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REVIEW

by Prof. Dr. Yordan Ivanov Uzunov,

retired, former Head of the Department of "Aquatic Ecosystems", IBEI-BAS

regarding the dissertation work of Mila Kirilova Aleksandrova-Ihtimanska – full-time doctoral student at the Department of Aquatic Ecosystems of IBEI-BAS, on the topic:

"Composition, structure and distribution of macrozoobenthos in the riparian zone of the Bulgarian section of the Danube River under conditions of anthropogenic pressure and impact",

presented for the acquisition of the ESD "Doctor" in the scientific specialty "Hydrobiology", in the professional direction 4.3. "Biological Sciences", field of higher education 4. "Natural Sciences, Mathematics and Informatics"

This review is being prepared in implementation of Order No. 16/14.02.2025 of the Director of IBEI-BAS in connection with the Decision of the Academic Council of IBEI-BAS, Minutes No. 37, item 6 of 07.02.2025, and the decision of the Scientific Jury from its First meeting, held on 26.02.2025, as well as in accordance with Art. 9 of the Regulations on the conditions and procedure for acquiring scientific degrees and for holding academic positions at IBEI-BAS, adopted by the Academic Council on 22.07.2014, last amended on 25.11.2022.

All documents related to the process and procedures of the doctoral studies and defense are properly drawn up.

(1) general characteristics of the dissertation work – volume and structure:

The main element in the **working hypothesis** is the (pre)sumption that changes in the composition, structure and distribution of macrozoobenthos (MZB) reflect the impact of various types of anthropogenic pressure, with the impact of hydro-morphological pressure being stronger than that associated with the load of nutrients and heavy metals (which should be proven).

The **general objective** of the dissertation paper is to outline the changes and assess the ecological potential of different sections and habitats, using multimetric indicators for ecological classification, through a study of the composition, structure and distribution of the MZB in the riparian zone of the Bulgarian section of the Danube River and a comparative retrospective analysis of available (historical?) data.

To achieve this goal, 4 **research tasks** have been formulated. The following are planned: assessment of the parameters of the existing anthropogenic pressures and impact in the riparian zone of the Bulgarian Danube sector (1); determination of model referent and differently influenced river sections (2); study of the species composition, structure and distribution of benthic invertebrate communities in the model sections (3); identification of the MZB parameters indicative for the different forms of pressure and impact (4).

The presented work contains 135 pages of text, as well as 13 annexes, 32 tables and 55 figures, which illustrate various aspects of the development and conclusions of the dissertation, which is structured according to the chapters/sections adopted by us: Introduction, Goals & Objectives, Literature overview, Materials & Methods, Results & Discussion, Summary of results, Conclusions, Contributions; a list of the literature sources used is also attached.

From a technical point of view, the dissertation work is excellently designed; the visual materials are readable, the objects are recognizable, the designations are sufficient and correctly formulated.

(2) literary awareness and theoretical preparation of the candidate:

The list of literary sources used contains 240 titles, of which 25 are titles/indexes of normative documents or standards, another 27 titles are in Cyrillic, and all the rest - in Latin. It should be emphasized that 64.5% of the sources were published after 2000, and 40.2% after 2010, which is a very good indicator of the relevance of the literature used. Of course, some of the titles (35.6%) inevitably discuss historical data, in which the doctoral student also sought support. The literature review covers several areas that correspond to the thus formulated goals and objectives of the dissertation work and are further developed in the research program. The appropriate and successful inclusion and citation of literary sources when discussing one's own data and results, rather than simply listing them in the literature review, also makes a very good impression. All this shows good awareness and skill in handling literary data by the doctoral student, which also supports her high theoretical training.

(3) methodical approach:

Samples have been collected from 29 stations in the riparian zone of the Bulgarian section of the Danube River between 844 r.km (Novo Selo) and 380 r.km (Silistra). The points were selected to reflect the effects of various pressures and impacts on the Danube biota: point and diffuse sources of pollution, nitrate vulnerable zones; places where a permit for the dragging f inert materials and

dredging of the riverbed has been issued, according to the current RBMP. Also noted were: the presence of bank fortifications; tributaries, ports, settlements and the city; infrastructure and others up to 5 km upstream. An important circumstance is the coordination of the selected target points with those of the national monitoring network (EEA), as well as with others from the Joint Danube Survey 3, conducted in 2013.

Based on the available information, the approach of Stoddard et al. (2006) was applied with an addition by Flotemersch et al. (2006a) in determining referent sections for the purposes of this study, by determining the anthropogenically influenced stations, and those that can be considered the least influenced (conditional referent sites).

The field studies were conducted at low water levels of the Danube (July, August and September) in the years 2012, 2013 and 2014. A total of 54 samples from the MZB were collected and analyzed, applying the multihabitat approach, compatible with the definitions/norms of the WFD for the assessment of the ecological status, respectively. potential (AQEM Consortium, 2002). A wide variety of bottom habitats/substrates was examined (megalithal, macrolithal, mesolithic, microlithal, akal, psamal, psamopelal, argylal, xylal, phytal, microalgae, submerged or semi-submerged higher aquatic vegetation (macrophytes), living parts of terrestrial plants, etc. All samples were fixed with 4% formaldehyde.

The species determination of the collected materials from the MZB was carried out using classical methods and specialized literature (determinants), as well as with the assistance of colleagues-specialists in individual taxonomic groups. Some of the physical and chemical indicators of the Danube waters and sediments were registered both *on site* (a total of 5 indicators) and *in lab* (various forms of biogens, as well as heavy metals, arsenic), with the measurements being carried out according to the relevant BDS and ISO standards.

The wide use of various statistical methods and procedures is striking, as their selection is well-argued and purposeful - for research and assessment of the composition, structure and distribution of the MZB (ANOSIM-test and SIMPER, Primer v.6 program), for visualization of the results (nMDS-ordination); for testing the null hypothesis (DistLM); for assessing the relationship between water levels and abundance (correlation analysis of the main taxonomic groups, PAST v. 4.03 program); for assessing the relationship between water level fluctuations and abundance (via Generalized Linear Model, RStudio v.4.3.0 program with the "mvabund" package), etc.

To determine the diversity of the communities and to reflect the degree of evenness, indices for average taxonomic difference (Δ+) and variation of taxonomic difference (Λ+) were calculated, and for this purpose a complete inventory taxonomic list of all taxa from the MZB in the section from 851 r.km to 130 r.km of the Danube River (including the Bulgarian section) was compiled based on literature data for the period 1952-2014 inclusive. It can only be regretted that the opportunity to include an organized set of data on MZB from other periods and other localities, which the reviewer has at his disposal, was missed in this array. The analysis was supplemented with data on individual species diversity and evenness of the communities by calculating the Shannon-Weaver and Pielou indices.

The ecological potential of the riparian zone of the Danube was assessed using two multimetric indices - the Flemish multimetric index for macroinvertebrate assessment for very large rivers with a catchment area of over 10,000 km2 (MMIF) and the modified rapid biological monitoring method (mRBA), which is regulated in national legislation (Regulation H-4/2013). In parallel, an assessment of the stations by physicochemical parameters was carried out in accordance with Regulation H-4/2013. A modification of the mRBA calculation methodology was developed and applied, which was tested through correlation analysis with a set of variables of the aquatic environment and on this basis a refinement of the EQR limit values was proposed.

As noted above, the results obtained from the application of this not small set of methodologies are presented in 13 annexes, 32 tables and 55 figures.

(4) significance and persuasiveness of the obtained results, interpretations and conclusions;

In fulfilling her tasks, the doctoral student consistently presents the obtained results in several sections:

- Taxonomic composition and distribution of the MZB in the riparian zone of the Bulgarian section of the Danube River, where a total of 133 taxa of species rank from 18 main taxonomic groups (2013) and 93 species from 16 taxonomic groups (2014) were established. Reliable, but low differences in the MZB from the island arms (2012) and the main channel (2013) were obtained. The conditional division of the Bulgarian-Romanian sector according to MZB indicators, as defined by JDS-3, is confirmed, with the DB Vardim station being taken as the border between sectors 5 and 6, as well as the thesis of Prof. B. Rusev (1960) that the composition of the MZB off the Bulgarian bank is sufficiently representative to characterize the entire general area,

- The analysis of the factors and variables of the aquatic environment and their impact on the MZB follows basic indicators of the aquatic environment such as: the importance of the bottom substrate for the distribution of the MZB; fluctuations in water levels and the dynamics of the composition and abundance of the MZB; the relationship of the various physical and chemical indicators with the composition and abundance of the MZB; as well as the integral indicators "location of the stations", "section of the river", "specific pollutants", which are subjected to statistical analysis to derive/prove their importance for the MZB. The low values, despite the good level of reliability, of most statistical results suggest the need either to accumulate more information (number of samples/cases for each station), or to develop an integral approach to assess the state and dynamics of the MZB community.
- A special section is dedicated to the diversity indices, which have significantly lower values in the main channel in 2013 and 2014, which is interpreted by the differences and sharp fluctuations in water levels during the individual seasons/years of study. The values of the Shannon-Weaver and Pielou indices show higher diversity and a more even structure of the stations in the islands arms when compared to those in the main channel.
- Similarly, analyses of taxonomic difference indices were conducted regarding the differences in the period before and after the commissioning of the Iron Gates Dam; specifically the situation in the island arms, as well as in the main riverbed for the current study period (2012-2014), and taxonomic differences were also assessed through primary and secondary aquatic organisms.
- The assessment of the ecological potential in the riparian zone of the Bulgarian section of the Danube was carried out on the basis of physical and chemical indicators, in accordance with the national regulatory framework, and not a single station in the main riverbed in 2013 and 2014 was fully assessed with "good" or "excellent" ecological potential for all studied parameters. Based on the conducted parallel assessments of the ecological potential using two multimetric indices, a proposal is made for a modification of the methodology for calculating the mRBA index; the limit values of mRBA and EQR for assessing the ecological potential have also been refined.

Based on the thus obtained, presented and statistically evaluated results of the study conducted on the topic of the dissertation, several conclusions have been drawn, which in summary are reduced to:

- The taxonomic composition and abundance of MZB in the riparian zone of the Bulgarian section of the Danube River is representative of the entire Bulgarian-Romanian section of the river and can be successfully used to characterize the entire sector. In the riparian zone of the main riverbed, the composition of the bottom substrate is largely influenced by the rip-rap type of coastal fortification, while the island arms are less influenced by human activity and it can be assumed that the composition and distribution of the bottom substrate reflect close to natural processes in the river continuum,
- Substrate formation/distribution plays a leading role in the distribution of MBA communities, with classes of primary aquatic organisms showing significant connectivity with a greater number of substrate types compared to secondary aquatic organisms.
- Higher water levels and their sharper fluctuations create less favorable conditions for the development of benthic organisms in the riparian zone. In conditions of low water levels, the communities of the stations in the arms of the islands have a more even structure than those in the main channel. The greater and longer the fluctuations of the water levels, the more significant the relationship with the change in the abundance/number of organisms. The hydrological regime has a strong impact on the concentrations of dissolved oxygen and phosphorus in the water, the incoming dissolved organic matter from the floodplain terrace. Pollution with heavy metals and arsenic in the water is equally high at the stations after tributaries and after cities. On the other hand, tributaries and cities have a stronger influence on the loading of water with nutrients than with heavy metals and arsenic.
- Despite the large number of species established in the contemporary period (2012-2014), the richness of senior taxa groups remains lower than observed in retrospect even during a period of strong impact, immediately after the construction of the Iron Gates Dam I hydroelectric complex. The taxonomic difference indices fail to distinguish the impact of different types of anthropogenic pressure on the composition of benthic invertebrate communities in the riparian zone of the Bulgarian section of the Danube River.
- The values of the taxonomic difference indices of the stations in the island arms highlight them as peculiar refugiums and places in which the biological and taxonomic richness of the primary aquatic benthic invertebrates manages to be preserved to the greatest extent close to the average expected for the region of the Bulgarian section of the Danube River.

- Changes in the taxonomic composition, abundance and distribution of representatives of the groups Gastropoda, Bivalvia, Malacostraca, Trichoptera and Chironomidae to a greater extent than those of other groups reflect changes in the condition of habitats and can be used as bioindicators.
- The assessment of the ecological potential according to the proposed modification of the mRBA calculation methodology showed that in low water conditions (the period during which standard hydrobiological monitoring should be carried out) the majority of the stations in the islands arms and a significant part of those in the main riverbed are assessed as having "good" ecological potential. The effectiveness of the index calculated using the proposed modification of the calculation methodology is also confirmed by the reliable correlations with a significant number of environmental variables that influence the composition, structure and distribution of MRB communities.

(5) critical comments on the dissertation:

I have the following critical comments on the dissertation:

- The authorship of the data contained in Annexes II, III and IV is not indicated; if they are borrowed from the RBMP or provided by the RIEW/BDDR, this should be duly cited;
- The data provided on the species composition of the MZB in Annexes V, VI and VII do not contain author names and years; there is a discrepancy/inconsistency with the names of some taxa/species provided in Annex X (full inventory taxonomic list….);
- I do not accept the use of the generalized term "biological diversity" instead of the concept of "species diversity" clearly defined in the regulatory framework.

(6) nature of the scientific contributions:

The first and such comprehensive, detailed, in-depth and competent study of the bottom communities of the Danube River for the last more than 30 years is the largest and most significant contribution of this dissertation.

As ***confirmatory contributions***, the following are outlined: updating the knowledge/concept of the contemporary composition, structure and distribution of bottom invertebrate communities in the riparian zone of the Danube; confirming the stronger impact of hydro-morphological pressure on the composition, structure and distribution of the MZB in comparison with that of the load with nutrients and heavy metals; analyzing the influence of various forms of anthropogenic pressure in the riparian zone of the entire Bulgarian section on the MZB;

According to the reviewer, the contributions related to the first in-depth study of the influence of water level fluctuations on the distribution of MZB in the riparian zone of the Bulgarian Danube sector are original; The first application of taxonomic difference indices with data on the composition of the MZB is also original, and their effectiveness in lotic ecosystems has been tested (a transfer from the study of MZB in marine ecosystems).

As ***scientific and applied contributions***, I present the developed proposal for a modification in the methodology for calculating the mRBA indicator/index, standardized in the national legislation for determining the ecological potential of type R6 rivers (Middle and Lower Danube), as well as the prepared assessment of the ecological potential of a number of points in the riparian zone of the Bulgarian section of the Danube River, highlighting the points/locations least influenced by anthropogenic pressure.

I accept the doctoral student's account of her contributions as objective and comprehensive.

(7) assessment of the quality of scientific works reflecting the research on the dissertation;

The titles of publications on the topic of the dissertation provided objectively reflect the course of the research and various aspects of the results obtained. In them, the doctoral candidate has the leading position of the main/leading initiator and co-author, as the main source of data, analyses, determination and subsequent statistical processing. An article with IF has been published, full-text reports have been presented at international and national scientific events.

(8) a motivated answer to the question to what extent the research on the dissertation is primaily the personal work of the doctoral student;

I categorically believe that the research on the dissertation is the personal work of the doctoral student; the participation of colleagues in the development has been expressed primarily in logistical support/ and collegial consultations on common topics.

(9) a motivated conclusion recommending the unequivocal award of the scientific and educational degree "doctor":

Based on the above analysis and assessments, I confidently propose to the esteemed Scientific Jury to award Mila Kirilova Alexandrova-Ihtimanska the scientific and educational degree "**DOCTOR**" in Hydrobiology (01.11.06).

Sofia, 12 May 2025

Reviewer: Prof. Dr Yordan UZUNOV