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REVIEW

of the PhD-thesis by Neila Ferimazowa from Azerbaidschan with the title
"Heterogeneity in photosynthetic performance as a result of metabolic, physiological and genetic regulation of photosynthesis"

"*Photosynthesis has been crystallized – what now?*" - At the end of 2003 the crystal structure of the cytochrome b6f-complex has been published, closing the last gap in our knowledge of the photosynthetic apparatus at atomic resolution. On this basis, together with the genomic advancements, we are now faced with new goals. A predominant one among them is to better understand the dynamics of the photosynthetic process at the different levels – from single cells, or even single chloroplasts to whole ecosystems. An invaluable tool in this enterprise is the ever improving, non-invasive technique of fluorescence imaging, for which Bohemia has become a leading center of progress within the last years.
The thesis of Neila Ferimazowa represents an impressive account of this approach. It comprises three examples of the application of the recently developed fluorescence kinetic microscope (FKM) – a highly appreciated achievement, in which she closely participated. These applications are 1) leave cells and protoplasts, 2) the filamentous, non-heterocystous cyanobacterium *Trichodesmium*, recently recognized in its dominance within the pelagic phytoplankton, and 3) the filamentous, heterocystous cyanobacterium *Anabaena*, a model system of cell differentiation. I presume, in contrast to the implication of the present thesis, that these three objects were not systematically chosen, but were at hand rather arbitrarily. However they provided a useful test for the FKM to prove its complementary power.

Ad 1) The outcome of the first investigation is, that the mesophyll cells of leafs reveal a complex pattern of oscillations as reflected by chlorophyll fluorescence, which differ from cell to cell. This certainly is an extension of previous notion. It is concluded that this complexity in oscillations and its heterogeneity from cell to cell constitutes "a most intriguing phenomenon of photosynthesis regulation" (p 63, but see title of the thesis already) or even "a true revolution" (p 53). I question this conclusion, which certainly reflects the scepticism of an outsider:
a) Isn´t the observed heterogeneity rather an expression of variation, reminiscent of the states of individual molecules in statistical thermodynamics? Since the time integral of these states represents the space integral of the molecular system, the state of the individual molecule is rather irrelevant. That addresses the question of what do we learn from individual variations with regard to the behaviour of the whole – from a population of chloroplasts within a cell, via populations of cells in tissues and organisms to populations of organisms in an ecosystem? In turn I´d like to point out that the finding that something is statistically more complex does not necessarily change (or even devalue) our older notions of the behaviour of a whole system.

b) In every network system, one step is ruling, is thus rate determining at a given state.This also holds for oscillatory behaviour. On p 10 ff. six possibilities for causing oscillations in green plant cells are elaborated. Which one is responsible for the dominant frequency of about 0,1 Hz in protoplasts and cells? What in our days of advanced cellular manipulations would be the experimental possibilities to pin that down? How do the oscillations compare to the well studied glycolytic pathway?
c) Why four species of *Brasiccaceae*? Certainly, *Arabidopsis* is justified, but are the results representative for higher plants in general, including model organisms of photosynthetic research, like spinach, pea, tobacco,maize, or *Chlamydomonas*?
d) What oscillations are known for plant physiology in general, which are of possible relevance?

Ad 2)
Is the meaning of "heterogeneity" the same for the 3 cases? In case 1 it concerns the variation in oscillation patterns from cell to cell, in the other two not oscillations but the differences in fluorescent states during adaptation/differentiation of the photosynthetic apparatus to nitrogen fixation is studied. For *Trichodesmium* several interchangeable fluorescent states in the cells of the filament could be identified, whose interplay somehow allows nitrogen fixation during bright sunlight. It remains to be elucidated:

a) What are the mechanisms of PBS-dynamics in *Trichodesmium*, and how do they compare to *Anabaena* and other cyanobacteria? More specifically, are the "bright" states confined to *T.*?
b) How is the Mehler reaction upregulated? Is this reflecting the old finding from David Krogmann´s group of the seventies that PS1 of cyanos is much more accessible to oxygen then of PS1 in higher plants (oxygen reducing factor ORF which seems to act like paraquat)? Next to H_2O_2, is the ascorbate/dehydroascorbate system involved?
c) With respect to evolution, why isn´t a simple day/night-cycle the better candidate for the ancestral strategy? Furthermore, what is the advantage of *Trichodesmium* to bother with a complex adaptation mechanism during the light period?

Ad 3)
In this investigation of Anabaena filaments during heterocyst differentiation after nitrogen step-down 4 phases could be discerned by different fluorescence states – perception, acclimation, stress and recovery. In addition, NPQ has been found elevated in heterocysts reflecting relative higher energization of the thylakoid membranes. Questions to be clarified:

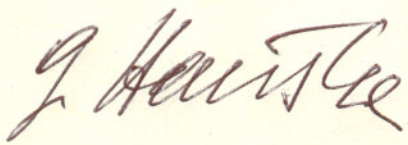
a) These 4 phases, how do they relate to previous divisions on physiological/genetic grounds of the differentiation process? For to answer this, it is highly desirable that the study should be extended to the many available differentiation mutants now.
b) It is a remarkable finding that PSII-efficiency does not only recover in vegetative cells, but also in heterocysts after the stress period. Does this resemble the situation in type I-bright cells of *Trichodesmium* with an upregulated Mehler reaction? A little more comparative discussion of *T.* and *A.* would be desirable.

Some further, exemplary criticism in detail, following the manuscript of the thesis:

- p5: Cellular "energy charge" proper is not equal to the ATP/ADP-ratio but rather to the ratio of ATP + ½ ADP over ATP+ADP+AMP (according to Atkinson in the sixties, I believe)
- Why is the LHCII-kinase, sensing the redox level of PQ, with regard to the state transitions in plants entirely left out from discussion (already on p5/6)?

- Furthermore, what is the kinetic reason that the quinol oxidations in PS, as well as in respiration are "pivotal" for regulation?
- Fig.1 on p7 contains some inconsistencies: a) For one O_2 there should be 2 NADPH formed, and 8 protons translocated in the Q-cycle; b) The number of protons translocated by the ATP-Synthase should be the same on both sides of the membrane, not change from 4 to 3 (the most reasonable value for chloroplasts actually is 4,7 these days, but only 3,3 for yeast mitochondria – on what basis?). Furhermore, the protons do not run through the ATP-forming site, as implicated in the figure.
- The rather elaborate description of nitrogenase on p24 ff. is not required in its details, remaining rather incomprehensive to the unprepared reader (difference between "P-loop" and "P-cluster"…), and in some details are even incorrect (12-15 ATP/N_2, "variable degree" of hydrogenase…)
- The last sentence on p24 is incomplete.
- The intriguing statement on p24 that nitrogenase and protochlorophyllide reductase share a common ancestor deserves some reasoning, as well as the one about FeS-cluster formation between protomers. What is another well known example for an 4F4S-cluster held between two proteins?
- Inconsistent abbreviation for the same entity: AR on p46 and AI on p47; furthermore in the text and in footnote 2 on p 47 it should read "Fig.10" not "Fig.1".
- citations are either missing from the reference list (Laisk et al. 1992, Lazar. 2005 both on p 10, Rohacek 2002 on p51), or do not correspond to each other with respect to the year of publication (Herrero et al. 2004 on p37 and 2001 in the list)
- The ms also contains several typos.
- Page number 62 is missing

The repeated efforts of Mrs. Ferimazowa to explain the theoretical background as wellas the rather sophisticated experimentation, in particular the operation of the FKM including the various fluorescence parameters which are so confusing to the outsider, are very valuable and highly appreciated by the reader. This includes the chapter "Results" on p63 ff, which constitutes a summary of the attached 3 papers. In addition it should be noted that Mrs. Ferimazowa is coauthor of 5 more original publications by now. To my judgement the pertinent literature is recognized comprehensively.
The English writing of the thesis is remarkably good, the consistent drop of using articles is an "Eastern" peculiarity which does not decrease the comprehensibility.
In conclusion, without any hesitation, I can recommend the present thesis for its defence – it represents a remarkable achievement, despite some formal shortcomings which do not exceed the extent as they usually occur also by native English speakers.



(Prof. Dr. Günter Hauska)

Report on the Doctoral Thesis by *Naila Ferimazova*

"Heterogeneity in photosynthetic performance as a result of metabolic, physiological and genetic regulation of photosynthesis.

In her Doctoral Thesis, Naila Ferimazova presents very interesting and from a scientific point of view extremely fruitful original results of her experimental work. The heterogeneity in photosynthetic performance of photosynthesizing organisms and their complex responses to changes in environmental parameters belong, at present, to pivotal themes of the basic research in the field of biophysics and ecophysiology of photosynthesis. The theme of Naila's work is very actual and, as well, complex due to involvement of many-sided effects in the metabolic, physiological, and genetic regulations of photosynthesis in samples investigated. Though many studies on this subject were published in a professional literature during the last two decades, as demonstrated clearly in the introductory part of her writing, the use of a modern and powerful chlorophyll (Chl) fluorescence imaging technique as well as mathematical data processing/analysis brought about quite new, original, and surprising findings.

During Naila's many year's stay in the Dept. of Autotrophic Microorganisms, Institute of Microbiology AS CR in Třeboň, she had to cope with many biophysical and biochemical analytical methods, sophisticated laboratory techniques, and serious mathematical models. Among all, she mastered the 2-D Chl fluorescence imaging technique represented by the improved laboratory version of a fluorescence kinetic microscope (FKM). It is obvious from the text and publications affiliated to this thesis that Naila Ferimazova performed a great piece of work on all three mentioned projects, under a guidance of her supervisor Dr. Ivan Šetlík, and worked with endurance and enthusiasm. She cooperated effectively in frame of both the institute team and the international one. Due to contacts with many foreign researchers, she acquired a valuable experience and practice in the photosynthesis research.

The thesis deals with results of three projects called expressively: (i) the "*Oscillations project*", on the photosynthetic oscillations in individual cells of leaves and isolated leaf cell protoplasts, (ii) the "*Trichodesmium project*", on the coordination of photosynthesis and nitrogen fixation in a non-heterocystous marine cyanobacterium, and (iii) the "*Anabaena project*", on the regulation of photosynthesis during heterocysts differentiation. The central part of Naila's work consists of three original papers, two of them were already published in high-rated international journals (Photochem. Photobiol., and Plant Physiol.), the 3rd one was submitted in a form of manuscript for publication. Her work is complemented with the short introduction, detailed literature review, methodological part, and references.

In this thesis and, especially, in its summary, the aims, necessary theoretical background, methodology, results, their discussion and proposed conclusions are clearly presented for all three mentioned projects. The text of thesis is written in a good English (excellent in the 'Summary'), the individual chapters are written comprehensibly, logically segmented and supported by high number of references. The part 'Literature review' is very detailed but it was probably compiled under time stress because it is shot through with many inaccuracies and text errors. Serious formal errors can be found among references cited within the text.

Some references are cited incorrectly (especially those for adopted Figures), many of them are missing in the enclosed list, many listed references are not used in the text! Not all abbreviations frequently used in the text (*e.g.* AI, AR, DAS, FY, LAS, MR, SP, SIP) are listed in the 'Abbreviations' page, as well.

Here, I give a short comment and ask a few questions to individual parts of Naila's thesis.

In the part 'Methods', the "reverse proportionality" between Φ_F and Φ_P is mentioned under conditions when the rate constant of thermal dissipation k_D remains constant (p. 43). Strictly speaking, the indirect proportionality is true only between quantum yields of photochemistry (Φ_P) and non-photochemical processes ($\Phi_N = \Phi_F + \Phi_D$). Thus, the declared statement is valid only if the *ratio* Φ_F/Φ_D remains constant (commonly, k_D could change in the light induction phase). Further to p. 46, pulses of the measuring radiation (MR) applied in PAM-fluorimeters should be of strictly constant *intensity* but not of " strictly constant *energy*" due to a definition formula for the quantum yield of fluorescence ($\Phi_F = I_F/I_A$). Concerning Φ_{Fm} (p. 47), in this state (*i.e.* within SP) the photochemistry "occurs" but photochemical processes are *saturated*. It means k_P tends to zero and thus $\Phi_P = 0$.

Very interesting results were obtained during the "*Oscillations project*" (Ferimazova *et al.*, Photochem. Photobiol. 76: 501-508, 2002). In this context, my **question** to Naila is: Superposition of a set of harmonic waves differing in their frequencies and exhibiting some *phase variability* (p. 87, Fig. 3.) can lead to an emergence of a "running wave" or, better, "running wave pocket" in a matter. Due to described spatial heterogeneity of photosynthetic oscillations in cells of palisade mesophyll, are the "waves of the photosynthetic activity" in a leaf tissue possible (in spite of damping) and/or did you observed it using the fluorescence imaging technique?

Important results were gained also in two other projects on regulation of photosynthesis and nitrogen fixation in the cyanobacterium *Trichodesmium* and during differentiation of heterocysts in the cyanobacterium *Anabaena*. As for me, the most interesting conclusion made in the manuscript by Ferimazova *et al.* is the consideration on the drift in the F_0-level which might heavily influence the shape of recorded Chl fluorescence kinetic curves (*e.g.* Fig. 10., p. 48) in their actinic and dark-relaxation phases. The corrections proposed in Fig. 3 of the manuscript are very striking. In this context, I would like to ask on influences of saturation pulses (SPs, SIPs) on F_0. In a case of stressed and/or damaged samples (plants), the intensive 1st SIP applied in DAS causes the noticeable and slowly back-relaxing shift of the F_0-value. **Question**: Did you observe this kind of effect in the case of nitrogen fixing cyanobacteria? Can you estimate the (half-time of) relaxation of the F_0-level in darkness?

Concerning the 3rd publication (manuscript), I did not find in the text any information on instrumental settings used in the experiments (intensity/flux density of light sources, time of the dark adaptation, sample temperature, *etc.*). These parameters can affect resulted fluorescence transients markedly. For example, too short time of dark adaptation (5 min for F_M, plus 90 s to F_0 determination, Fig. 10., p. 48) might be insufficient for the correct determination of both levels due to exponential relaxation kinetics of the activated non-photochemical processes. The change in the F_0-level will be probably the reason of "paradoxical" *negative* values of the non-photochemical fluorescence quenching (NPQ) as mentioned in the text and clearly seen in Figs. 4A, 4B (the manuscript). In this case, the

better fluorescence parameter is the "Schreiber's" $q_N = 1 - F_V'/F_V$ as far as the F_0'-value can be determined. For this possibility, equipment of the fluorescence kinetics microscope with far-red light source is necessary.

Finally, some petty mistakes should be corrected, *e.g.*: (p. 5) "triplet formation" might be reformulated as "formation of Chl molecules in a triplet state", (p. 42) "excited electron" means "electron in excited state of Chl *a* molecule", (p. 42) "its basic orbital" is "the ground energy state of Chl *a* molecule", (p. 44) Φ_{Fv} means the *maximum* variable Chl fluorescence yield, (p. 47) "Fig. 1." mentioned in text should be Fig. 10., (p. 58) the announced "Fig. 6." is missing, *etc*.

In spite of a number of formal errors, I did not find any discrepancies between the data acquired and their interpretation. The large volume of the systematic work was performed by the author. Presented results are very interesting, valuable, original and accepted by the scientific community, *e.g.* the paper by Ferimazova et *al.* (2002) Photochem. Photobiol. 76: 501-508, was cited 7-times to the present date. Complexity of the three projects, interpretation of results acquired and number of techniques mastered should be also taken into account. It is obvious that measurements of the photosynthetic performance on a single-cell level are still unique. The aims set in the thesis were successfully fulfilled.

I am convinced that the work written by Naila Ferimazova fulfils all the requirements set on the Doctoral Thesis. Thus, **I recommend the Doctoral Thesis by Naila Ferimazova to the defense procedure**.

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Posudek na disertační práci s názvem
„Heterogeneity in photosynthetic performance as a result of metabolic, physiological and genetic regulation of photosynthesis“,
vypracovanou Mgr. Nailou Ferimazovou.

Předložená disertační práce je napsána v anglickém jazyce, má 82 označených stran plus další nezbytné úvodní strany, obsahuje 12 obrázků a okolo 239 citací v seznamu literatury. Práce je založena na 2 již publikovaných článcích a na 1 rukopisu článku připraveného k publikaci, jejichž přetisky jsou přiloženy na konci práce.

Faktická stránka disertační práce
Přehled dosavadních znalostí z literatury tvoří hlavní část práce (strany 3 – 40). Vzhledem k zaměření práce, je tato část rozdělena na 2 hlavní celky a to na „fotosyntetické oscilace“ a na „sinice fixující dusík“. V části „fotosyntetické oscilace“ autorka podala detailní přehled stávajících vysvětlení pro fotosyntetické oscilace, opírající se jak o experimentální výsledky, tak o teoretické modelování tohoto jevu. V části „sinice fixující dusík“ autorka detailně shrnula metabolismus těchto sinic a dále pak uvedla dosavadní znalosti o ne-heterocystních sinicích rodu *Trichodesmium* a heterocystních sinicích rodu *Anabena*, s kterými prováděla vlastní experimenty.
Protože spojujícím článkem disertační práce je metoda fluorescenčního imagingu, další velkou část práce tvoří popis metodiky fluorescenční indukce (strany 41 – 61). V této části autorka nejdříve uvedla souhrn a vzájemné souvislosti mezi fluorescenčními parametry použitými ve vlastních měřeních. Dále pak autorka podala chronologický přehled předchozích typů přístrojů pro měření fluorescenčního imagingu a detailně popsala fluorometr FluorCam pro měření fluorescenčního imagingu, který používala při vlastních měřeních.
V části výsledky autorka s odkazem na přiložené vlastní publikace stručně (strany 63 – 67) shrnula dosažené výsledky na téma fotosyntetické oscilace (1 již publikovaný článek) a na téma „sinice fixující dusík“ (1 již publikovaný článek, kde se zkoumaly sinice rodu *Trichodesmium* a rukopis 1 článku připraveného k publikaci, kde se studují sinice rodu *Anabena*).

Formální stránka disertační práce
Disertační práce je rozdělena do jednotlivých kapitol podle standardního členění a celková forma práce je standardní. Je však vidět, že práce byly asi psána velmi rychle, což se projevilo například častými dvojitými mezerami mezi slovy, existenci znaků ve větách, které tam nemají co dělat, různými odsazeními pro začátek nového odstavce a různě velkými fonty pro psaní speciálních znaků. Také velmi zajímavě, věta začínající na konci strany 24 nikde nekončí! Také věta začínající na konci strany 27 podivně přechází na stranu 28. Hlavním nedostatkem však je, že v seznamu použité literatury chybí mnoho citací.

Otázky k disertační práci
K předložené disertační práci mám několik otázek a prosím Mgr. Nailu Ferimazovou o jejich zodpovězení.

- Na základě vlastních výsledků a známé literatury, kterou hypotézu o vzniku fotosyntetických oscilací autorka upřednostňuje? A myslí si autorka, že oscilofor („zdroj“ oscilací) je pouze jeden nebo jich je více? A lze nějakou metodou (experimentální či teoretickou) zjistit, do jaké míry je daná reakce osciloforem?
- I když dochází ke změnám F_0, proč nemůže mít v tomto případě parametr $(F_M' - F_T') / F_M'$ svůj standardní význam, když v něm hodnoty F_0 nijak nefigurují (popis k obr. 6, strana 99, článek II)?

- Při odůvodnění existence driftu F_0 v rukopise článku III (strana 120) z dat AAVB 0h (obr. 3) předpokládáte, že velká redukce PQ poolu znamená velké nefotochemické zhášení, čili pokud to chápu správně, tak Vámi předpokládaný sled událostí je: nárůst PQ_{red} => nárůst $[H^+]_{lumen}$ (= nárůst ΔpH) => narůst nefotochemického zhášení. Jenže nárůst PQ_{red} nemusí být nutně spojen s nárůstem ΔpH, protože H^+ mohou být výrazně použity na formování elektrické složky (Δψ) protonmotivní síly (články Kramera v TIPSech 2003 a 2004 a PNASech 2004 a 2005) a tudíž nevést k nefotochemickému zhášení. Navíc, využití H^+ na formování Δψ nebo ΔpH se může v čase rychle a výrazně měnit, čímž by asi šlo také vysvětlit některé Vaše výsledky (změny v sadě AAVO 0h v obr. 3). Neuvažujete také možné nefotochemické zhášení oxidovaným PQ poolem, které by mohlo taky vysvětlit některé data. Co si autorka myslí o této mé argumentaci? Dále pak, mnou výše zmíněné fakta tedy říkají, že naměřené křivky by se snad daly vysvětlit i bez zavedení driftu F_0. Jaký je tedy hlavní důvod, proč zavádíte drift F_0?

Závěr

Vzhledem k zmíněným formálním nedostatkům doporučuji, aby autorka provedla příslušné opravy a pro knihovnické účely dodala nové opravené výtisky její disertační práce.

I přes zmíněné formální nedostatky, celkově hodnotím disertační práci Mgr. Naily Ferimazové pozitivně. Je vidět, že pracovala systematicky a že v daných problematikách má hluboké znalosti. Její disertační práce také ukázala, že vývoj a aplikace nové metodiky (fluorescenční imaging) může pomoci k lepšímu poznání jak fotosyntetických oscilací, tak i funkce a regulace fotosyntézy sinic. Disertační práci Mgr. Naily Ferimazové proto doporučuji k obhajobě.

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