubations. Because the DIC concentration in the cultures varied depending on the time of the day, \(^{13}\)C-DIC atom fractions varied during our incubations, although the amounts of added NaH\(_{13}\)CO\(_3\) were the same (Supplementary Table 1).

**TEM Analysis**

At the end of each isotope-labeling incubation, cells were collected and centrifuged at 2,700 rpm for 10 min at room temperature. One microliter of the pellet was mixed with 1 \(\mu\)L of 20% bovine serum albumin and transferred to a formvar-coated 100 mesh TEM grid. After removing the excess liquid with a filter paper, the grids were frozen in liquid ethane cooled with liquid nitrogen. Freeze-substitution was carried out in a 2% mixture of OsO\(_4\) in 100% acetone (v/v) sequentially at three temperatures: \(-90°C\) (for 96 h), \(-20°C\) (for 24 h), and 4°C (for 10 h). Temperature was increased at a rate of 5°C h\(^{-1}\) (from \(-90\) to \(-20°C\)) and 3°C h\(^{-1}\) (from \(-20\) to 4°C). After freeze-substitution, the samples were washed three times in acetone and infiltrated sequentially in a 2:1, 1:1, and 1:2 (v/v) mixture of acetone and low-viscosity Spurr resin (EMS) for 1 h in each step. Finally, the samples were incubated overnight in a 100% resin, transferred to embedding molds, and allowed to polymerize. Thin sections (200 nm) were cut with a diamond knife, placed on Cu-indexed TEM grids (rinsed in 30% ethanol), and contrasted for 20 min in saturated ethanolic uranyl acetate (EMS, Hatfield, United States; concentration 13 g/100 mL 50% ethanol; solution filtered before use through a 0.45 \(\mu\)m pore size filter). Images were taken using a JEOL 1010 TEM at 80 kV.

**NanoSIMS Analysis**

Nanoscale secondary ion mass spectrometry analyses were performed on two types of samples: (i) thin sections that were first imaged by TEM (as described above) and (ii) whole cells collected on polycarbonate filters. For downstream analysis of samples initially imaged by TEM, the lowest primary ion beam current (0.5 pA) was used to achieve the highest lateral resolution afforded by the instrument (\(\sim 50\) nm). However, because the samples were very thin (\(\sim 200\) nm), the number of imaged frames was rather low (20–50) before the cell material was sputtered away. This sometimes resulted in a poor signal-to-noise ratio (SNR) in the final secondary ion images, and thus, a low number of cells for which good quality complementary TEM
and nanoSIMS images are available. Additional measurements were therefore performed on cells deposited on filters, because the imaging could be done with a stronger beam (2 pA) and over a larger area and many more frames (>200). However, the improved throughput and SNR came at the expense of a lower spatial resolution (see section "Results"). For nanoSIMS analysis of whole cells, the cells were filtered onto polycarbonate filters (2.5 cm diameter, 0.2 µm pore size, Millipore), washed three times, air-dried, and stored at room temperature. Chemical fixation was not performed thus avoiding dilution of the isolate label. Just prior to nanoSIMS analysis, filters were sputter-coated with a 10-nm gold layer, cut into small circular pieces (5 mm diameter) suitable for the nanoSIMS sample holder, and imaged with a Neoscope II JCM-6000 scanning electron microscope (JEOL, Japan) to check sample quality (cell integrity and cell density).

Nanoscale secondary ion mass spectrometry measurements were performed with the NanoSIMS 50L instrument (Cameca, France) operated at Utrecht University. Areas of interest were first pre-sputtered with Cs+ ions until secondary ion yields stabilized. Subsequently, the primary Cs+ ion beam was scanned over the sample (areas between 10 µm × 10 µm and 30 µm × 30 µm in size, dwell time of 1 ms pixel−1) while detecting secondary ions 12C−, 13C−, 16O−, 12C14N−, 12C15N−, 31P−, and 32S−. To increase the overall signal, the same area was imaged multiple times, and the resulting ion count images were aligned and accumulated.

**NanoSIMS Data Processing and Quantification of Rates**

Nanoscale secondary ion mass spectrometry data were processed with the Look@NanoSIMS software (Polerecky et al., 2012) to quantify 13C and 15N atom fractions, denoted as x(13C) and x(15N) (Coplen, 2011), in regions of interest (ROI’s) corresponding to cells or inclusion bodies (polysaccharide granules or cyanophycin inclusions). After drawing ROIs manually, x(13C) in the ROI was determined from the total counts of secondary ions 12C− and 13C− accumulated over the ROI pixels as x(13C) = 13C−/(12C− + 13C−). Similarly, x(15N) in the ROI was determined from the total counts of 12C15N− and 12C14N− accumulated over the ROI pixels as x(15N) = 12C14N−/(12C14N− + 12C15N−).

The C- and N-specific rates of 13C and 15N assimilation into whole cells (kC and kN, respectively) were calculated as:

\[
k_C = - \frac{1}{t} \ln \left[ 1 - \frac{x(13C) - x(13C)_{ini}}{x(13C)_S - x(13C)_{ini}} \right]
\]

(1)

\[
k_N = - \frac{1}{t} \ln \left[ 1 - \frac{x(15N) - x(15N)_{ini}}{x(15N)_S - x(15N)_{ini}} \right]
\]

(2)

while the C- and N-specific rates of 13C and 15N incorporation into polysaccharide granules (pC or pN, respectively) and cyanophycin inclusions (yC and yN, respectively) were calculated as:

\[
p_C = \frac{1}{t} \frac{x(13C) - x(13C)_{ini}}{x(13C)_S - x(13C)_{ini}}
\]

(3)

\[
p_N = \frac{1}{t} \frac{x(15N) - x(15N)_{ini}}{x(15N)_S - x(15N)_{ini}}
\]

(4)

In Eqs 1–4, x(13C)_S and x(15N)_S are atom fractions of the C and N source, respectively, and x(13C)_{ini} and x(15N)_{ini} are the initial atom fractions of C and N in the ROI, respectively. The isotope labeling of the C and N sources, x(13C)_S and x(15N)_S, was assumed to be constant during the incubation and was

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**TABLE 1 | Culture conditions and properties of Cyanothece 51142 during N2 and NO3 growth.**

| Culture | Collection time | N2 | NO3 | SC-N2 |
|---------|----------------|----|-----|-------|
|         |                | Turbidostat | Turbidostat | Semi-continuous batch |
| Culturing strategy | Daily average | 0.22 ± 0.07 | 0.31 ± 0.05 | 0.26 ± 0.09 |
| Specific growth rate (d−1)a |            | 2.92 ± 0.07 | 3.03 ± 0.09 | 2.99 ± 0.13 |
| Cell diameter (µm) | Dawn | 109 ± 14 | 144 ± 9 | 111 ± 9 |
| Cell a (fg cell−1) | Dawn | 2725 ± 23 | 2515 ± 159 | 3076 ± 127 |
| Cell C (fmol cell−1) | Dawn | 198 ± 23 | 209 ± 13 | 256 ± 13 |
| Cell C (fmol cell−1) | Dawn | 534 ± 69 | 584 ± 30 | 550 ± 1.4 |
| C:N (w:w) | Dawn | 4.45 ± 0.58 | 4.30 ± 0.35 | 5.60 ± 0.22 |
| C:N (mol: mol) | Dawn | 5.19 ± 0.08 | 5.02 ± 0.08 | 6.53 ± 0.26 |

Shown are mean ± SD values for three replicate cultures.

a Determined by exponential fits of the OD720 signal from the on-board sensor in the turbidostats (Zavrel et al., 2015a) or by total volume displaced during daily dilution of semi-continuous batch culture.

b Measured at 16:00.
c Mean and SD calculated from three values measured 2, 7, and 14 h after the start of the light phase.
calculated as described above (Supplementary Table 1). $x^{(13)}C_{ini}$ and $x^{(15)}N_{ini}$ were determined by averaging data obtained from cells that were not exposed to the labeled substrate (i.e., control cells; $x^{(13)}C_{ini} = 1.052 \times 10^{-2}$, SD($x^{(13)}C_{ini}$) = 0.007 $\times 10^{-2}$, and $x^{(15)}N_{ini} = 3.75 \times 10^{-3}$, SD($x^{(15)}N_{ini}$) = 0.04 $\times 10^{-3}$, $n = 30$).

A cell or an inclusion body was considered significantly enriched in $^{13}$C if the 95% confidence interval of its estimated mean $^{13}$C atom fraction did not overlap with that of the control cells, i.e., if $x^{(13)}C_{ini} \pm 2 \times SE(x^{(13)}C_{ini})$ did not overlap with $x^{(13)}C_{ini} \pm 2 \times SE(x^{(13)}C_{ini})$. Here, the standard errors were calculated as $SE(x^{(13)}C_{ini}) = SD(x^{(13)}C_{ini})/\sqrt{n}$ for the control cells, and $SE(x^{(13)}C) = x^{(13)}C \times PE(x^{(13)}C)$ for each individual cell or inclusion body, where the relative Poisson error was calculated from the total counts of $^{12}$C$^-$ and $^{13}$C$^-$ in the cell or inclusion body as $PE(x^{(13)}C) = [1 - x^{(13)}C] \times [1/^{13}C^- + 1/^{12}C^-]^{1/2}$ (Polerecky et al., 2012). The same approach but using the total counts of $^{12}$C$^{14}$N$^-$ and $^{12}$C$^{15}$N$^-$ was applied to determine significant enrichment in $^{15}$N.

Note that the C- and N-specific rates of $^{13}$C and $^{15}$N assimilation into whole cells, polysaccharide granules, and cyanophycin inclusions have units of per time (i.e., h$^{-1}$ or day$^{-1}$) and give the rate of $^{13}$C and $^{15}$N assimilation rates normalized to the C and N content of the ROI [i.e., mol C (mol C)$^{-1}$ h$^{-1}$ for $k_C$, $p_C$, and $y_C$, and mol N (mol N)$^{-1}$ h$^{-1}$ for $k_N$, $p_N$, and $y_N$]. Evaluation of the variability in assimilation rates among cells and intracellular inclusions required considering how the measured $x^{(13)}C$ and $x^{(15)}N$ in the ROI were linked to cell growth and metabolism of internal C and N pools (e.g., synthesis and degradation of polysaccharides and cyanophycin inclusions, or recycling of N from existing proteins during cyanophycin synthesis). These considerations are summarized in the Discussion section (Section "Interpreting Isotopic Enrichment Imaging Data"). The assumptions underlying the rate calculations presented above are further explored and discussed in Polerecky et al. (In Revision).

RESULTS

Identification of Intracellular Inclusions

Prominent intracellular inclusion bodies identified in TEM images of *Cyanothecae* 51142 included carboxysomes, cyanophycin inclusions, polyphosphate bodies, polysaccharide granules, and thylakoid membranes (Figure 1). Some, but not all, of these inclusions could be reliably identified in nanoSIMS images when the accumulated secondary ion counts $^{12}$C$^{14}$N$^-$, $^{32}$S$^-$, and $^{31}$P$^-$ were combined into RGB overlays. Specifically, carboxysomes, which were identified in TEM images as dark areas with a characteristic hexagonal shape (Figures 1A,C, arrows labeled “c”), had relatively higher CN$^-$ and S$^-$ but lower P$^-$ counts than the surrounding cell material. These differences caused carboxysomes to appear yellow-green in the RGB overlays (Figures 1D,F). Cyanophycin inclusions, which were identified through their oval shape and darker appearance in the TEM images, had markedly higher CN$^-$ counts, while the S$^-$ and P$^-$ counts were not different from the surrounding cell material. The relative enrichment in CN$^-$ counts gave cyanophycin inclusions an orange-to-red appearance in the RGB overlays (Figure 1, arrows labeled “cy”). Polyphosphate bodies had higher P$^-$ counts and lower CN$^-$ and S$^-$ counts relative to the surrounding cell material and appeared as bluish spots in the RGB overlays (Figure 1, arrows labeled “p”). Although polysaccharide granules could be identified in TEM images as bright oval shapes (Figure 1A, arrows labeled “ps”; see also Deschamps et al., 2008), their identification from the nanoSIMS images was not reliable. For example, most polysaccharide granules were associated with localized decreases in CN$^-$ counts (Figure 1D). However, the contrast between the polysaccharide granules and the cell matrix was low, and similar decreases in CN$^-$ counts sometimes occurred even when there was no obvious presence of polysaccharide in the TEM images. Neither could variability in S$^-$, P$^-$, C$^-$, or O$^-$ counts (C$^-$ and O$^-$ data not shown) be used to distinguish polysaccharide granules in the nanoSIMS images. Similarly, thylakoid membranes were not identifiable using the nanoSIMS images although their visibility in the TEM images was often good (Figures 1A–C, arrows labeled “t”). Conversely, nucleoids were clearly observed as the violet-colored regions in the RGB overlays due to P counts being markedly higher than the surrounding cell material and CN$^-$ and S$^-$ counts that were similar to the surrounding cell material (Figures 1D–F, arrows labeled “n”); however, direct nucleoid identification in the TEM images was not possible.

Carbon and Nitrogen Assimilation Rates and Allocation Patterns

N$_2$ Culture

Daytime C fixation in *Cyanothecae* 51142 grown under diazotrophic conditions in turbidostat mode ("N$_2$ culture") was observed in all but one of the 126 cells imaged (Figures 2A,B). The C-specific rates of $^{13}$C assimilation in whole cells, $k_C$, were highest in the morning and declined on average by about 80% in the afternoon (Table 2). $^{13}$C enrichment was highest in polysaccharide granules and lower and diffusely spread throughout the cell matrix (Figures 3A,B). The C-specific rates of $^{13}$C assimilation in polysaccharide granules, $p_C$, varied (CV≈32%), with 61% of the variance explained by differences among cells and 39% of the variance explained by differences within cells (Supplementary Figure 1A). Moreover, the relative area of the cell sections covered by polysaccharide granules varied among cells (range: 0.08–0.28, CV≈43%) and was significantly positively correlated with the $k_C$ values ($R = 0.68$, $p = 0.002$; Supplementary Figure 2B).

The majority of cells in the N$_2$ culture fixed N$_2$ during the night (Figure 2C), with the exception of one cell (out of 104 imaged) that fixed N$_2$ in the morning (green circle in Figure 2A; "asynchronous diazotrophic" cell in Figure 3A). During the early night, cells grouped into two clear subpopulations exhibiting different activities: P1 showed significant N$_2$ fixation and accounted for 83% of cells, while P2 showed no significant N$_2$ fixation and accounted for 17% of cells (compare green and red circles in Figure 2C). P1 and P2 showed low but significant C fixation during the early night incubation (Figure 2C). The $k_C$ values in P1 and P2 did not differ at night [ANOVA,
Correlative microscopy of thin sections of *Cyanothece* 51142 cells. Shown are examples of TEM images (A–C) and the corresponding nanoSIMS images (D–F). The dashed polygon in panels (D–F) shows the boundary of the TEM image aligned within the NanoSIMS image. Shown are images from samples collected at 09:30 following a morning incubation (07:30–09:30) from the N$_2$ culture (A,D) and NO$_3$ culture (B,E), and from a sample collected at 07:00 following a night-time incubation (21:45–07:00) from the NO$_3$ culture (C,F). Arrows in the images point to sub-cellular structures identified as carboxysomes (c), cyanophycin inclusions (cy), polyphosphate bodies (p), polysaccharide granules (ps), thylakoid membranes (t), and nucleoid (n). NanoSIMS images are shown as RGB overlays of secondary ion counts $^{12}$C$_{14}$N$^{-}$ (red), $^{32}$S$^{-}$ (green), and $^{31}$P$^{-}$ (blue). Note that the hues are not comparable among the images because, for each overlay, the contrast for the three color channels was modified so as to enhance the overall visibility of the intracellular variability. In addition to P$^{-}$, the polyphosphate bodies had markedly increased O$^{-}$ ion counts (data not shown).

TABLE 2 | $^{13}$C and $^{15}$N assimilation rates in *Cyanothece* during N$_2$ and NO$_3$ growth.

|                | Morning                  | Afternoon                | Night                   | Night + day               |
|----------------|--------------------------|--------------------------|-------------------------|---------------------------|
|                | (07:30–09:30)            | (14:45–17:15)            | (early 21:45–00:45)     | (late: 02:00–07:00)       | (21:45–17:15)             |
| $k_C$ (d$^{-1}$) | N$_2$ culture            | 1.70 ± 0.35              | 0.39 ± 0.08             | 0.022 ± 0.006             | 0.007 ± 0.005             | 0.090 ± 0.015             |
|                | NO$_3$ culture           | 0.84 ± 0.15              | 0.42 ± 0.07             | 0.0045 ± 0.0019           | –                         | –                         |
|                | SC-N$_2$ culture         | 0.74 ± 0.63              | 0.14 ± 0.03             | 0.0012 ± 0.0008           | –                         | –                         |
| $k_N$ (d$^{-1}$) | N$_2$ culture            | 0.016 ± 0.164            | 0.0024 ± 0.0032         | 1.34 ± 0.79               | 0.012 ± 0.047             | 0.078 ± 0.044             |
|                | NO$_3$ culture           | 3.19 ± 0.91              | 1.31 ± 0.18             | 0.32 ± 0.18               | –                         | –                         |
|                | SC-NO$_3$ culture        | 0.24 ± 0.28              | 0.0005 ± 0.0034         | 0.077 ± 0.063             | –                         | –                         |

Shown are mean ± SD values of $k_C$ and $k_N$ for cells measured by nanoSIMS. Values for individual cells are shown in Figure 2.

F(1,77) = 1.64, $p = 0.204$] and were about 1−1.5% of morning $k_C$ values. The $k_C$ and $k_N$ values did not correlate in subpopulation P1 ($R = 0.043$, $p = 0.73$), and $k_C$: $k_N$ ranged from 0.008 to 0.035 (mean = 0.016, SD = 0.008, CV≈50%) among the cells. During the late night, only about 3% of cells fixed N$_2$ (green pluses in Figure 2C), and the average $k_N$ was about 1% of the average $k_N$
in the early night (Table 2). Significant C fixation was observed in 5% of cells, and these cells again grouped into subpopulations depending on whether they also fixed N\textsubscript{2} (3%) or not (2%) (compare green and red pluses in Figure 2C). The remaining 95% of cells showed no detectable C nor N\textsubscript{2} fixation during the late night (black pluses in Figure 2C).

The C and N\textsubscript{2} fixation patterns observed separately during the day and night were reflected in cells incubated with isotopes over the full night + day cycle (Figures 2D, 3D). The majority of cells (~87%) fixed both C and N\textsubscript{2} (P3, orange circles in Figure 2D), whereas the remaining 13% of cells had k\textsubscript{C} values similar to P3 but showed no significant N\textsubscript{2} fixation (P4, red circles in Figure 2D).

Average k\textsubscript{C} and k\textsubscript{N} values were similar for the majority of cells incubated for the full night + day cycle, but these values were only about 5% of the peak k\textsubscript{C} and k\textsubscript{N} values observed during the morning and early night, respectively (Table 2). These dramatic shifts in metabolism over the day caused k\textsubscript{C} in the morning to overestimate specific growth rate (\(\mu = 0.22 \text{ day}^{-1}\)) by 7.7-fold. Theoretically, the average night + day k\textsubscript{C} value should estimate \(\mu\), but was only 0.09 day\textsuperscript{-1}. Similarly, k\textsubscript{N} at night overestimated \(\mu\) by up to 6-fold, and the average night + day k\textsubscript{N} was only 0.08 day\textsuperscript{-1}. The discrepancies between \(\mu\) and the average k\textsubscript{C} and k\textsubscript{N} from the night + day (~20 h) incubations were likely caused by differences in incubation conditions, including gas flow and medium exchange in the turbidostat that were not possible in the isotope labeling incubation. Finally, k\textsubscript{C}:k\textsubscript{N} varied widely among cells (range: 0.6–3.3, mean = 1.24, SD = 0.78, CV = 63%).

Cyanophycin inclusions showed the greatest 13C and 15N enrichment compared to other inclusions within individual cells from the early night incubation (Figures 3C, 4A–C). Similar to the data clustering observed for the whole cells, individual cyanophycin inclusions grouped into two clear subpopulations, one with significant 15N enrichment (P1) and one with no significant 15N enrichment (P2) (compare green and red circles in Figure 4B). There was no significant correlation between the C- and N-specific 13C and 15N assimilation rates in cyanophycin granules, \(y_C\) and \(y_N\), in P1 (\(R = 0.10, p = 0.10\)), and \(y_C:y_N\) were highly variable between cyanophycin granules among cells (range: 0–0.09, mean = 0.015, SD = 0.016, CV = 107%) and within individual cells (Figure 4A). In one cell where we could clearly
FIGURE 3 | Images of isotopic enrichment in Cyanothece 51142. Shown are overlays of $^{13}$C (red) and $^{15}$N (green) enrichments measured in cells from three parallel cultures ($N_2$, diazotrophic culture grown in turbidostat mode; $NO_3$, culture grown on nitrate in turbidostat mode; SC-$N_2$, diazotrophic culture grown in semi-continuous batch mode). Cells were incubated during time intervals indicated above the images. Images shown in panels (A,B,E–G) were obtained from thin cell sections analyzed by TEM [panels (A,E,G) correspond to panels (A–C) in Figure 1], whereas panels (C,D,H–J) show images of cells collected on polycarbonate membrane filters. Additional images of filtered cells are shown in Supplementary Figure 2. In each image, the intensity (“brightness”) of the red and green color scales linearly with the $^{13}$C and $^{15}$N enrichment, respectively, with black indicating no enrichment. Note, however, that because the scaling for the red and green colors was optimized independently for each image to enhance the visibility of the intracellular heterogeneity, the intensities of the red and green colors are not comparable among the images. In panels (A,C,E–G,I,J), examples of cyanophycin inclusions (cy) and carboxysomes (c) are marked with dashed-line and solid-line arrows, respectively. In panels (A,B,E–G), areas and spots with pronounced $^{13}$C enrichment (red) correspond to polysaccharide granules. Black areas in panels (F,G) correspond to voids (v) due to artifacts associated with the preparation of the thin cell sections.

resolve all relevant intracellular structures, we observed low but significant $^{15}$N enrichment in the carboxysomes and a slightly greater $^{15}$N enrichment in the nucleoid in addition to the strong $^{15}$N enrichment in the cyanophycin inclusions (Figure 4C).

$NO_3$ Culture

Cyanothece 51142 grown under non-diazotrophic conditions in turbidostat mode (with $NO_3$ added; “$NO_3$ culture”) showed daily patterns of C fixation that were similar to the $N_2$ culture. Morning $k_C$ was 2.7-fold greater than $\mu$ of 0.31 day$^{-1}$. In the afternoon, $k_C$ decreased on average by 50% and reached about 0.5% of the morning values during the night (Figures 2E–G and Table 2). Cells in the $NO_3$ culture always assimilated newly fixed C into polysaccharide granules (Figures 3E–G), whereas in the $N_2$ culture the highest $^{13}$C enrichment was observed in polysaccharide granules during the day but in cyanophycin inclusions during the night (compare Figures 3A–C).

The daily patterns of N assimilation and intracellular allocation were more complex in the $NO_3$ culture than in the $N_2$ culture. Values of $k_N$ were highest in the morning (10.3-fold greater than $\mu$) and decreased by about 60% in the afternoon and by 90% during the night (Figures 2E–G and Table 2). In the morning, $^{15}$N was often accumulated in what appear to be newly synthesized cyanophycin inclusions (Figure 3E and Supplementary Figure 3D, arrows 1) or was added to existing cyanophycin inclusions as manifested by a $^{15}$N-rich “shell” surrounding a $^{15}$N-poor core (Figure 3E, arrow 2). In many cells, $^{15}$N enrichment was clearly present.
in the cell matrix but not in existing cyanophycin inclusions (Supplementary Figure 3D, arrows 3). In the afternoon, $^{15}$N enrichment was distributed relatively homogeneously within the cell matrix, and $^{15}$N enrichment in cyanophycin inclusions was sometimes greater but more often lower than in the cell matrix (Figures 3E,F). In the morning, $k_C$ and $k_N$ were significantly correlated ($R = 0.43$, $p = 0.045$), whereas no significant correlation was observed in the afternoon ($R = -0.02$, $p = 0.90$). For both morning and afternoon incubations, $k_C/k_N$ varied among individual cells from 0.15 to 0.5 (mean = 0.31, SD = 0.08, CV = 26%).

Night-time NO$_3^-$ assimilation resulted in $^{15}$N being homogeneously enriched within the cell matrix, but carboxysomes were notably more enriched in $^{15}$N than the cell matrix, and cyanophycin inclusions showed no $^{15}$N enrichment (Figure 3G). This intracellular N allocation pattern was observed in all cells (Supplementary Figure 3F) despite the large intercellular variability in $k_N$ values. In contrast to the N$_2$ culture, $k_C$ and $k_N$ in the NO$_3^-$ culture were strongly correlated during the night ($R = 0.94$, $p < 10^{-4}$; Figure 2G) even though the newly assimilated $^{13}$C and $^{15}$N were allocated into different cell compartments (Figure 3G). Night-time $k_C/k_N$ values varied only slightly in the NO$_3^-$ culture among individual cells (range 0.01–0.03, mean = 0.015, SD = 0.005, CV = 33%).

**SC-N$_2$ Culture**

In the majority of *Cyanothece* 51142 cells grown under diazotrophic conditions in semi-continuous, fed-batch mode...
These asynchronous diazotrophic cells, fixed N\textsubscript{2} values were observed during the morning and night, respectively (Figures 2H–J and Table 2). As in the N\textsubscript{2} culture, the highest \textsuperscript{13}C and \textsuperscript{15}N enrichments were detected in the polysaccharide granules and cyanophycin inclusions, respectively (Figures 3H–J). In the afternoon, there was no measurable N\textsubscript{2} fixation, and \textit{k}_C values decreased by about 80\% compared to the morning values (Figure 2I).

There were, however, two notable differences in the behaviors of the N\textsubscript{2} (turbidostat) and SC-N\textsubscript{2} cultures. Firstly, in the SC-N\textsubscript{2} culture there was a large subpopulation of cells (~46\%) that fixed N\textsubscript{2} during the morning (subpopulation P6 in Figure 2H). In these asynchronous diazotrophic cells, \textsuperscript{13}C and \textsuperscript{15}N enrichments were concentrated in cyanophycin inclusions (Figure 3H), and \textit{k}_N and \textit{k}_C as well as \textit{y}_C and \textit{y}_N were significantly negatively correlated (\textit{R} = −0.81, \textit{p} < 10^{-4}, Figure 2H; \textit{R} = −0.36, \textit{p} < 10^{-5}, Figure 4D, respectively). Additionally, the average \textit{k}_N measured in these morning N\textsubscript{2}-fixing cells was 6.4-fold higher than the average \textit{k}_N during the night time. Consequently, the average \textit{k}_N for the SC-N\textsubscript{2} culture in the morning was about 3.1-fold higher than at night (Table 2). The second notable difference was that cells from the SC-N\textsubscript{2} culture incubated in the afternoon had some cyanophycin inclusions that were significantly more enriched in \textsuperscript{13}C compared to the polysaccharide granules and cell matrix (Figures 3I, 4F).

**Intercellular Heterogeneity**

In all cultures and incubations, \textit{k}_C and \textit{k}_N were markedly heterogeneous among individual cells (Figure 2). Intercellular heterogeneity in day-time \textit{k}_C values was similar with CV = 16–22\% (Figure 5) across the turbidostat cultures (N\textsubscript{2} and NO\textsubscript{3} cultures). Heterogeneity in night-time \textit{k}_N values was also similar between the turbidostat cultures but was about 3-fold greater (CV = 53–57\%) than heterogeneity in day-time \textit{k}_C values. Heterogeneity in day-time \textit{k}_N in NO\textsubscript{3} cultures decreased from the morning to the afternoon (CV declined from ~30 to 15\%). Moreover, heterogeneity in \textit{k}_N in the N\textsubscript{2} culture during the night time was higher than in the NO\textsubscript{3} culture during the morning.

The semi-continuous diazotrophic batch culture (SC-N\textsubscript{2}) showed considerably greater intercellular heterogeneity than the turbidostat cultures (N\textsubscript{2} and NO\textsubscript{3}), especially for rates of N\textsubscript{2} fixation (Figure 5). In the SC-N\textsubscript{2} culture, the differentiation of cells into subpopulations in the morning was reflected in the high heterogeneity in \textit{k}_C (CV = 85\%) and \textit{k}_N (CV = 155\%). In the afternoon, heterogeneity in \textit{k}_C in the SC-N\textsubscript{2} culture decreased to a level similar to the N\textsubscript{2} culture (CV = 20\%).

**DISCUSSION**

**Interpreting Isotopic Enrichment Imaging Data**

The data yielded from nanoSIMS analyses hold valuable information about metabolic strategies used by cells across time and space. However, knowledge about cells’ activities that influence their isotopic composition at the end of an SIP incubation is critical to properly interpret nanoSIMS data. Carbon and nitrogen assimilation in *Cyanothece* mainly occurred during short and intensive periods either in the few hours after dawn or during the night. These periods of rapid assimilation activities fueled the majority of the cells’ C and N needs for growth and were followed by long periods of very low assimilation rates. Our results also show a wide range of C- and N-specific rates of \textsuperscript{13}C and \textsuperscript{15}N assimilation within and

![FIGURE 5](image-url) | Intercellular variability of C and N assimilation rates in *Cyanothece* 51142. Shown are the coefficients of variation (CV) of C-specific (A) and N-specific (B) rates of \textsuperscript{13}C and \textsuperscript{15}N assimilation for whole cells, \textit{k}_C and \textit{k}_N, derived from data shown in Figure 2.
between whole cells and among inclusions. Here, we critically evaluate the factors that can lead to variations in these measured rates and discuss several important, and to our knowledge previously unrecognized, considerations for using the spatially resolved $^{13}$C and $^{15}$N enrichment data obtained by nanoSIMS to infer rates of substrate assimilation. We first focus on principles of labeling as applied to subcellular structures, such as inclusion bodies, and then discuss these principles in the context of whole cell assimilation rates. A more comprehensive and mathematical analysis of these considerations can be found in Polerecky et al. (In Revision).

$^{13}$C or $^{15}$N enrichment depends upon the amount of labeled C or N added to a structure during an incubation relative to the amount of unlabeled C or N present prior to the incubation. Any structure that is newly synthesized during an incubation will have $^{13}$C and $^{15}$N enrichments that match those of the enriched C and N sources. However, C or N that is added to an existing structure during the incubation will cause the average $^{13}$C and $^{15}$N enrichment measured in the structure to be lower than in the C and N sources. The deviation between structure enrichment and source enrichment will decrease with incubation time as a function of the rate of biosynthesis but increase with the initial C and N content of the structure. Consequently, variation in the initial C and N content of structures will lead to apparent differences in $^{13}$C and $^{15}$N enrichments among structures present in an incubation even though the rates of biosynthesis and accumulation of $^{13}$C and $^{15}$N may have been the same.

$^{13}$C or $^{15}$N enrichment of a structure will also vary if the C and N used for its biosynthesis are derived from unlabeled sources of C and N, including the turnover of cellular macromolecules, in addition to the labeled sources external to the cell. One way to detect the relative importance of internal macromolecular recycling is to quantify the ratio of C- and N-specific rates of $^{13}$C and $^{15}$N incorporation into a structure (e.g., $y_{C\rightarrowYN}$ for a cyanophycin granule). Because the C:N ratio of many compounds comprising cell structures is well defined (e.g., cyanophycin has a C:N ratio of two), their biosynthesis will preserve the $^{13}$C:$^{15}$N signature of the C and N sources (except for the minute deviations linked to kinetic isotope fractionation). Consequently, if only the external, labeled pools of C and N are utilized for biosynthesis, the ratio of the C- and N-specific rates of $^{13}$C and $^{15}$N assimilation must be equal to 1. Any departure of this ratio from 1 implies that some of the C or N in the structure originated from a different source (i.e., with a different 13C:15N signature than that of the externally supplied sources).

Similar reasoning is needed when analyzing the isotopic enrichment of whole cells. Average $^{13}$C or $^{15}$N enrichment of a cell depends upon the amount of labeled C or N taken up during an incubation relative to the amount of unlabeled C or N present in the cell prior to the incubation. Consequently, intercellular variability in the amounts of C and N storage compounds will lead to apparent differences in the cellular $^{13}$C and $^{15}$N enrichments among cells even if the rates of $^{13}$C and $^{15}$N assimilation into cells were same. The influence of varying storage compound content can be revealed by analyzing the ratio between the C- and N-specific rates of $^{13}$C and $^{15}$N assimilation into whole cells, $k_{C\rightarrowK_{N}}$. For an individual cell, this ratio will be 1 (or very close to 1, if the subtle effects of kinetic isotope fractionation are included) provided the cell is in balanced growth, and the externally supplied labeled pools of C and N were the only sources of C and N assimilated by the cell. Any deviation from 1 indicates that (1) the cell assimilated an additional, unlabeled external source; (2) a storage product was preferentially synthesized over another (e.g., polysaccharides over cyanophycin); or (3) the cell recycled internal, unlabeled stores of C or N.

The foregoing analysis of enrichment sources, biosynthesis, and initial C and N content highlights that $k_{C\rightarrowK_{N}}$ can reveal the presence, synthesis, or mobilization of intracellular C and N stores. With these ideas in mind, we evaluate the isotope enrichment results for Cyanothece 51142 cultures grown with different N sources across a day–night cycle to understand their C and N assimilation processes and allocation strategies.

Roles of Internal C and N Recycling in Cyanophycin Synthesis

The majority of C used for cyanophycin synthesis at night in cells growing diazotrophically originated from recycling of existing C compounds within the cells. Cyanophycin synthesis involved some $^{13}$C (external C source) assimilation, but the $k_{C\rightarrowK_{N}}$ and $y_{C\rightarrowYN}$ values were much lower than 1 (Figures 2C, 4A,B). Thus, the bulk of the CO$_2$ incorporated into cyanophycin was likely derived from polysaccharide catabolism needed to simultaneously provide energy and reducing power (ATP and NADPH) for N$_2$-fixation. Nevertheless, cyanophycin synthesis was detected via their enrichment in $^{13}$C rather than $^{15}$N in about 17% of cells (Figure 3C). $^{13}$C is assimilated via two CO$_2$ fixation steps leading to synthesis of the non-ribosomal peptide, cyanophycin, which is comprised of aspartate and arginine (Flores et al., 2019). Specifically, CO$_2$ is incorporated via (i) pyruvate carboxylase or phosphoenolpyruvate carboxykinase yielding oxaloacetate which is transaminated by glutamate to form aspartate and (ii) carbamoyl phosphate synthase together with ornithine transcarbamoylase operating to generate arginine (Zhang et al., 2018).

The differences in $y_{C\rightarrowYN}$ values between and within N$_2$-fixing cells in the same culture (Figures 3C, 4A,B) were also caused by variations in the relative contributions to cyanophycin synthesis of $^{15}$N assimilated during the incubation and unlabeled N assimilated prior to the incubation. Unlabeled N may originate from efficient recycling of N in polyamines, including degradation of arginine via the recently described AgrE/PutA pathway (Burnat et al., 2019; Lee and Rhee, 2020). We hypothesize that the enrichment patterns observed in the N$_2$ cultures at night were caused by a variable fraction of cyanophycin-N that originated from active N$_2$ fixation (P1) or from protein degradation (P2). These findings suggest that diazotrophy demands internal N redistribution at night in all cells (as indicated by the similar $^{13}$C enrichment in cyanophycin inclusions), but distinct subpopulations emerge depending on their rates of N$_2$-fixation.

Some cyanophycin inclusions in cells from the SC-N$_2$ culture showed pronounced $^{13}$C but no $^{15}$N labeling.
During the afternoon incubation (Figures 3I, 4E,F). We speculate that these cells are part of the asynchronous diazotrophic morning subpopulation that fixed N₂ into cyanophycin (P6, Figure 3H) but that later synthesized cyanophycin using internal (and unlabeled) N sources. This idea is supported by the observation that about half of the population fixed N₂ in the morning and exhibited pronounced 13C enrichment in cyanophycin granules (Figures 3H, I). These observations suggest that cyanophycin synthesis in *Cyanothece* can occur throughout the entire light period, with cyanophycin-N derived either from N₂-fixation or internal N (e.g., via protein degradation). Our high-resolution imaging shows that cyanophycin effectively collects, stores, and redistributes N to facilitate ongoing protein synthesis and catabolism.

**Cyanophycin Biosynthesis Is Prioritized, but N Can Flow Directly to Protein During Growth on NO₃**

Whether growing diazotrophically or with NO₃, *Cyanothece* prioritized cyanophycin synthesis when the rates of N assimilation were at their highest. When N assimilation rates were lower, most cells growing on NO₃ assimilated N into the cell matrix and carboxysomes, suggesting that N was used directly for protein synthesis without prior storage in cyanophycin. Night-time NO₃ uptake into carboxysomes indicates that this new N was used immediately for the synthesis of RubisCO to maintain its content throughout the diel cycle (Nassoury et al., 2001). How cyanophycin synthesis is prioritized over protein synthesis is not clear, but our data suggest that once the cell has acquired sufficient N storage into cyanophycin, this storage step can be bypassed in favor of direct incorporation into proteins.

Night-time assimilation of N in the NO₃ culture was accompanied by small but detectable assimilation of CO₂, indicative of pyruvate carboxylase activity during the night. Surprisingly, this new C was directed into polysaccharides (Figure 3G). Typically, the pyruvate carboxylase reaction is considered important to ensure availability of oxaloacetate for citrate synthase to initiate the TCA cycle that produces NADH and amino acid precursors. However, in NO₃-grown cells, existing polysaccharides appear to have supplied all of the C for protein synthesis (because no 13C was incorporated into the carboxysomes coincident with 15N; see above). These results suggest that the amphibolic nature of the glycolytic/gluconeogenic pathway is directional with respect to the flow of newly fixed C into polysaccharides: newly fixed C flows through gluconeogenesis into polysaccharides simultaneous to glycolytic catabolism of “old” C stored in polysaccharides for use in protein synthesis. Glycolytic and gluconeogenesis pathways are strictly controlled so that they cannot be both highly active at the same time, which would create a futile cycle. The highly sensitive detection of labeled C and N afforded by the stable isotope probing and nanoSIMS technologies combined with TEM allowed us to view these unexpected cell activities that occur at very low levels but that support the careful modulation of C and N storage and re-mobilization in *Cyanothece*.

**Intercellular Heterogeneity in C and N Metabolism**

Within-population heterogeneity in 13C and 15N enrichments (k_C and k_N), such as that observed in N₂ and SC-N₂ treatments, has been reported in previous nanoSIMS-based studies (Foster et al., 2013; Mohr et al., 2013; Masuda et al., 2020), although the causes remain poorly understood (Ackermann, 2015). Intercellular heterogeneity has been attributed to stochastic gene expression or state switching in fast growing bacteria and yeast (Elowitz et al., 2002; Blake et al., 2003; Raj and van Oudenaarden, 2008; Raser and O'Shea, 2013; Sanchez et al., 2013; Damodaran et al., 2015). *Cyanothece* metabolism is strongly regulated by circadian rhythms, and thus, the intercellular heterogeneity observed in our study is at least partly associated with the regulation of C and N fixation determined by the light period and cell cycle (Caudron and Barral, 2013; Bach and Taucher, 2019).

We find that the metabolism of internal C and N storage compounds is another mechanism contributing to cell-to-cell heterogeneity in isotopic enrichment. Dual-label stable isotope probing combined with sub-cellular resolution imaging enabled us to identify internal recycling of N during cyanophycin synthesis, which led to variation in k_N values during night-time N₂ fixation (CV≈55%; Figure 2C) and to the wide ranges of y_N (CV≈72%; Figure 4B) and y_C/N (Figures 4A,B) values.

The intercellular heterogeneity in k_C values during morning C fixation could be caused by the variable polysaccharide content among cells (see Section “Interpreting Isotopic Enrichment Imaging Data”). The limited cellular volume probed by the nanoSIMS measurement also likely contributes to an apparent population heterogeneity. These alternatives are supported by the variable content and uneven distribution of polysaccharide granules within cells (Figures 1, 3A,B,E–G) and the fact that a large fraction of the variability in k_C values (~50%) was explained by the areal coverage by polysaccharide granules (Supplementary Figure 1B).

Differences in the turnover rates of storage inclusions with different C:N contents (cyanophycin vs. polysaccharides) may underlie the three-fold mismatch between the cell-to-cell variation in k_N and k_C. Other cyanobacteria and pico-eukaryotes have exhibited similar differences in k_N and k_C (Berthelot et al., 2019; Masuda et al., 2020). One explanation is that day-time acquisition of C reserves was insufficient to fuel N-fixation and other night-time metabolisms (Dron et al., 2013). However, predawn cells were never completely depleted of polysaccharide granules (data not shown), making it unlikely that C-reserves limited N₂ fixation. The large difference in cellular inclusion content also suggests that their subcellular metabolism influences k_N and k_C. Cyanophycin comprised ~3% of cellular N in the N₂ culture, whereas polysaccharides comprised ~30% of cellular C (Table 1). Moreover, the early night y_N values were considerably higher than the morning p_C values, suggesting that the turnover rate of cyanophycin is considerably faster than polysaccharide turnover at times of highest N and C assimilation, respectively.
Thus, it appears that during diazotrophy, cells retain large pools of C storage with slow turnover rates and small pools of N storage with high turnover rates to manage their C and N demands. This strategy could result in a greater range of enrichment in cyanophycin inclusions compared to the larger and less dynamic pool of polysaccharides. A high turnover rate of cyanophycin also helps explain why variation in $k_3$ values in NO$_3$-grown cells was higher in the morning, when the cells assimilated N into cyanophycin, and lower in the afternoon, when the cyanophycin pool was bypassed. Differences in the turnover rates of N-rich proteins might also explain the large variation in $k_N$ in NO$_3$-grown cells during the night when N assimilation again bypassed cyanophycin.

Differences in the timing of N$_2$ fixation revealed a surprising amount of within-population cell-to-cell heterogeneity in diazotrophic cultures. While the majority of cells fixed N$_2$ at night as expected (Mitsui et al., 1986; Gallon, 1992; Tuit et al., 2004; Wilson et al., 2017), subpopulations in both the turbidostat-grown and semicontinuous batch cultures fixed N$_2$ in the morning. Asynchronous diazotrophy has been suggested to occur when the amount of N$_2$ fixed at night is insufficient to support growth in the following day (Dron et al., 2013; Rabouille et al., 2014; Rabouille and Claquin, 2016). In *Cyanothece* 51142, asynchronous diazotrophy coincided with the diel maxima in population-level C fixation (Figure 2), but single-cell analysis revealed that morning N$_2$ fixation was limited to cells whose C fixation rates, and thus presumably intracellular O$_2$ concentrations, were low compared to the rest of the population. This behavior may be associated with prolonged deactivation of PSII through the early morning hours (Rabouille and Claquin, 2016). How these activities are regulated is not yet known.

Semi-continuous, fed-batch cultures are exposed to a wider range of nutrient and light concentrations compared to turbidostat cultures. These variations could result in a greater range of cell physiologies within a population. Together with previous reports of N$_2$ fixation in UCYN occurring during a subjective dark phase under continuous light (Colón-López and Sherman, 1998; Pennebaker et al., 2010; Dron et al., 2013), our findings suggest that the timing of N$_2$ fixation is not only regulated by the circadian rhythm or light/dark cycle but also by the cell's ability to balance N and light energy demands. The greater range of heterogeneity within the SC-N$_2$ culture compared to the N$_2$ culture is also consistent with the idea that cell-to-cell metabolic heterogeneity facilitates rapid population adjustment to environmental changes (Ackermann, 2015; Schreiber et al., 2016) such as those present in coastal environments (Rippka, 1988).

**CONCLUSION**

Dual labeling combined with nanoSIMS imaging enabled a much richer and more complex view of cell activities than previously observed using measurements of bulk activities. Specifically, we observed significant cell-to-cell variation, which we attribute to differences in (1) the degree to which internal storage compounds are used as sources of C and N for cyanophycin synthesis, (2) the turnover rates of different storage pools, (3) the range of environmental conditions experienced by a population over a day–night cycle, and (4) the timing of N$_2$ fixation. The intercellular heterogeneity potentially reflects adaptive mechanisms that allow *Cyanothece* to thrive in dynamic environments.

Additional details of C and N metabolism were also elucidated by evaluation of $^{13}$C and $^{15}$N labeling patterns across the day–night cycle. Cyanophycin synthesis is a highly effective N-scavenging pathway that assimilates N from protein degradation as well as external sources (NO$_3$ or N$_2$). Whether growing on N$_2$ or NO$_3$, cells prioritize cyanophycin synthesis when N assimilation rates are highest. In NO$_3$-growing cells, N assimilation switches from cyanophycin synthesis to RubisCO synthesis, suggesting that there is a cyanophycin requirement that, once met, can be bypassed in favor of protein synthesis. In NO$_3$-grown cells, night-time CO$_2$ was assimilated into polysaccharides simultaneous with catabolism of polysaccharides used for protein synthesis, suggesting that one way these cells control C is to maintain a directional flow of new carbon entering the cell: CO$_2$ → gluconeogenesis → polysaccharides → protein.

**DATA AVAILABILITY STATEMENT**

The original contributions presented in the study are included in the article/Supplementary Material; further inquiries can be directed to the corresponding author.

**AUTHOR CONTRIBUTIONS**

OP organized the experimental part of this study conducted during the 10th Group for Aquatic Productivity (GAP) workshop in August 2017. LP, TM, ME, and KH designed the study. TM, ME, and LP performed the SIP experiment. MK and LP performed the nanoSIMS analysis. MV performed the TEM analysis. LP, KH, ME, and TM drafted the manuscript, and all authors provided input during writing of the manuscript. All authors contributed to sampling and data interpretation.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2021.620915/full#supplementary-material

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