Opponent´s review of the Doctoral (Ph.D.) Thesis

Jose Romel F. Malapascua, MSc.

Photosynthesis Monitoring in Microalgae Mass Culture

Institute of Microbiology, Centre Algatech, Třeboň & Faculty of Science, University of South Bohemia, České Budějovice, Czech Republic. Supervisor: prof. RNDr. Jiří Masojídek, CSc.

Opponent: doc. RNDr. Jan Pokorný, CSc., ENKI, o.p.s.

The thesis has 81 pages and is based upon 7 author´s publications. Titles and abstracts of all the 10 publications are given in the summary of the Ph.D. Thesis. Author´s contribution to the individual papers and journal IF are given in the Thesis. Full text of the publications has more than 140 pages. I have got the publication from the supervisor in electronical form. The papers were reviewed by specialists. Therefore, I will not review and assess relevance of methods used, statistical evaluation, etc.

The Thesis is divided into 4 Chapters according to the objectives formulated on the page 4. The general objective is to correlate changes of physiology and photochemical activity and growth rate/productivity of microalgae strains to find suitable conditions of cultivation. Chl fluorescence techniques are used to monitor the physiological status of microalgae mass cultures.

The general objective is dealt with in the **Chapter 1.:** The State-of the-art: Microalgae Mass Culture. Author describes briefly principles of microalgae mass cultivation in terms of environmental conditions and nutrient availability as well as development and progress from beginnings in 1960s.

Comments and questions to the Chapter 1: photosynthesis transforms only small part of the solar radiation. On the page 10, Fig 1, a schematic diagram of the photosynthetic light-response curve is shown. Which spectrum of radiation is considered (light, photosynthetic active radiation, narrower spectrum)? Please explain briefly the term "dissipation" in conditions of algae cultivation (Fig 1., page 13).

Page 11, par 3: "Microalgae can take up CO_2 by diffusion and several microalgae species have active carbon uptake systems which take up HCO_3^-. "According *to my "old knowledge" from 1980s, microalgae increase by their photosynthesis pH above 8,3, i.e. they use* HCO_3^-. *Bicarbonate uptake was explained by the interface effect of alga surface – surrounding water which results in pH decrease on the alga surface and dissociation of bicarbonate. Macrophytes which are able to utilize bicarbonate have a special enzyme.*

Page 12, par. 1: what ratio of nitrogen/phosphorus is considered as optimal for the cultivation of microalgae.

Page 12, par 12: "Dissolved oxygen concentration equivalent to 3 – 4 times …etc.". *3-4 times air saturation is equivalent of 60 – 80% oxygen atmosphere. Does it mean that concentration*

of dissolved oxygen 2 – 3times air saturation does not negatively affect production process of biomass and can be tolerated?

Chapter 2. Microalgae Cultivation Systems. Both open reservoir systems and closed or semi-closed vessels (photobioreactors/PBRs) are discussed in terms of optimal use of photons. Light/dark turnover of the photosynthetic unit is considered in the range 1 – 10ms. Daily productions of biomass per m^2 for different cultivation systems are given and compared with other feedbacks and cultivation costs.

I have several questions to the Chapter 2, not knowing their answer.

What light/dark frequencies (time period in euphotic and dark zone) were found for dense phytoplankton in eutrophic waters like our fishponds?

In terms economy, how important are water losses caused by evaporation from open outdoor systems and namely from TLCs?

In the paper "Photobioreactors with Internal Illumination" you describe also the PBR installed and tested in Nové Hrady (Fig 1). Illumination was provided by Fresnel lenses which concentrated solar radiation several times. What conclusions were made from this experiment?

Plant physiology textbooks show photosynthetic light curves of shade adapted and sun adapted plants. Is it possible to draw roughly such a curve for algae culture at beginning and at the end of experiment lasting for example two weeks? (On basis of case studies on pages 20 to 28).

Chapter 3. Photosynthesis Monitoring in Microalgae Cultures: Chl Fluorescence Techniques. The Chapter is based on the paper Malapascua et al. (2014)

The fluorescence induction curve of the cyanobacterium *Arthrospira platensis (Spirulina platensis* in Fig. 3.1) shows a distinct inflection of the photochemical phase in comparison with eukaryotic algae (*Chlorella sorokiniana, Trachydiscus minutus*). *How can be this effect interpreted in terms of competition among algae and cyanobacteria in phytoplankton communities. Does it partly explain domination of cyanobacteria in eutrophic waters with dense phytoplankton?*

Fig. 3.2: A series of stepwise increasing irradiance intensities (0 to 2000 micromol photons $m^{-2}.s^{-1}$ *were applied ... What source of light was used, solar energy? What type of quantum meter was used, i.e. what spectrum range was measured?*

Page 33: "When the nitrate limited culture was exposed to high irradiance at midday, the J and I inflections were clearly visible, suggesting over-reduction of the Q_A *and* Q_B *electron acceptors ....". Do you assume possibility of direct reduction of nitrate in PSII?*

Chapter 4. Growth of Microalgae under Unfavorable Conditions. Text is based mostly on the paper Jerez et al. (2016).

Fig 4.5: *amounts of lipid, protein and starch are given as % dry weight. In some cases, the sum of lipid + starch + proteins is substantially lower than 100%. Which other compound is missing to get 100% dry weight?*

Page 45: "This experiment clearly illustrates that Chl fluorescence …can alternate/substitute for the monitoring of O_2 production, as the former technique is easy to measure and reliable." *Biomass production can be easily calculated from O_2 measurements (180 grams of starch is equal to 192 g of oxygen produced). How can be primary production calculated from fluorescence measurements?*

Page 46: Chl fluorescence diagnostic was tested in outdoor trials with Chlorella vulgaris culture in the presence of selenium. Relevance of the diagnostic was proved. Are there some practical outputs of these experiments?

After reading "Summary and Conclusions" I have the following more general questions:

Cultivation of microalgae in former Czechoslovakia started more than 50 years ago aiming at production of algae biomass as a food alternative. Contemporary witnesses remember "algae burgers" in the Hotel Jordan/Tábor. How important are cultivated microalgae as a food alternative in the world, now?

In worked with different types of cultivators of microalgae in the world and you describe and evaluate them in your papers. Which type of algae cultivation you would recommend to our conditions of temperate zone? If you are advisory board of Algatech what scientific and commerce strategy you would recommend?

Final assessment of the Doctoral Thesis

As a whole, the thesis submitted by **Jose Romel F. Malapascua** clearly fulfils the requirements for the PhD degree (topicality, research methods and procedures, results, achievements and practical use): it demonstrates that the candidate has trained to and now possesses the capacity of carrying out scientific research in an adequate manner. For my part, I warmly recommend the thesis as being worthy of the award of the degree of Ph.D.

Following a successful defence of the doctoral thesis I recommend the granting of the Ph.D. degrees.

Doc. RNDr. Jan Pokorný, CSc.

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ENKI, o.p.s. Třeboň, Dukelská 145, Czech Republic

universität wien

Fakultät für Lebenswissenschaften

Department für Limnologie
Ao. Univ.-Prof. Mag. Dr. Michael Schagerl
Althanstraße 14 (UZA I)
A- 1090 Wien

T+43-1-4277-76414
F+43-1-4277-876414
michael.schagerl@univie.ac.at
http://www.univie.ac.at/phycology/

Prof. Dr. Frantisek Vacha
Dean of Faculty of Science
University of South Bohemia
Ceske Budejovice

Review of PhD – Jose Romel Malapascua Vienna, 25.9.2018

Spectabilis, dear Prof. Vacha, dear examination committee,

I appreciate inviting me as one of the PhD referees of Joes Malapascua´s work, who did his studies on photosynthesis monitoring in microalgae mass cultures.

The thesis addresses a topic, which has become recently a burning issue, as companies are increasingly interested in mass culturing of microalgae. This fact is due to valuable compounds synthesized by microalgae, which could be downstream processed (e.g., PUFA, biofuels). This goal still seems far-off, but new technologies such as LED-illumination or processor-controlled cultivation systems are a big step forward and probably will pave the way in a few years. Part of processor-controlled cultivation is a fast online measurement technique, which provides information about the health state of organisms.

The thesis focuses on strains grown photoautotrophically, with special emphasis on open systems. The key factor for successful cultivation is detailed knowledge of the microalgae´s physiological state. Full medium provided with high irradiance supply will result in high growth rates, optimized to gain maximum biomass. Nutrient depletion may result in enrichment of valuable compounds, but for the expense of productivity. High biomass together with high amounts of valuable compounds can only be achieved by a two-step cultivation (fast growth followed by nutrient depletion).

Mr. Malapascua worked with a highly sophisticated, non-invasive method, which was developed in the 1980-ies, as electronic parts essential for the measurements become available on the market. This so-called fluorescence technique provides detailed

information of photosynthetic processes, which is a core element of plant metabolism. With standard devices, information is mainly gained from photosystem II (PSII), as PSI is almost not fluorescing. Recently, affordable instruments such as the "fluorpen" (PSI company) became available, which makes measurements quite easy. The non-invasive measurements usually take seconds to a few minutes and can be performed in-situ, which makes them very attractive even for non-biologists. Although the measurements are easily to obtain, it does not mean that their interpretation is simple. Mr. Malapascua did hard and excellent work, which is also reflected in 8 publications in renowned international journals and two additional book chapters. They already passed the international review process and they are available to the scientific community. This fact can be considered in itself as an indication of the high PhD quality.

The thesis is highly topical and presents the potential of fluorescence measuring techniques for mass cultures. Specifically, cultiviation of microalgae on a thin-layer cascade (TLC) system was studied and compared with other methods. The TLC technique established in Trebon turned out to be a promising and affordable system compared to closed photobioreactors and yielded much higher biomass compared to other open systems such as raceway ponds. Summarizing up, Jose Malapascua used appropriate, highly sophisticated methods in an innovative way.

The thesis can be seen as a summary of the profound work done by Mr. Malapascua, the way of presentation however does not reflect adequately his excellent studies and his efforts put into experiments. I was searching for detailed measuring protocols, which according to the objectives (p4) were a main purpose of the work. They are however not set out in the thesis. I additionally visited the homepage provided for downloading the thesis to make sure that I did not miss parts of the thesis. There might be some specific regulations of the University of South Bohemia such as maximum number of pages, which could explain, why the published papers are lacking in the official document, but this fact makes a check on the plausibility of the presented results a challenge. If the thesis is a cumulate work of the papers, I expect the papers being part of the official document. If the thesis is seen as a separate work, then much more in-depth information needs to be included in the document to make methods applied comprehensible. The current version is in between both options. Because of the condensed presentation, also some relevant literature was not discussed in depth. The general introduction and discussion/conclusion are of good quality, but they could go more in-depth. Additionally, I am also missing a very basic introduction in this field of research for non-physiologists. A brief and simple presentation of sometimes highly sophisticated topics to non-biologists is not trivial, but it will help the candidate to inspire others for basic science.

From the thesis provided, a few specific questions arose – some of them are very basic, others a bit more advanced - which might be adressed in the defense talk (I therefore address the candidate directly in the next paragraphs):

(1) Please provide basic information of the OJIP-curve; relate the plateaus to physiology so that non-physiologist will be able to follow your explanations.

(2) In chapter 1 (pp9 and 10), PE-curves based on oxygen release over time are presented and explained. You however used a different technique for obtaining RLCs. Is it justified to conclude from "traditional" PE-curves obtained eg. with oxygen release or 14C-incubation to results obtained from RLCs?

(3) In the legends of figures of chapter 2, following information is provided: "...columns labelled by the same letter differ significantly from each other..." (eg. figs 2.1, 2.3, 2.4). In chapter 4, the legend states: "...different letters denote significant differences..." (eg. Figs 4.3, 4.5). Please confirm this contradicting explanation (the latter explanation is commonly used).

(4) According to dozens studies and also to your explanations (p9), microalgae often become light-saturated at around 150 – 200 µmol photons m-2 s-1. At this point, the actual fluorescence yield will be close to zero, as photons are used for photosynthesis/non photochemical quenching, which are alternative pathways for absorbed energy. Relative electron transport rates (rETR), which you defined as "rETR = incoming irradiance x actual fluorescence yield" of your experiments are rather high compared to other studies (I would have expected values of about 20-40). In fig 2.2, rETR max of *Arthrospira* was about 150 units, reached at 1000 µmol photons m-2 s-1. rETRs of *Chlorella fusca* were 800 (fig. 3.4)! How can this enigma be explained?

(5) How can you explain an actual fluorescence yield of 0.60 at 1000 µmol photons m-2 s-1, which was obtained for *Chlorella fusca* (Fig. 2.6, day 14). The maximum dark fluorescence yield is about 0.75 to 0.80 for many plants and drops significantly with increasing irradiance supply – please provide an explanation (measuring artefact?).

(6) The onset of light saturation Ek is usually much below 500 µmol photons m-2 s-1. *Chlorella fusca* grown in TLCs even were in the linear light-limited slope at 1000 µmol photons m-2 s-1. What could have happened here? The results raised in paragraphs 4 to 6 suggest that rETRs might not be the correct parameter for such comparisons. Instead of it, the absolute ETR considering true absorption and not incoming irradiance would have been the unit of choice. Please justify, why you kept the rETR and why it could be used for your applications.

(7) You use the word "adaptation" for processes to adjust to the current environmental conditions (e.g. p31). Can the terms "adaptation" and "acclimation" be used in the same context? Can they be replaced arbitrarily?

(8) In an interesting study dealing with depletion of certain nutrients and the subsequent reaction of microalgae, *Chlorella fusca* showed changes in both the cell size and number (N- and S-starvation, Figs. 4.1b,c). If both cell size and cell number decrease, the biomass must also decrease as a consequence. You however showed stable biomass (Fig. 4.1a). Please explain.

(9) From exactly this experiment (fig 4.1c), the cells of *Chlorella* became smaller after 6 to 8 days especially during S-depletion. The cell wall of *Chlorella* is thick and quite rigid and therefore shrinkage of the cell volume is very unlikely. The only conclusion will be that smaller cells must be a result of cell division. Cell division can however be excluded because also the cell number decreased with time. Please justify/explain.

(10) You focused on the interplay of photochemical versus non-photochemical quenching (fig. 4.4), and used the terms Y(II) + Y(NPQ) + Y(NO) = 1. The paper you cited (Jerez et al. 2016), referred to Komkamp & Forster (2003) as an explanation to these terms. To my knowledge, these parameters were not explained in Komkamp & Forster (2003). Please provide a short explanation of the parameters that the audience will be able to follow your defense talk. I suggest answering the raised points (1), (7), and (10) in the beginning of the talk.

(11) Figs 4.1 to 4.6 present means and SE with a sample size of 2. You moreover tested the groups (n=2) for significant differences with two-way repeated measures ANOVA. Do you think that this method is appropriate from a statistical perspective?

(12) In another experiment, you tested potential CO2-limitation on *Chlorella vulgaris* (p45). You assumed that cells became carbon limited because of moderate pH increase from 7.3 to8.4. At this pH range, carbon is still available as hydrogencarbonate, which obviously can be taken up by *Chlorella*. As inorganic carbon was not analysed quantitatively, also other reasons could have caused decreased productivity. One explanation could by increased light respiration due to imbalance of the carbon/oxygen (Rubisco also acts as oxygenase at high partial pressure of oxygen). How can you be sure that it really was carbon depletion, which was responsible for lower productivity?

(13) In the same experiment, you used oxygen differences (O2-concentration at the end of the TLC minus start of the TLC). The open culture device has a very high surface to volume ratio and the surface is moreover directly exposed to the atmosphere. How did you consider gas exchange with the atmosphere?

Summarizing up, Jose Malapascua overcame various challenges and he also did a very good job in both laboratory and outdoor experiments. He focused on method development and developed measuring protocols for certain applications and microalgal species. This is essential for interpretation of the results, because "algae" as a functional group are not necessarily related to each other. The consequences are very different reactions/acclimations to environmental/culture conditions. I appreciate this important piece of work, which certainly expands our knowledge of using fluorescence techniques for both applied research and industrial mass cultivation of algae. In my opinion, the PhD thesis fulfils the criteria for obtaining the PhD degree and I therefore recommend paving the way for the final step, which is the PhD defence. I rate the work and the papers as excellent (1), the thesis as good (2).

Respectfully yours

Ao. Univ. Prof. Mag. Dr. Michael Schagerl – Vienna, 25.9.2018

Opponent´s review of Ph.D. Thesis
Candidate: Jose Romel F. Malapascua, M.Sc.
Title: Photosynthesis Monitoring in Microalgae Mass Cultures

Topicality of thesis

Application of non-destructive methods for evaluation of important characteristics of microalgae physiology and growth in microalgae mass cultures is still an important topic. Presented thesis is focused mainly on the application of Chl ***a*** fluorescence techniques, both PAM Chl-a fluorescence (mainly so called rapid light-response curves) and measurements of rapid phase of fluorescence induction. The Chapters II, II and IV contain results from numerous experiments that considerably contributed to the knowledge about the optimization of microalgae growth conditions in different cultivation systems, monitoring of photosynthesis in cultures using Chl fluorescence techniques and growth under unfavourable conditions (in relation to enhancing production of valuable compounds).

Chapter I - The State-of-the-art: Microalgae Mass Cultures

Thesis is generally well written, contains valuable information related to the topic. English is mostly correct, both stylistically and grammatically. The amount of not-precise statements or jerky passages is not high (yet there are some). The first chapter briefly (on 9 pages) and mostly in a general way summarizes information on the microalgae mass cultures (including main factors that determine the biomass production, methods used for monitoring of functional state of algae culture).

Although the information is relevant I miss a deeper and detailed information, particularly regarding the fluorescence techniques that are used to monitor photosynthetic performance of mass cultures (measurements of individual fluorescence values and interpretation of individual parameters and/or survey of mechanisms that are behind). See the comments bellow.

Chapters II- IV

Detailed description of the results related to the published papers is presented in the three chapters (II-IV, 36 pages) that represent the most important part of the thesis. I really appreciate that Mr. Malapasqua devoted time to collect almost all data (figures) on the both OJIP and PAM chlorophyll fluorescence measurement and information on algae culture growth and rephrased the description of the results presented in the VII appendixes (papers) into chapters focused on: A, comparison of advantages and disadvantages of the different algal bioreactors; B, application of the fluorescence techniques in monitoring of the PS II function and of the efficiency of absorbed light utilization in PS II photochemical reactions - that can be a good proxy of the photosynthetic activity and biomass production; C, impact of unfavourable conditions (mainly nutrient limitation/starvation and selenium accumulation) on the physiology and growth of algae cultures. I would appreciate if numbers of given Annexes were accompanying references of the papers of candidate and co-authors in the text of Thesis and in the legends of Figures, as this could improve the orientation (it was done vice versa - at the beginning of each Annex a Chapter(s) of the thesis where the published results were described is mentioned – but this was not as much useful). Another moderate criticism is that section Material and Methods (description of the measuring protocols) is missing and legends of the Figures did not contain all the necessary information. Thus one should often look in Annexes to find this information (e.g regarding measuring protocol of rapid LRC etc.).

Chapter 5

I appreciate also that the main achievements were surveyed (4 pages) that clearly documented the fulfilment of the main objectives of the thesis.

Comments and questions.

1, On page 14 you wrote: "….. and the electron transport rate (ETR) through PSII, used as a proxy of the photosynthetic capacity and productivity." Then you have mentioned that estimation of the physiological state of cyanobacteria based on Chl fluorescence should be done with caution. I completely agree with this statement but I am persuaded that critical assessment of the factors that can affect the measurements

(estimated values of some parameters mentioned here or later in the text) would be useful for green algae as well.
This is especially valid for ETR. Neither here nor further in Chapter III (related to monitoring of photosynthetic parameters) did not I find formula for the estimation of ETR (or rETR – I also did not find an explanation, why you use the term relative ETR). In Material and Methods sections in individual papers it is stated that "rETR was calculated as EPAR x Y(II), where EPAR is the incident PAR irradiance and Y(II) is the actual quantum yield of PSII". ***According my opinion this formula does not correspond to "relative ETR" but rather to "ETR not corrected for ….. " . I purposely did not fill out which two other factors are usually taken into account when calculating ETR from Y (PSII). Could you specify them and explain why they are not used when determining ETR in algal cultures?***

2, On page 41 you discuss the partitioning of absorbed excitation energy within PS II between PSII photochemistry (YPSII) and non-photochemical looses (separated into Y(NPQ) and Y(NO) – Fig. 4.4.). I have not found any explanation of the meaning (interpretation) of the two components of nonpohotochemical deexcitation processes in the thesis, just the description in the abbreviation (page 3). ***Is the given explanation of the Y(NO) complete? Do you think that term "energy quenching" is physically correct term?***

3, Do you think that "blue-light driven" chloroplast movement can distort or affect the estimation some fluorescence parameters also in microalgae?

4, In the case study 2.2. you have analysed diurnal changes in rapid LRC of cyanobacteria Arthrospira grown in Open Circular Ponds (OCP) and Thin-Layer Cascades (TLC) at suboptimum (25 ^{0} C) and optimum temperatures (33 ^{0} C) - Figs. 2.2. and 2.5. ***Do you have some idea why at the suboptimum temperature rETR values gradually (but considerably) decreased (particularly at the higher PAR irradiances) after midday only in the OCP (Fig.2.2.)?*** Moreover, the NPQ values obtained on the same samples (OCP at 25 ^{0} C) revealed a lack of increase of NPQ with increasing PAR irradiance already at midday (Fig. 2.5.) that was connected with extreme abundance of Y(NO) – Annex 1). These results are inconsistent with my knowledge about interrelation between Y(PSII) and NPQ [Y(NPQ), Y(NO) at different irradiances measured with terrestrial plants - the reduction of Y(PS II) [or ETR] is in every case accompanied by the increase of NPQ [Y(NPQ)]. ***Can you comment on this or suggest some explanation?***

Final evaluation

I am persuaded that extremely rich set of published papers (8 papers in journals with IF, 2 of them in Algal research with IF around 4, and two chapters in monography „Algal Biorefineries Volume 2: Products and Refinery Design", published by Springer International) represents valuable contribution to the knowledge in the field of algae mass cultures. The considerable contribution of the candidate to the papers is documented by the fact that he was 2times first author, 3times the second and 3times the third one. I am persuaded that demands for the Ph.D thesis in Biophysics have been fully accomplished.

I strongly recommend presented disertation thesis for defence. Further on, after successful defence, I recommend to award Jose Romel F. Malapascua, M.Sc. the title Ph.D. in Biophysics.

In Ostrava 24. 09. 2018

Assoc. Prof. Dr. Vladimír Špunda, CSc.
Department of Physics
Faculty of Science
University of Ostrava