IGB • Alte Fischerhütte 2 • 16775 Stechlin - Neuglobsow • Germany

To Prof. RNDr. Libor Grubhoffer, CSc,
Dean of the Faculty of Biological Sciences
University of South Bohemia
Branisovska 32
370 05 Ceske Budejovice

www.igb-berlin.de

Leibniz-Institut für
Gewässerökologie
und Binnenfischerei
im Forschungsverbund
Berlin e.V.

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PS: Review for the PhD thesis of Jan Jezbera, RNDr.

Dr. Hans-Peter Grossart
Leiter der Nachwuchsgruppe
Aquatische Mikrobielle Ökologie

Limnologie Geschichteter Seen

Alte Fischerhütte 2
16775 Stechlin – Neuglobsow
Germany

Tel: +49 (033082) 699 91
Fax: +49 (033082) 699 17

hgrossart@igb-berlin.de

Dear Prof. RNDr. Libor Grubhoffer,

please receive my review for the PhD thesis: "Protozoan food preferences studied by means of in situ hybridization techniques" by Jan Jezbera. The PhD thesis of Jan Jezbera offers a nice overview on protistan bacterivory in aquatic ecosystems and in particular on its impact on bacterioplankton communities. The thesis is well and carefully written (only minor typos) and considers all literature linked to these topics. The introduction leads the reader well into the subject, the specific characteristics of the study areas, and the major aims of the study.

By using state of the art methods Jan Jezbera is able to demonstrate that small choanoflagellates which are attached to large diatoms can account for a substantial portion of the overall protozoan bacterivory -at least at certain time points. These findings need to be taken into account for further studies on the same or similar study objects and their ecological consequences are yet largely unknown.

The use of the CARD-FISH method for quantifying and better phylogenetic characterization of bacteria inside protozoan food vacuoles is a smart way to better elucidate the protozoan impact on bacterial communities. The method allows to simultaneously study the extent and preferences of protozoan bacterivory by individual organisms. This allows for the first time to really address ecological important questions, e.g. do different protozoans show preferences in their food choice and what consequences does this have for bacterial growth and community structure. Jan Jezbera can nicely

demonstrate that protozoans indeed show differences in their food preference which has major ecological consequences. For example, Actinobacteria seem to be more resistant to protozoan bacterivory than other bacterial groups. In contrast, bacteria of the R-BT065 subcluster of the beta Proteobacteria are frequently found in food vacuoles of protests and, thus, may contribute substantially to carbon cycling through the microbial loop.

I did not discover any major pitfalls of the thesis and it was a great pleasure for me to review the highly innovative work of Jan Jezbera. All chapters of the thesis have been submitted or already published in international peer-reviewed journals. Since all of his results are presented in an "easy to read" fashion I rate the thesis as excellent.

Yours sincerely,

H.P. Grossart

Hans-Peter Grossart

UNIVERSIDAD NACIONAL AUTÓNOMA DE MÉXICO

DR. MIROSLAV MACEK
profesor titular
UNAM FES IZTACALA
UNIDAD DE INVESTIGACIÓN INTERDISCIPLINARIA DE CIENCIAS DE LA SALUD Y LA EDUCACIÓN
PROYECTO DE INVESTIGACIÓN EN LIMNOLOGÍA TROPICAL
UIICSE/PILT

IZTACALA

Los Reyes Iztacala, April 15, 2006

University of South Bohemia
Biological Faculty
TO WHOM CORRESPONDS

The present set of five articles summarised as the PhD thesis

Jan Jezbera: Protozoan food preferences studied by means of in situ hybridization techniques

reflects very solid series of consecutive investigations leading to prove an original hypothesis:

protists could be selective in discrimination within the bacterial food of similar size and shape. The thesis (100 pp in total) consists of 13 pages of general introduction & references, and of four already published articles and one accepted manuscript. It is well organized and, it should be pointed out, with very clear definition of the Thesis gain for the general science: An improvement of a use of CARD-FISH method for direct hybridization of protistan vacuoles content and an analysis of species-specific results.

Although the method of FLB is used already about twenty years, till now there is a lack of well statistically supported data obtained from various water habitats. Studies on the protistan bacterivory *in situ* but on a bacterial species-level are still scarce and well obtained results as presented in the Thesis, are comparable only with the results published by the Catalonian group (Medina Sánchez *et al.* 2005). Last but not least is necessary to stress that the results very well document usefulness of the FLB method if correctly applied. Comparing dilution experiments with a direct method was shown that, even though with some scepticism, there are very good examples of bacterivorous protists, for which the same feeding rates were obtained applying different investigation methods.

I liked the Thesis very much, however, I have got several doubts on the analysis of results, which

Av. de los Barrios 1
Los Reyes Iztacala, Tlalnepantla
54090 Edo. México
MÉXICO

☎ +5255 / 56231 296 ext. 427 ó 118
+5255 / 5390 76 04
mirek@campus.iztacala.unam.mx

could be divided into two main groups: species specific feeding behaviour and evolutionary taxonomy.

Feeding behaviour

Comparison of uptake rates (as ingestion of bacteria per cell per time) is a very good measure for comparison between different water reservoirs from the point of view of organic carbon fluxes and, certainly, looking for possible growth rates of ciliates (according to Fenchel 1986). However, to deduce more on the ciliate ecology in the habitats with different bacteria concentration, clearance (clearance rates) should be discussed. Are there significant difference between clearances in Římov and Orlík reservoirs? Are there differences in clearance rates between old studies in Římov reservoir comparing to the new ones?

For a long time, picophytoplankton potential ingestion rates published from Římov in 1995 were the only direct data; during last years, APP data were added from many other places. Do you think, the ingestion of APP of significantly higher individual biomass was affecting different results of feeding upon FLB in the reservoirs of different trophy?

Prostome ciliates behaviour could be specified generally as a raptorial. In your data, however, ingestion of FLB by (urotrichas) appeared as common, even though of low importance. Was it connected with minute, supposingly prostomes, or was it general for all sizes of prostomes? To my knowledge, Müller has published bacterivory of minute urotrichas, however, it was not mentioned again (even not by Weisse).

Tintinnids contain species of quite different behaviour although all could be defined as coarse to fine filter feeders. *Codonella* could be separated without problems, however, genera *Tintinnidium* and *Tintinnopsis* are not separable by fluorescence microscopy. Could you specify at least the cell size and shell shape of *Tintinnidium* spp. from the reservoirs? Are they observed with chlorophyll-bearing food?

Epistylis is probably more coarse- than fine-filter feeder comparing to vorticellids, however, have you got any idea of their growth rates? You have mentioned, in the case of HNF, that the attached species had better success due to their defence against metazooplankters and high feeding efficiency. Could you comment possible effect of colonization of phytoplankton (both diatoms and cyanobacteria) within vorticellids? What was, if you estimated it, the feeding activity of free swimming, plankton vorticellids?

Among scuticociliates, *Cinetochilum margaritaceum* is one of the most ubiquitous. However, free-

Av. de los Barrios 1
Los Reyes Iztacala, Tlalnepantla
54090 Edo. México
MÉXICO

☎ +5255 / 56231 296 ext. 427 ó 118
+5255 / 5390 76 04
mirek@campus.iztacala.unam.mx

bacteria are not its dominant source of feed. Do you know from your CARD-FISH experiments, if the ciliate depends on heterotrophic bacteria (flocs or aggregates) or on APP?

Taxonomy

Protistan taxons grouping in the thesis follows already passed Foissner´s system without a strict separation of choreotrichs from oligotrichs. Particularly in the case of the genus *Halteria* it is not an "oligotrichous ciliate" anymore. According to your experience, does the *Halteria´*s behaviour reflect the behaviour of Stichotrichida in other aquatic environments?

Choanoflagellates are leaving their position within "flagellates". Could you tell me your opinion, if it could be related to the peculiarities in their behaviour?

To my opinion, Mgr. Jan Jezbera presents the Thesis, which may be accepted without any doubt to obtain a PhD degree.

MIROSLAV MACEK

Av. de los Barrios 1
Los Reyes Iztacala, Tlalnepantla
54090 Edo. México
MÉXICO

☎ +5255 / 56231 296 ext. 427 ó 118
+5255 / 5390 76 04
mirek@campus.iztacala.unam.mx

Review of the doctoral thesis of Mgr. Jan Jezbera entitled

Protozoan food preferences studied by means of in situ hybridization techniques

The work of Mgr. Jezbera deals with different aspects of protistan grazing preferences and grazing rates in the aquatic environment. The main methods used are epifluorescence microscopy and Fluorescent *in situ* hybridization (FISH) in combination with various *in situ* manipulations of natural microbial populations mostly in Římov reservoir, Czech Republic.

The submitted thesis has 99 pages, consists of an introductory chapter, summary of main results and five enclosed publications. Paper I (Jezbera et al. *Hydrobiologia* 504: 115, 2003) deals with the distribution of bacterial production and bacterial mortality along the longitudinal transect through Římov and Orlík reservoirs. Attention was mostly focused on mortality due to heterotrophic nanoflagellate and ciliate grazing determined using fluorescently labeled bacteria. The study showed that in Římov and Orlík protists removed 35% and 70% of the bacterial production, respectively. Paper II (Šimek et al. *Aquat Microbiol Ecol* 36: 257, 2004) was dedicated to the study of the ecological role of diatom-attached choanoflagellates as pelagic bacteriovores. Again, fluorescently labeled bacteria were used to determine rates of bacteriovory in this specific group. Overall, choanoflagellate *Salpingoeca* sp. was found to be responsible for 11 to 64% of total protistan grazing in Římov reservoir, which makes it one of the most important grazers in this environment. In Paper III (Jezbera et al. *FEMS Microbiol Ecol* 52: 351, 2005) the author applied the method of FISH to analyze the composition of bacterial prey and assess the grazing preference of protists in laboratory studies as well as under natural conditions of Římov reservoir. Prey selectivity was found in two tested flagellate species whereas cilitate *C. glaucoma* showed no clear grazing preference. In paper IV (Šimek et al. *Appl Environ Microbiol* 71: 2381, 2005) the authors analyzed the effects of top-down (protistan grazing) and bottom-up (transplantations) manipulations on bacterial community in Římov reservoir. Using FISH it was found that 10-50% of total bacteria belonged the R-BT065 subcluster of beta-*Proteobacteria*. The detail analysis of growth rates of individual bacterial groups in Římov reservoir and by CARD-FISH analysis

demonstrates that R-BT065 subcluster exhibited the fastest growth rate and likely it was preferentially removed by the grazers. Another life strategy was displayed by relatively slowly growing *Actinobacteria*, which benefited from their resistance to grazing. The study published in Paper V (Jezbera et al. *Environ Microbiol.* Accepted) follows the direction elaborated in paper IV and gives the detailed analysis of food vacuole contents of heterotrophic nanoflagellates. The analysis suggests that nanoflagellates remove preferentially *Cytophaga/Flavobacteria* group whereas *Actinobacteria* were negatively selected.

The thesis is a high quality work, which fully meets the international standards. The major part of the results was published in high quality international journals. The strong point of the thesis is a robust experimental support elaborated in several detailed studies, which provides a solid scientific base for firm conclusions.

In spite of that I am obliged to raise some comments and questions:

Introduction, page 8. The sentence gives an impression that FISH probes are targeted only to 16S rRNA, whereas some of the probes (BET42a, GAM42a, HGC69a) used in the study are designed against 23S rRNA.

Paper I. Can you give a general comment on use of dead and stained bacteria for grazing experiments?

Paper I. Why is X-axis description always from the right to the left?

Paper II. Do you think that glucose is the best substrate for aquatic bacteria? Why not to use some more suitable carbon sources (acetate, glutamate, leucine or ideally a mixture of carbon sources), which are taken up by much broader variety of bacteria then glucose? (Just a quibble: In the standard biochemical nomenclature Glu is an abbreviation for Glutamate, Glucose is abbreviated as Glc).

Paper III. What was the percentage of the bacterial cells hybridized with EUB338 probe? Have you observed higher percentage of EUB338 cells in standard or CARD-FISH protocol?

Paper III, IV a V. In a paper of Yeates et al, Microbiology 149: 1239-1247, 2003, the authors reported a significant cross-talk between GAM42a and BET42a probes (the probes differ only in one nucleotide). Do you have similar experience? Could this fact affect your data?

Paper III, IV, V Did you use antisense EUB338 probe as a negative control?

Paper III and V. Why don't you use probes against alpha-*Proteobacteria*? Alphas probably represent only minor fraction in freshwater environment, but they cannot be completely ignored.

Paper III, IV and V. Do you think that CARD-FISH approach is a way to go? I would assume that new CCD cameras can register even weak signals from standard FISH probes, so there is no need for complicated, labor-intensive and expensive CARD-FISH protocols.

Paper IV. Why is there an increase in cell size in the <0.8 treatment?

Paper IV. The dominant and rapidly growing of R-BT065 cluster is likely to be a subject of heavy viral attack. This would be consistent which the "virus kills the winner" theory.

Paper V. Why are abundances in the control experiment going down? Why is there hardly any difference in all the UNF treatments? (I do not see the pronounced increase in +P treatment advertised in the first sentence of the Results).

Paper V. In +P+GLU, <5 treatment there is a marked increase in bacterial productivity, which is not compensated by grazing, but there is no change in bacterial numbers. Why?

Paper V. In paper IV you have shown that the doubling times in grazer free experiments are significantly less then 24 hours. Do you think that sampling rate once per day is appropriate in populations, which grow in hours?

Paper V. Selectivity index. In theory, the selectivity indexes should mean <1 negative selection, >1 positive selection. For the entire community it should be on average one.

However, in Fig. 4 it looks that, on average, the data are bellow 1. In some treatments (+GLU, <5; +P, <5) basically all the points are bellow 1. Why is this so?

Paper V. The data suggest that *Cytophaga/Flavobacteria* are preferentially grazed. In contrast, this group displays the slowest growth rate in the population (paper IV). How would you explain this?

Paper V. Do you think that different bacterial species might differ in the rates of their digestion? If yes, then the inspection of the food vacuole contents would be biased towards slowly digestible or non-digestible species.

Papers I-V. Which conclusions drawn from Římov data would you consider as universally applicable for other aquatic environments?

The text of the thesis contains some grammatical and spelling errors, which could have been avoided by better proof reading. The combination of sanserif and serif fonts on page 5 is a typographic error. It is a pity that the author did not use the opportunity to enclose some high quality color microscopic images, which would not only illustrate the submitted work but also aid the reader to get a better feeling of the presented data.

In summary, the work of Mgr. Jezbera presents important and new results and proves his ability to conduct productive scientific research. The submitted doctoral thesis meets high international standards. I have no doubt that Mgr. Jan Jezbera fully deserves the title *Doctor of Philosophy* Ph.D.

Třeboň, April 13th, 2006

Mgr. Michal Koblížek PhD

Inst. of Microbiology CAS

Opatovický mlýn

379 81 Třeboň