

# Carbon metabolism shapes FtsZ levels and cell division in the cyanobacterium *Anabaena* PCC 7120

Corresponding Author: Professor Cheng-Cai Zhang

This manuscript has been previously submitted at another journal. This document only contains information relating to versions considered at Communications Biology.

**This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.**

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

This work addresses a fundamental question in cyanobacterial biology, specifically the coordination between carbon metabolism and cell division and growth. The approach of perturbing the tricarboxylic acid (TCA) cycle by introducing an additional carboxylation module (PCEM) into *Anabaena* sp. PCC 7120 is original and well-executed. The findings presented are interesting and contribute to our understanding of how metabolic signals influence key cellular processes.

Major comments:

- There is a mistake in Figure 1C, so it is not clear which strain is which for the PCR experiment. Also, if the last two positive PCRs in P3+P4 correspond to C1 and C2, why do they show different sizes? And, which colony (C1, C2) was selected for further experiments? Were they checked by sequencing? If so, this should be stated in Methods section.

- Since the authors chose an inducible system for gene expression, did they check if the genes inserted were being expressed under non-induced conditions?

- According to Laurent et al., 2005; the addition of a 2-oxoglutarate analog (DFPA) could elicit heterocyst differentiation even with NH<sub>4</sub> added to the medium. Could the authors comment on why gPCEM strain doesn't show heterocysts under nitrate conditions despite the increased amount of 2-oxoglutarate? Also, based on Laurent's work, did the authors consider using DFPA to test if the cells show the same increase in FtsZ levels by artificially activating NtcA?

- The levels of 2-oxoglutarate under SL conditions are similar (t-test: no significance) between WT and gPCEM strain (Fig 2). How do the authors explain that gPCEM strain presents an elevated number of heterocysts under this condition (Fig S2)? Specially the number of contiguous heterocysts is quite elevated. Also, Fig 3 and Fig 4 show differences in growth, cell length and width when comparing gPCEM vs WT strain under SL condition.

- In Fig S2B, could the authors comment on the decision of representing absolute instead of relative frequency of intervals? Also, it is essential to include in the legend the number of intervals counted, and the number of cells in S2A.

- In Figure S3A, the authors showed that the deletion (without any insertion) of genes *alr2634*-*all2640* did not cause morphology defects in the cells under LL and SL conditions. Why didn't they check the same under HL conditions, where the phenotype of gPCEM strain is more evident? In the same line, why didn't they evaluate the cell morphology of the cells expressing the components individually (like in Fig. S3D) under high light conditions? I think that performing these control experiments would support better their conclusions.

- Since the graphs for length and width have different axis scales along the manuscript, it would be helpful to show the mean or median values in the text. For instance, in line 461 when comparing length and width of the WT strain under different light intensities. Anyways, I would advise to maintain the same axis, and adjust them to facilitate the visualization of the dot plots.

Minor comments:

- The authors state  $p < 0.1$  as \* significantly different. However, normally it is commonly accepted for biological studies 0.05 or 0.01 as the minimum significant difference. Still, this doesn't change any important statement of the work.
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#### Reviewer #2

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This is a very comprehensive study that nicely establishes a link between metabolism, the transcriptional regulator NtcA, FtsZ and cell morphology in the model filamentous cyanobacterium *Anabaena*. The work looks to have been done to a high standard and is very well-presented. I could find just one thing that doesn't seem to fit and needs some explanation:

The summary model in Fig. 7 suggests that smaller cell size in the wild type in high light results from mildly enhanced expression of FtsZ. However, Fig. 5C suggests that transcription of FtsZ does not increase in high light in the wild type, and Fig. 5A suggests that protein levels of FtsZ in the wild type do not increase during high light growth, although the accompanying cell images show the cells getting smaller. So it looks like the authors' model can account for differences between WT and gPCEM, and the work reveals a metabolic input into cell size control, but it can't explain the HL effects on cell size in the wild type, which must have a different cause.

#### Reviewer #3

##### (Remarks to the Author)

Review on Manuscript Number: COMMSBIO-25-5640-T

Corresponding Author: Professor Zhang "Carbon metabolism shapes FtsZ levels and cell division in a cyanobacterium"

Cyanobacteria are important players in the global carbon cycle. Moreover, some strains such as *Anabaena* 7120 serve also as model to study cell differentiation among prokaryotes. Finally, cyanobacteria are recently regarded as green cell factories that will certainly play an important role in the future sustainable bioeconomy. Despite their great importance, many basal aspects in the cyanobacterial biology are unresolved. The authors provide for the first time evidence that cell division in cyanobacteria and carbon metabolism are directly linked. They showed that manipulation of the signal metabolite 2-oxoglutarate directly impact cell division via a network regulating the expression of the cell division protein FtsZ including the transcriptional factor NtcA. The authors used an *Anabaena* strain expressing a CO<sub>2</sub>-fixing module leading to higher 2-OG levels under specific conditions. Using this strains and many control strains overexpressing single genes or altering the ntcA levels they could provide comprehensive evidence for a model, how carbon metabolism is regulating cell division among cyanobacteria. The experimental design is very nice and includes all necessary controls to justify the drawn conclusions.

I have only rather small minor points, which may help to improve the manuscript further.

Line 216: no change in 2-OG under SL conditions but heterocyst frequency differs from WT, please discuss this point!

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Line 238: this mutant shows similar reduction of 2-OG at SL as in pGCEM strain at LL, but there are changes visible! Please discuss this seemingly contradiction! may be that higher malate compensates for it?

Line 534: another possibility to explain the findings might be that HL results in relative CO<sub>2</sub> limitation because of higher growth, which is then replenished by the PCCEM module that is not necessary at LL and SL conditions!?

Line 576: where are these data, please add a reference on NtcA at different light intensities

#### Version 1:

##### Reviewer comments:

#### Reviewer #1

##### (Remarks to the Author)

I have reviewed the authors' responses to my comments and those from other referees. I think the authors have addressed my concerns with sufficient care and clarity. Therefore, I have no further requests.

Reviewer #2

(Remarks to the Author)

The authors have provided a satisfactory response to the points I raised on the first submission. I have no further modifications to suggest.

Reviewer #3

(Remarks to the Author)

I would like to thank the authors for the careful revision.

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Reviewers' comments:

**Reviewer #1 (Remarks to the Author):**

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**Response to the above general comments:**

We thank the reviewer for the overall positive assessment of our manuscript.

Major comments:

- There is a mistake in Figure 1C, so it is not clear which strain is which for the PCR experiment. Also, if the last two positive PCRs in P3+P4 correspond to C1 and C2, why do they show different sizes? And, which colony (C1, C2) was selected for further experiments? Were they checked by sequencing? If so, this should be stated in the Methods section.

**Response**

We sincerely apologize for the error in Figure 1C. We have now corrected this figure in the revised manuscript. Regarding the different band sizes observed for colonies C1 and C2 in the P3+P4 PCR, this is indeed due to gel electrophoresis artifacts caused by the GelRed stain, rather than actual size differences in the PCR products. The PCR products of both colonies were verified by sequencing, confirming they contain the correct construct. We have now clarified this in both the Methods section (lines 652-654) and the figure legend (lines 166-170). Since C1 and C2 show similar phenotypes and genotypes in our preliminary tests, we randomly selected C2 for further experiments. We added the description to the revised manuscript, as seen in the lines 166-170.

- Since the authors chose an inducible system for gene expression, did they check if the genes inserted were being expressed under non-induced conditions?

## Response

For all strains containing the inducible expression system, we conducted rigorous parallel control experiments comparing phenotypes with and without inducer treatment. The results demonstrated that the non-induced conditions consistently exhibited phenotypes identical WT controls (with the sole exception of WT::OE<sub>CT</sub>-PCEM strain, which is specifically presented in Figure S3). We maintain that even if trace-level leaky expression occurred, since the phenotypes are not changed, it would not affect our main conclusions. Therefore, to emphasize the induced effects more prominently, we chose not to include these control data in the main figures in the previous version. In the revised manuscript, we provided the micrographs of the gPCEM strain under non-induced and different light conditions, see figure 3B in the revised manuscript.

- According to Laurent et al., 2005; the addition of a 2-oxoglutarate analog (DFPA) could elicit heterocyst differentiation even with NH<sub>4</sub> added to the medium. Could the authors comment on why gPCEM strain doesn't show heterocysts under nitrate conditions despite the increased amount of 2-oxoglutarate? Also, based on Laurent's work, did the authors considered using DFPA to test if the cells show the same increase in FtsZ levels by artificially activating NtcA?

## Response

Thanks for this insightful question. We would like to stress a few things about the use of DFPA and the consequences of its accumulation reported by Laurent et al. Firstly, DFPA was designed to provide in vivo evidence for the signaling function of 2-oxoglutarate. This question is not the focus of the current manuscript. Secondly, DFPA was synthesized by fluorine chemistry, which itself is still a challenge for its synthesis and characterization. Thirdly, the use of DFPA was coupled to the expression of a 2-oxoglutarate transporter. Therefore, DFPA can be transported into cells and accumulate to a high level to mimic the accumulation of 2-OG signals observed under nitrogen-deprived conditions. In this study, while the gPCEM strain exhibits elevated 2-OG levels under high light in BG11 medium, the magnitude of this increase is insufficient

to reach the threshold required for heterocyst differentiation, unlike the rapid and pronounced 2-OG changes triggered by nitrogen starvation. As reported by another publication by one of us (Li et al., Microbiology, 2003, 149:3257), simply increasing 2-oxoglutarate by expressing the transporter, is not enough to trigger heterocyst formation under  $\text{NH}_4$  conditions. Therefore, the current results are in line with the knowledge we have on the function of 2-oxoglutarate.

- The levels of 2-oxoglutarate under SL conditions are similar (t-test: no significance) between WT and gPCEM strain (Fig 2). How do the authors explain that gPCEM strain presents an elevated number of heterocysts under this condition (Fig S2)? Specially the number of contiguous heterocysts is quite elevated. Also, Fig 3 and Fig 4 show differences in growth, cell length and width when comparing gPCEM vs WT strain under SL condition.

#### Response

Thanks. While the difference in 2-oxoglutarate (2-OG) levels between WT and gPCEM under standard light (SL) conditions did not reach statistical significance ( $p=0.09$  by t-test,  $n=3$ ), we consistently observed a modest but reproducible increase in 2-OG in the gPCEM strain (mean  $\pm$  SEM: WT  $1.11 \pm 0.18$  vs gPCEM  $1.47 \pm 0.09$  normalized units). This biologically relevant difference, though not statistically significant in our experimental setup, may explain the observed phenotypic variations. The slight elevation in 2-OG could enhance the nitrogen-depletion response pathway, leading to the observed increase in heterocyst frequency (WT  $9.4\% \pm 0.32\%$  vs gPCEM  $11.7\% \pm 0.62\%$ ) and higher contiguous heterocysts, and leading to subtle, unstable cell length and width changes. We clarified this in the revised manuscript, see lines 214-216 in the revised manuscript.

- In Fig S2B, could the authors comment on the decision of representing absolute instead of relative frequency of intervals? Also, it is essential to include in the legend

the number of intervals counted, and the number of cells in S2A.

#### Response

Thanks for these comments. We have changed the absolute to relative frequency in Figure S2B to better facilitate comparisons across datasets, see the line 30 in the revised supplemental file. For S2A, we have included the number of cells in the revised supplemental file, see line 30.

- In Figure S3A, the authors showed that the deletion (without any insertion) of genes *alr2634-all2640* did not cause morphology defects in the cells under LL and SL conditions. Why didn't they checked the same under HL conditions, where the phenotype of gPCEM strain is more evident? In the same line, why didn't they evaluate the cell morphology of the cells expressing the components individually (like in Fig. S3D) under high light conditions? I think that performing these control experiments would support better their conclusions.

#### Response

We apologize for not including these important controls initially and thank the reviewer for identifying this opportunity to strengthen our conclusions. We added the morphological characterization of the  $\Delta$ *alr2634-all2640* deletion strain and the cells expressing the components of PCEM individually under high-light conditions in Figure S3A and Figure S3D in the revised supplemental file. These results confirm that they all show similar morphological features to WT under HL, which strengthens our conclusions.

- Since the graphs for length and width have different axis scales along the manuscript, it would be helpful to show the mean or median values in the text. For instance, in line 461 when comparing length and with of the WT strain under different light intensities. Anyways, I would advise to maintain the same axis, and adjust them to facilitate the visualization of the dot plots.

#### Response

Thanks for these comments. We have added the mean values to all graphs

showing cell length and cell width measurements in the revised manuscripts (see figures 3C,4B,6E in the revised manuscript). We maintained the axis for cell length and cell width, respectively, while adjusting their presentation to optimize visualization of the dot plots (see Figures 3C,4B,6E in the revised manuscript).

Minor comments:

- The authors state  $p < 0.1$  as \* significantly different. However, normally it is commonly accepted for biological studies 0.05 or 0.01 as the minimum significant difference. Still, this doesn't change any important statement of the work.

Response

Thanks for these comments. We have changed the significant statement as follows in the revised manuscript: \*,  $p < 0.05$ ; \*\*,  $p < 0.01$ ; \*\*\*,  $p < 0.001$ , \*\*\*\*  $p < 0.0001$ , n.s.: not significant. See Figures 2,3C,4B,5C,6D,6E,6F and the corresponding figure legend in the revised manuscript.

- FigS5: PftsZ2 should be PftsZ1.

Fixed.

- FigS6: Number 6 is missing.

Added.

- Line 72: A reference is missing for this sentence?

Added.

## Reviewer #2 (Remarks to the Author):

This is a very comprehensive study that nicely establishes a link between metabolism, the transcriptional regulator NtcA, FtsZ and cell morphology in the model filamentous cyanobacterium *Anabaena*. The work looks to have been done to a high standard and is very well-presented. I could find just one thing that doesn't seem to fit and needs some explanation:

Response to the above general comments:

We thank the reviewer for the overall positive assessment of our manuscript.

The summary model in Fig. 7 suggests that smaller cell size in the wild type in high light results from mildly enhanced expression of FtsZ. However, Fig. 5C suggests that transcription of FtsZ does not increase in high light in the wild type, and Fig. 5A suggests that protein levels of FtsZ in the wild type do not increase during high light growth, although the accompanying cell images show the cells getting smaller. So it looks like the authors' model can account for differences between WT and gPCEM, and the work reveals a metabolic input into cell size control, but it can't explain the HL effects on cell size in the wild type, which must have a different cause.

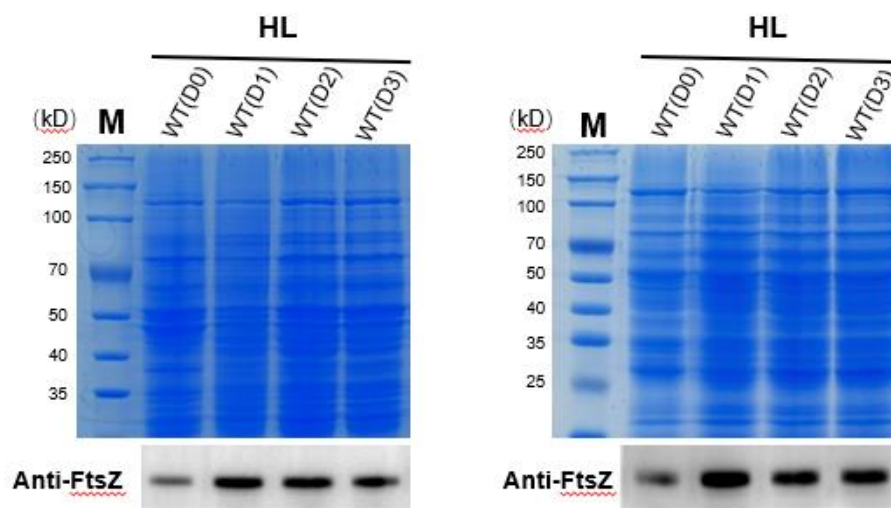
### Response

We appreciate the reviewer's careful examination of our data. In Fig. 5C, the transcriptional changes of FtsZ across all strains under different light conditions were analyzed independently, with data normalized to 1 for WT within each light condition (now clarified in the revised manuscript, Line 386). This normalization approach explains why no increase in FtsZ transcription was observed for WT under HL in this panel. Importantly, in Fig. S7B, we specifically analyzed the transcriptional fluorescence of *ftsZ1* and *ftsZ2* in WT across light conditions. The results demonstrate that *ftsZ2* is significantly ( $p < 0.0001$ ) upregulated under HL compared to standard light, confirming that FtsZ transcription does increase in WT under HL.

Regarding the changes in FtsZ protein levels in the WT under high light conditions, in two independent preliminary experiments, where we analyzed WT separately, we consistently observed an increase in FtsZ protein levels under HL (see attached Figures below). The lack of a clear visible increase in WT FtsZ levels in Fig. 5A is likely due to the overwhelming signal from the gPCEM strain, where FtsZ accumulates abundantly. This high signal intensity may have masked the subtler but reproducible increase in WT. To resolve this, we specifically quantified FtsZ levels in WT alone across light conditions in the original Fig. S7D. The results confirm that protein levels of FtsZ in the wild type

do increase under high light.

These results collectively support our model that the HL-induced cell size reduction in WT is mediated, at least in part, by enhanced FtsZ expression.



### Reviewer #3 (Remarks to the Author):

Review on Manuscript Number: COMMSBIO-25-5640-T

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Cyanobacteria are important players in the global carbon cycle. Moreover, some strains such as *Anabaena* 7120 serve also as model to study cell differentiation among prokaryotes. Finally, cyanobacteria are recently regarded as green cell factories that will certainly play an important role in the future sustainable bioeconomy. Despite their great importance, many basal aspects in the cyanobacterial biology are unresolved. The authors provide for the first time evidence that cell division in cyanobacteria and carbon metabolism are directly

linked. They showed that manipulation of the signal metabolite 2-oxoglutarate directly impact cell division via a network regulating the expression of the cell division protein FtsZ including the transcriptional factor NtcA. The authors used an *Anabaena* strain expressing a CO<sub>2</sub>-fixing module leading to higher 2-OG levels under specific conditions. Using this strains and many control strains overexpressing single genes or altering the ntcA levels they could provide comprehensive evidence for a model, how carbon metabolism is regulating cell division among cyanobacteria. The experimental design is very nice and includes all necessary controls to justify the drawn conclusions.

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I have only rather small minor points, which may help to improve the manuscript further.

Line 216: no change in 2-OG under SL conditions but heterocyst frequency differs from WT, please discuss this point!

Response:

Thanks. While the difference in 2-OG levels between WT and gPCEM under standard light (SL) conditions did not reach statistical significance ( $p=0.09$  by t-test,  $n=3$ ), we consistently observed a modest but reproducible increase in 2-OG in the gPCEM strain (mean  $\pm$  SEM:  $1.11 \pm 0.18$  vs gPCEM  $1.47 \pm 0.09$  normalized units). This biologically relevant difference, though not statistically significant in our experimental setup, may explain the observed phenotypic variations. The slight elevation in 2-OG could enhance the nitrogen-depletion response pathway, leading to the observed increase in heterocyst frequency (WT  $9.4\% \pm 0.32\%$  vs gPCEM  $11.7\% \pm 0.62\%$ ) and higher contiguous heterocysts. We clarified this in the revised manuscript, see lines 214-216 in the revised manuscript.

.Line 229. This conclusion is not entirely true, because at SL 2-OG was similar but the heterocyst phenotype is similar as in HL with increased 2-OG

Response:

We appreciate the reviewer's close attention to this point. As we addressed in the previous response (and have now clarified in the revised manuscript, Lines 214-216), while the 2-OG increase in gPCEM at SL did not reach statistical significance ( $p=0.09$  by t-test,  $n=3$ ), we consistently observed a reproducible modest elevation of 2-OG in gPCEM at this condition (mean  $\pm$  SEM:  $1.11 \pm 0.18$  vs gPCEM  $1.47 \pm 0.09$  normalized units). Therefore, we maintain the conclusion.

Line 238: were cells grown at ambient CO<sub>2</sub> or high CO<sub>2</sub>? the extra carbon-fixing module could have an advantage under ambient CO<sub>2</sub> conditions!

Response:

Thanks for this question. All experiments in this study were conducted under ambient atmospheric CO<sub>2</sub> conditions ( $\sim 0.04\%$  CO<sub>2</sub> in air) without supplementation. These conditions represent ecologically relevant growth conditions for cyanobacteria. Allows proper assessment of the carbon-fixing module's physiological advantage under carbon limitation. We clarified this condition in the method section, see line 628 in the revised manuscript. Whether the extra carbon-fixing module could have an advantage under ambient CO<sub>2</sub> conditions needs further study. We agree that comparative studies under different CO<sub>2</sub> conditions would be valuable for assessing industrial applicability. This direction has been added as our future work.

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Line 274: higher gene copies on plasmid than after chromosomal integration, could you please quantify the difference?

Response:

We acknowledge the reviewer's request. In our study, we employed the replicative plasmid PCT, which was derived from the well-characterized high-copy-number plasmid pDU1. As rigorously demonstrated in Yang et al. (2013, Research in Microbiology) - which we have now cited in the revised

manuscript - the pDU1 plasmid backbone maintains approximately 160-470 copies per chromosome in *Anabaena* sp. PCC 7120 under standard BG11 growth conditions. We added the reference and clarified this in the revised manuscript, seeing line 645-648 in the revised manuscript.

Line 238: this mutant shows similar reduction of 2-OG at SL as in gPCEM strain at LL, but there are changes visible! Please discuss this seemingly contradiction! may be that higher malate compensates for it?

Response:

Thanks for this comment. Since we could not identify the specific strain referenced at Line 238, we infer that you are inquiring about the 2-oxoglutarate (2-OG) levels in the  $\Delta$ suc-gPCEM strain under SL conditions. Yes, similar to the 2-OG levels observed in the gPCEM strain under SL conditions, the  $\Delta$ suc-gPCEM strain under SL showed a visible ( mean  $\pm$  SEM: WT  $1.11 \pm 0.18$  vs  $\Delta$ suc-gPCEM  $0.87 \pm 0.27$  normalized units) but statistically non-significant reduction in 2-OG ( $p = 0.32$  by t-test,  $n = 3$  biological replicates). We clarified this in the revised manuscript, seeing lines 330-333 in the revised manuscript. This lack of significance may be attributed to substantial variation between replicates. Importantly, even if the 2-OG reduction in  $\Delta$ suc-gPCEM (SL) were changed significantly, the absence of a comparable cell elongation phenotype would still be biologically plausible for the following reasons: (1) The 2-OG decrease in  $\Delta$ suc-gPCEM (SL) was milder than in gPCEM (LL) (mean  $\pm$  SEM: gPCEM (LL)  $0.25 \pm 0.11$  vs  $\Delta$ suc-gPCEM  $0.87 \pm 0.27$  normalized units), potentially insufficient to trigger the same morphological response. (2) The two strains exhibited divergent profiles of other TCA cycle metabolites (e.g., malate), suggesting that compensatory mechanisms may differentially modulate downstream effects. (3) As discussed in lines 537-544 in the original manuscript (lines 563-570 in the revised manuscript), we think in gPCEM (LL, significantly reduce 2-og), the changed TCA metabolites (2-OG and others) may directly or indirectly influence the FtsZ amount at posttranscriptional

levels and Z-ring assembly (not by *ftsZ* transcription) through an unknown mechanism.

Line 534: another possibility to explain the findings might be that HL results in relative CO<sub>2</sub> limitation because of higher growth, which is then replenished by the PCEM module that is not necessary at LL and SL conditions!?

**Response:**

We appreciate the reviewer's insightful suggestion regarding CO<sub>2</sub> limitation as a potential explanation for our observations. This alternative hypothesis is indeed plausible, and we discussed it in parallel with our proposed light-dependent NADPH mechanism in the revised manuscript as follows (see lines 543-560 in the revised manuscript):

**Original:** The PCEM module displays varying efficiency in carbon source input in *Anabaena* under various light conditions, affecting the TCA cycle differently (Fig. 2). This indicates that the PCEM-TCA coupled metabolic pathway in the gPCEM strain is light-dependent. This effect may be explained by the effect of light intensity on the production of NADPH in *Cyanobacteria* 48,49, which is a reducing power required for CO<sub>2</sub> fixation by PCEM. High light intensity may enhance the production of NADPH through photosynthesis in *Anabaena*, conditions in favor of the PCEM-TCA coupled metabolism. In contrast, low light intensity limits the energy supply and substrates available for the PCEM-TCA coupled pathway, ultimately affecting its overall activity. In addition, the TCA pathway may be directly or indirectly regulated by light so that carbon metabolism operates in response to photosynthetic activities. On the other hand, the artificial and synthetic PCEM pathway itself is unlikely to be regulated by light, consistent with the observation that consumption of propionyl-CoA (Fig. 2), the first substrate of this pathway, is similar under different light conditions tested.

**Modified:**

The PCEM module displays varying efficiency in carbon source input in

Anabaena under various light conditions, affecting the TCA cycle differently (Fig. 2). This suggests that the PCEM-TCA coupled metabolic pathway in the gPCEM strain is light-dependent. This effect may be explained by the effect of light intensity on the production of NADPH and CO<sub>2</sub> limitation in Cyanobacteria 48,49, NADPH is a reducing power required for CO<sub>2</sub> fixation by PCEM. High light intensity may enhance the production of NADPH through photosynthesis in Anabaena, conditions in favor of the PCEM-TCA coupled metabolism. In contrast, low light intensity limits the energy supply and substrates available for the PCEM-TCA coupled pathway, ultimately affecting its overall activity. Furthermore, HL-induced faster growth may lead to relative CO<sub>2</sub> limitation of cyanobacterial cells, which could be alleviated by the carbon input through the activity of the PCEM module - a function not required under low light (LL) or standard light (SL) conditions when CO<sub>2</sub> is sufficient. In addition, the TCA pathway may be directly or indirectly regulated by light so that carbon metabolism operates in response to photosynthetic activities. However, the artificial and synthetic PCEM pathway itself is unlikely to be regulated by light, consistent with the observation that consumption of propionyl-CoA (Fig. 2), the first substrate of this pathway, is similar under different light conditions.

Line 576: where are these data, please add a reference on NtcA at different light intensities

Added.