Statement of the Master thesis reviewer

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Thesis title: Comparison of photophysiological characteristics of two strains of the cyanobacterium *Trichodesmium* in relation to nitrogen fixation
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Review of the master thesis **Comparison of photophysiological characteristics of two strains of the cyanobacterium *Trichodesmium* in relation to nitrogen fixation** by **Bc. Anežka Terezie Míšková.**

The submitted Thesis aims to analyze an important question of oceanic nitrogen fixation. Namely, it is known that cyanobacteria of the *Trichodesmium* genus seem to use nitrogenase alongside the photosynthetic apparatus. Most other nitrogenase-active cyanobacteria separate these two biochemical pathways spatially or temporally because nitrogenases are highly sensitive to molecular oxygen, a byproduct of oxygenic photosynthesis. It has been postulated previously that the nitrogen-fixing cells (diazocytes) uncouple the major photosynthetic light-harvesting antenna to help downregulate photosynthesis, resulting in a marked increase in fluorescence yield of the diazocytes. Therefore, the Thesis aims to analyze the problem by a combination of bulk and single-cell time-resolved fluorescence methods (TCSPC and FLIM) and by Lugol staining, which is used to detect the diazocytes.

The Thesis is written in English and contains 52 pages of main text, split into 9 chapters, plus Bibliography, List of abbreviations and Attachment. For unclear reasons, some chapters are numbered and other are not. The text lacks the standard "Aims" chapter but the expected information is included in the very first short chapter, called Introduction. The Thesis is graphically well prepared and it does not suffer from major graphical inconsistencies as sometimes happens in qualification works. In terms of overall structure, there are 20 pages of Introduction (5 chapters), three pages of Methods, 20 pages of Results, eight pages of Discussion and a page of Conclusions. The included bibliography is impressive as it contains almost 100 references, of which 20 are to good scientific literature from the last 10 years. Thus, as far as structure and formal requirements are concerned, the Thesis is of high quality. Although overall the writing and language is good and understandable, with few typographic or factual errors, in terms of content the Thesis leaves a lot to be desired. It was very difficult for me to orient myself and understand the described work, its justification and its results.

The Introduction suitably covers the topics of photosynthesis, nitrogen fixation, the specifics of *Trichodesmium* and the theory of fluorescence. Considering the methods used by the student, description of microscopy and FLIM theory is lacking. As already mentioned above, the Methods section, while covering most of the necessary information in a scientific article fashion, is quite short, which is not wrong *per se* but stands out in comparison with the rest of the Thesis. Some information necessary for evaluating the results, such as laser power, light energy density in sample plane, spectral resolution, exposure times etc., is missing.

The Results chapter contains a lot of data but the text does not help the reader very much. The text jumps right in with little introduction on why the experiments were conducted in the specified way and what the reader should expect. The first sub-chapter on pigment analysis is inexplicably filled with statistical analyses and accompanying numbers but it is not clear why the analysis was conducted

and how it connects to the rest of the work. Likewise, the part about Lugol staining does not help the nonspecialist reader very much. The images are not very well explained and it is not clear what to focus on. In text, one paragraph uses units in %, another uses μl/ml. The bulk of the chapter is dedicated to fluorescence lifetime results. The part on TCSPC is quite clear, provides good analysis of the *Trichodesmium* fluorescence features and compares data from the cells of one strain (IMS101) in diazotrophic and 'normal' regime. In contrast, the next section on FLIM (6.3.2) again drops the reader in a vortex of numbers extracted from 'the first experiment' which was not introduced previously. The whole Results chapter seems to be very raw and in need of more writing and editing.

In contrast to many other theses I've seen, the Discussion chapter here is quite long and detailed. A bit surprisingly, more experimental results from TCSPC, which seem to belong to the Results, are introduced. Apart from a few segments, most of the chapter is difficult to follow and understand and I admit I got lost in the text a few times. The discussion of photosynthesis, fluorescence, diazotrophy and stress is not very convincing.

The Conclusion follows the tune of most of the text, it is not well focused, forcing the reviewer to hunt for the actual conclusions, which are scattered in bits and pieces throughout the text.

In the following text, I first list a few specific shortcomings of the text to support my overview above and then follow up with a list of questions which the student should answer during the defense.

a) Although the references are generally well prepared, there are a few omissions. Fig. 1.4 is taken from a web site but it is referenced as "MCB, H. (2007)". The source of Fig. 4.1 is stated as "Edinbur*ch* Instruments". Source of Fig. 1.2a contains the proper reference of Campbell et al. (1996), oddly conjoined with a reference to an Alzheimer paper. The well known book of Lakowicz is cited erroneously as the reference implying a full book is actually just a chapter. Publisher information is missing for Gilbert & Baggott (1991), likewise for Gruber (2008).

b) Some of the figures are only briefly introduced in text yet contain quite a lot of detail in captions. Most style guides recommend the figures and information in them be well incorporated in text so that the reader is not surprised by the amount of information in figure and/or its legend, good examples are Figs. 1.1, 1.2 or 4.2. Some figures lack reference in text altogether (Figs. 2.2, 3.1, 3.2). Speaking of figures, a full page is dedicated to the scheme of the Calvin-Benson cycle (Fig. 1.4), which is hardly justified as the accompanying text covers just 4 lines and the photosynthetic carbon reactions are not mentioned later anywhere in the text.

c) There are puzzling references to "first" and "second" experiment throughout the text. It seems some of the experiments were carried out twice and one experimental set was not very satisfactory. But much better information should be provided to the reader to understand the situation.

d) On p. 49, there is somewhat confusing discussion of FLIM image intensity and detected lifetime. This relates to a more general theme — the FLIM images are mostly just described in words but a much more elaborate numerical analysis would be needed to support the analysis and conclusions. For example, in table 6.1, FLIM signal is classified into 'regular', 'bright' and 'dark'. There is however no histogram of observed lifetimes on which this is based. Likewise, no data on intensity distribution are provided. The chosen classification terms unfortunately mix these two metrics together.

e) On p. 50, it is suggested that short fluorescence lifetime could indicate N_2 fixation powered by cyclic electron transport. While this could be true, one would expect proper introduction of the issues etc. What could have been one of the main themes of discussion is only briefly mentioned.

f) In the first experiment, phycobilin content of phycoerythrin was analyzed. There were very small differences, which were analyzed extensively by statistical methods and are then a bit confusingly discussed more on p. 52. It is not clear why this analysis was carried out and considering that the observed effect size was very small, and potential influence of the PUB:PEB ratio difference on the fluorescence experiments is also small, elaborate statistical considerations are quite unnecessary.

To conclude, here are some **questions** the student should answer during the defense:

1) You did not provide any theory or specifics on how the FLIM data are obtained. Please very briefly introduce how lifetimes are measured in a microscope and what are the benefits and drawbacks in comparison to bulk TCSPC. Please also provide information on the duration of the experiment, illumination intensity and expected effects on the live cells, so that we can compare the influence of experimental conditions on the cells in bulk TCSPC and in (your) FLIM.

2) On pages 10 and 11, you mention that the nitrogenase is irreversibly inhibited by O_2 and that some species solve the problem by separating photosynthesis and nitrogen fixation in time. Is their (inactive) nitrogenase also inhibited by oxygen? If yes, how is it activated if the inactivation is irreversible? Is this process costly for the cell?

3) On p. 21, you write that $NaNO_3^-$ was added regularly to the cell culture to achieve mean concentration of 100 μM. How was the concentration calibrated/detected? How do you know when and how much to add?

4) In Fig. 6.1, the biggest difference among the strains seems to be around 670 nm. Is that allophycocyanin or something else? There is apparently a trend from IMS to NIBB+. You do not comment on this feature, can you provide an explanation of why the cultures differ so much here?

5) Concerning Fig. 6.11, you write *"In the N_2-fixing Trichodesmium (IMS) the average lifetime increased during midday while in the NO_3^--consuming sample it decreased."* This interpretation is based on just the values detected at 13 hours? Considering that one of the shortest lifetimes is detected at 12 hours and the second longest at 9 hours, I do not think that this result is trustworthy. Can you convince me that your text is correct?

6) Your 'bright cell', as shown in Fig. 6.19, seems to be emitting mostly from the cell wall rather than from the cell interior as in the case of many other bright cells. Could this be an artifact caused by limited resolution? Do you believe the signal comes from chlorophyll/PBS? Please comment more on this observation.

Overall, it is clear that the Thesis is a product of a lot of experimental work as well as of a considerable writing effort. It is a pity that the final text does not leave the reader with a comparably good impression. Regardless, it is my opinion that the student fulfilled the requirements expected at the Master level and I **recommend the Thesis for defense**.

In České Budějovice, on January 12, 2023. Radek Litvín