REVIEW

by **Prof. George Angelov Miloshev**,
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on the dissertation work of

Radina Nikolaeva Nikolova

on the topic:

"Structural and functional characteristics of soil microbial communities in response to long-term pollution with heavy metals"

for awarding of the educational and scientific degree "**Doctor**"
in the field of higher education 4. "Natural Sciences, Mathematics and Informatics",
professional field 4.3. "Biological Sciences", scientific specialty "Molecular Genetics"
Institute of Molecular Biology "Roumen Tsanev", Bulgarian Academy of Sciences

1. Grounds for preparing the review

This review was prepared in accordance with Order No. 143-OB of 28.08.2025 of the Director of the Institute of Molecular Biology "Academician Roumen Tsanev" for the appointment of a Scientific Jury to conduct a public defense of a dissertation on the topic "Structural and functional characteristics of soil microbial communities in response to long-term pollution with heavy metals", presented by Radina Nikolaeva Nikolova, a full-time doctoral student at the section "Regulation of Gene Activity". The dissertation of doctoral student Radina Nikolaeva Nikolova

was presented at an extended meeting of the section, held on 29.07.2025. After discussion, it was directed to a public defense procedure.

The review was prepared in accordance with the requirements of Art. 9, Art. 30, 3 of the Regulations for the Application of the Law on the Development of Academic Staff in the Republic of Bulgaria

and the Scientific council meeting No.13 of 28.08.2025

2. Data on the Dissertation

The work on the dissertation was carried out under the scientific supervision of Assoc. Prof. Galina Radeva and Prof. Anelia Kenarova.

The dissertation is presented in a volume of 170 pages of main text, containing rich visualization - 27 figures, 33 tables. In addition, a detailed Appendix is presented, including 12 tables, a list of abbreviations and cited literature, including more than 300 sources.

The author Radina Nikolova presents results of complex studies, combining:

o classical microbiological analyses;

o enzyme tests and functional profiles;

o molecular genetic methods (PCR, qPCR, clone libraries);

o targeted amplicon sequencing of the 16S rRNA gene of bacteria and the ITS region located between the 18S rRNA and 26S rRNA genes in fungi;

o bioinformatic analyses and statistical models.

Publications on the topic, scientific activity and participation in projects complement the overall assessment of this serious research work.

3. Relevance and significance of the topic

Soil contamination with heavy metals is a global environmental problem that poses a constant risk to human health and the environment. The scientific community recognizes the microbiome as a sensitive and early indicator of changes in the health and functionality of soils. However, in Bulgaria there is a lack of studies that use a metagenomic approach to analyze microbial communities in permanently contaminated soils.

This dissertation addresses the problem, for the first time on a national scale combining classical and modern metagenomic techniques, analyzing both bacterial and fungal microbial communities, assessing structural and functional adaptations in chronic heavy metal pollution, and integrating physicochemical factors with topography and biotic interactions.

The topic has a pronounced ecological, scientific and social significance, and the results have potential for application in biomonitoring, ecosystem management, risk assessment and bioremediation.

The relevance of this scientific work is undeniable, and the approach is modern and scientifically justified.

4. Goals and objectives of the study

The main goal of the dissertation is a comprehensive clarification of the structural and functional characteristics of soil microbial communities in conditions of long-term heavy metal pollution, through the integration of molecular, microbiological, biochemical and bioinformatics methods.

The tasks are precisely formulated and logically follow the goal. These tasks include: determination of the concentrations of the main heavy metals in the soils; characterization of key physicochemical indicators; determination of the abundance of bacterial and fungal communities; taxonomic analysis using classical (clone libraries) and metagenomic techniques; assessment of

functional potentials (KEGG, Ecoplate, enzymes); determination of the influence of heavy metals and abiotic factors on the structure and function of the microbiome.

The goal and tasks are correct, measurable, achievable and adequate to the scientific specialty.

5. Methodology and methods used

The implementation of the formulated research tasks is based on the application of modern standards and systematic approaches to research. This implies the use of the most modern methods for conducting the study and evaluating the results obtained, the use of specialized scientific equipment, the application of specialized statistical methods for verification and interpretation of the results obtained.

The methodology is one of the strongest elements of the dissertation. The author Radina Nikolova uses a wide range of modern techniques. The rich set of methods and techniques deserves their detailed listing. Methods and techniques include:

<u>Microbiological and biochemical approaches:</u>

• colony-forming unit (CFU) analysis

• enzymatic activities of six enzymes (DHA, BGL, ALP, AsP, ApS, Ur)

• determination of functional profiles using Biolog EcoPlate™

<u>Molecular genetic methods:</u>

• PCR, qPCR

• TOPO TA cloning

• RFLP analyses

• creation of 16S rDNA clone libraries

• targeted amplicon sequencing (16S rRNA and ITS)

Sequencing and bioinformatics:

• sequencing using Illumina MiSeq

• phylogenetic reconstructions

• OTU classifications

• functional profiles using KEGG Orthology

Statistical analyses:

• PCA, MDS, CCA

• PERMANOVA

• SIMPER

• correlation models.

For the chosen goals and tasks, the methods in this study are precisely selected, adequately conducted and statistically well justified.

6. Structure and logical sequence

The dissertation is structured clearly and correctly, divided into chapters and sections:

Introduction → Literature review → Aim and objectives → Materials and methods → Results → Discussion → Conclusions → Contributions → Application.

The presentation is consistent, the style is scientific, and the graphic material is clear and informative. The text is well edited, with minimal linguistic errors.

7. Main scientific results

The results in the dissertation are presented logically and consistently, which allows for their full understanding and supports critical and in-depth discussion. Among the most significant results are:

- High levels of heavy metal contamination were found in soil samples taken around “KCM 2000”. These levels are above the maximum permissible concentrations according to Regulation No. 3/2008. “On the norms for permissible content of harmful substances in soils”. The soils have high levels of contamination with the metals Pb, Zn, Cd, Cu and the metalloid As. Of particular interest are the elements Pb, Zn and Cd, as they are present in high concentrations, exceeding the maximum permissible, in all studied samples. The pollution index (NPI) was calculated, which allows categorization of the samples according to the level of their pollution. Correlations between heavy metals and between their concentration and the distance from the pollution source ("KCM 2000") were established. Values of the biologically available forms of Pb, Zn and Cd in the soils were determined.

- By using two independent methods (CFU and qPCR), significant changes in the abundance of microbial communities were established. A significant decrease in bacterial abundance was observed in soils heavily contaminated with TM (heavy metals). Interestingly, no dependence on soil pollution was observed in fungal communities.

- The taxonomic profile of the bacterial and fungal communities was reconstructed using two independent genetic-molecular approaches: 16S rRNA clone libraries and targeted amplicon sequencing (16S and ITS). Analysis of the clone libraries revealed the presence of new uncultivable bacterial species.

- A thorough analysis of the taxonomic distribution by division, class, genus and species of the bacterial communities was carried out using the above methods. A detailed statistical analysis showed that bacterial diversity was greater in soils contaminated with TM.

- Significant correlations were found between the level of TM contamination and the fungal classes Eurotiomycetes, Sordariomycetes, and Pezizomycetes.

- Key bacterial taxa associated with resistance to heavy metals were revealed, such as: *Actinobacteriota*, *Firmicutes*, *Proteobacteria*, and also for fungi from certain divisions such as Ascomycota and Basidiomycota.

- The influence of heavy metals on functional characteristics has been proven through:
  - ✓ association of higher enzyme activities with slightly contaminated soils, which suggests that pollution has an inhibitory effect on the biological activity of soil microbial communities;
  - ✓ reduced enzyme activities;
  - ✓ reduced functional diversity;
  - ✓ significant changes in metabolic profiles.

- Predictive mechanisms for adaptation have been proposed through KEGG analyses of potential resistance genes.

All results listed so far are reliable and new and provide scientifically significant data on the ecology of microbial communities under contamination with heavy metals and metalloids.

8. Scientific and applied scientific contributions

The achieved contributions of the dissertation work are correctly formulated. After critical assessment, the contributions can be synthesized as:

Scientific contributions

1. For the first time in Bulgaria, metagenome has been applied
2. The structure of bacterial and fungal communities has been characterized by combining clone libraries and high-throughput sequencing.
3. Key bacterial taxa with a potential role in soil resistance to Pb, Zn and Cd contamination have been revealed.
4. Statistically significant relationships between abiotic factors, heavy metals and microbial diversity have been established.
5. Potential adaptive mechanisms, including KEGG pathways related to bacterial resistance to TM, have been identified.

Applied contributions

1. Functional profiles of microbial communities have been created, which can be very useful for biomonitoring.

2. The potential for soil bioremediation through identified resistant bacterial taxa has been determined.

3. An integrated approach for determining microbial communities has been developed, which can be successfully applied in future ecological assessments.

The data obtained can support future expert analyses and the creation of plans for managing contaminated sites. Of these contributions, the discovery of taxa resistant to pollution with some heavy metals and the creation of functional profiles of microbial communities, which can be useful for biomonitoring, stand out as extremely important.

Overall, the contributions are original, well-founded and significant.

9. Publications on the topic of the dissertation

The best assessment of the level of a scientific work is the number and quality of scientific publications on it. On this dissertation, the author Nikolova presents 3 scientific publications, in which she is the first author. Participations in 9 scientific forums related to the dissertation topic

are also presented. A detailed examination of the publications shows that they reflect the most important and significant part of the results obtained in this work. Therefore, it can be assumed that the most important part of the results has been published and accessible to the international scientific community. Moreover, a total of 27 independent citations of the publications in the international scientific literature have been noted so far.

In addition, some of the results have also been reported at international scientific meetings.

10. Critical notes and recommendations

Any peer reviewing should include constructive criticism. My most important remarks are:

1. The discussion could focus on a comparison with more European metagenomic studies in order to emphasize the regional specificity of Bulgarian soils.

2. Some figures are presented in the dissertation with low resolution, which makes it difficult to read and interpret the data in them.

3. It would be useful in future studies to include:

– analyses of the mobile fractions of all heavy metals;

– shotgun metagenomics for precise determination of true functional genes;

– determination of the influence of seasonal dynamics on the abundance and diversity of microbial communities.

4. The Ecoplate™ system is informative, but does not cover the entire functional diversity of the microbiome. An extension to metabolomic approaches would add valuable information on the impact of microbial communities on heavy metal pollution.

These criticisms and recommendations are essentially minor and do not reduce the scientific value of the dissertation.

11. Questions related to the dissertation

I have a question for the doctoral student:

Can we assume those characteristics of the studied fungi that, generally speaking, make them more resistant than bacteria to heavy metal contamination?

12. Conclusion

The dissertation of Radina Nikolaeva Nikolova represents a complete, original and scientifically significant study that:

- meets all the requirements of the Low on the Development of the Academic Staff in the Republic of Bulgaria;
- demonstrates excellent methodological preparation;
- contains new scientific contributions;
- proves the author's ability for independent scientific research;
- the Dissertation abstract was prepared in accordance with the requirements of the Low on the Development of the Academic Staff in the Republic of Bulgaria and fully reflects the content of the dissertation. The Dissertation abstract contains 69 pages. In addition to a brief presentation of the dissertation, it includes the scientific contributions, as well as a list of publications on the dissertation.

Based on the presented work, the quantity and quality of the methods used, the results of the research and finally the undeniable contributions of the scientific work, I propose to the Scientific Jury:

To award the educational and scientific degree "Doctor" to Radina Nikolaeva Nikolova.

30.11.2025 Prof. George Miloshev