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OPPONENT'S OPINION
on the Ph.D. thesis
titled: "**Microbiology in relation to nuclear waste repository safety**"
by author: MSc. Rojina Shrestha

The relevance of this topic is undoubtedly important. This topic is a new area and direction of research in the Czech Republic. The prevailing opinion of experts in the field of radioactive waste management is that radioactive waste should eventually be isolated from the human environment until it has lost most of its hazard as a result of radioactive decay. The preferred means of achieving this for high level waste and spent fuel is by deep underground disposal. For waste of low activity and short half-life, surface or near surface disposal is the generally preferred approach and it has already been adopted in many countries. Most low-level radioactive waste is typically sent to land-based disposal immediately following its packaging for long-term management. This means that for the majority (~90% by volume) of all of the waste types produced by nuclear technologies, a satisfactory disposal means has been developed and is being implemented around the world. The globally accepted strategy for the management and treatment of high level and long-lived radioactive waste is to dispose of the waste in a deep and stable geological formation.

It should be noted that scientists mainly have studied the physicochemical aspects that have been described in detail to ensure the long-term safety of the repository. However, the effect of microorganisms was until recently rather underestimated, although it is well known that microorganisms can survive and propagate under environmental conditions, including nuclear waste repositories. The different groups of anaerobic microorganisms with diverse types of metabolism present in the groundwater or buffer material. These microbial groups may influence and compromise the long-term safety performance of the repository.

The author of the thesis has tried to improve the knowledge about the microbial effect processes on radioactive waste disposal. The main attention was focused on microbial activity and survivability under different repository relevant conditions. Moreover, the effect of variable doses of irradiation on the microorganisms, the evolution of anaerobic microbial ecosystem with and without added nutrients, and microbial interactions with cementitious material were studied.

It is difficult and complicated to provide research about microbial influence on corrosion of carbon steel under anaerobic conditions. It takes a lot of effort and diligence. However, the author has obtained original results that could be applied in future studies. The most of obtained experiments were carried out under a strictly anaerobic atmosphere in an argon-purged glove box with gaseous oxygen concentration lower than 1 ppm. The author has applied a multidisciplinary approach, combining advanced microscopy methods and other methods: electron microscopy, electrochemical impedance spectroscopy analysis with molecular biology-based methods such as NGS and qPCR. This approach resulted in obtaining important results for the PhD thesis. Following chemical analysis were performed: ion-chromatography and spectroscopy methods.

Microbiological part of the research was focused on anaerobic microorganisms, including sulfate, iron, and nitrate-reducing bacteria that were mostly detected in the samples. It should be noted that bacteria present in bentonite were resistant to the 19,656 Gy total absorbed dose of Gama

radiation at the constant dose rate of 13 Gy/hr. Also, interesting results have been obtained about the bacterial influence on the corrosion rate of carbon steel and it was compared to samples in sterile conditions. Particularly, abundance of *Methyloversatilis* genus population positively correlated with corrosion rates. The originality of the research is also supplemented by the presence of mackinawite, a corrosion product usually attributed to the activity of sulfate-reducing bacteria that was confirmed by Raman spectroscopy. Author has also demonstrated that the presence of concrete strongly reduced the relative abundance of bentonite bacteria in studied samples, especially the growth of SRB that was limited in the concrete environment. This research represents important information for the industry because these effects might have a negative impact on the repository safety.

The PhD thesis has classical structure and is divided into three main parts: "Literature overview (introduction and background of the study)", "Experimental part (microbial activities and their community structure in relation to repository relevant condition)", and "Conclusions". The literature overview is divided into four subchapters. The first one is a brief introduction into nuclear power plants and radioactive waste disposal concepts in Europe, including Czech Republic. The second subchapter is an overview of microorganisms in a deep geological environment. Similarly, the third subchapter is about the possible effect of microbial processes on deep geological repositories while the fourth subchapter explains the effect of deep geological repository conditions on microbial processes.

The experimental part is the key part of the thesis and is based primarily on published articles or manuscripts under preparation. This part is divided into four chapters and comprises both a methodical description of the experiments and results with comments. In the first chapter, the author characterizes microbial communities present in groundwater and bentonite sources in the Czech Republic by molecular biological tools. Different water sources were analyzed to choose the most relevant to the deep geological repository and to use as inoculum for further studies. Differences in microbial community structure between raw and commercial homogenized bentonite were also described. The second chapter of this experimental part, the author dedicated to explore the survival of indigenous microorganisms in bentonite subjected to ionizing radiation. The third chapter was focused on the corrosion of carbon steel influenced by microorganisms present in groundwater and divided into two subchapters: The first one is corrosion in groundwater and the second one is corrosion in synthetic bentonite pore water inoculated by groundwater. Corrosion in groundwater was performed for eight months while the corrosion in synthetic water determined the microbial corrosion run for twenty-six months. The experimental part is finalized by the fourth chapter that describes the effect of aged cementitious material in suspension on the development of microbial communities under repository relevant conditions.

The last part summarizes the most important findings of the PhD thesis and is presented in the Conclusions. The results summarized demonstrate gradual knowledge development since that time and all of them are relevant to the Czech waste disposal concept. The author has used the multidisciplinary approach combining most advanced molecular genetic techniques together with the specialized microscopic and chemical analyses to determine relative abundance and microbial community structure and estimate the possible microbial effects on the repository-like environment. It should also be noted that microbial activity issues in the geological repositories of radioactive waste is a relatively new scientific topic in the Czech Republic.

My questions to author, comments and recommendations are followings:

- What are mechanisms of microbial resistance to radiations, in particular to the 19,656 Gy total absorbed dose of Gamma radiation? Why did this Gamma radiation not completely eradicate bacteria present in bentonite?
- Author states that: "Bacteria also strongly influenced the corrosion rate of carbon steel compared to samples in sterile conditions". What was the corrosion rate? Have the kinetic parameters of corrosion been determined, in particular the corrosion rate? Has the

corrosion rate been compared with the variety and species composition of sulfate-reducing bacteria?

- Author used a lot of illustrative materials (about 65 figures and 19 tables). Have these figures been modified from other authors or created by the author of the thesis? If the author has used figures from literature papers, does the author have permission and agreement from origin authors?
- A large number of the results obtained are devoted to sulfate-reducing bacteria. Could the author explain the process of dissimilatory sulfate reduction? What species of sulfate-reducing bacteria were detected?
- Author states that: "the presence of concrete, although rich in specific indigenous **microflora**, strongly reduced the relative abundance of bentonite bacteria in studied samples and especially the growth of SRB was limited in the concrete environment". What does it mean: "concrete environment"? Did the author use cultivation methods for isolation of SRB? Which media could be used for this purpose? How can the author explain that the presence of concrete strongly reduced the relative abundance of bentonite bacteria in studied samples and especially the growth of SRB?
- The term "microflora" is not entirely correct and it is outdated, it was used by Antonie van Leeuwenhoek that microscopically evaluated unicellular organisms and called them microflora. From a microbiological point of view, it is more correct to use the term "microbiome" or "microbiota".
- What are the perspectives for future research? What practical recommendations the author can give to other researchers and future studies?

I think that the author achieved the overarching aim of the thesis and an appropriate knowledge about the influence of microbial processes on radioactive waste disposal with the implication for the safe performance of the waste disposal system. The microbial communities present in different groundwater sources and bentonite from the Czech Republic and to select a suitable source that represents the typical environment and microbial community pertinent to the waste repository were characterized in detail. Also, the microbial activities and its community structure in relation to repository relevant conditions including survivability of microorganisms subjected to different levels of radiation, the effect of concrete on microbial propagation, microbially influenced corrosion of metal and effect of radionuclides on the anaerobic microbial community were investigated.

In conclusion, the depth of the issue proves that the author is very well oriented in the field. The author mastered scientific methods of the work, conducts research at the international level, has published a number of studies and has relevant expertise. The above comments, questions and suggestions do not reduce the level and quality of the research and I recommend awarding the author with the PhD title.

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Report of PhD thesis of MSc. Rojina Shrestha "Microbiology in relation to nuclear waste repository safety"

The PhD thesis of MSc. Rojina Shrestha "Microbiology in relation to nuclear waste repository safety" conducted at the Technical University of Liberec presented the following structure: Literature review, Experimental part including results and discussion, and general conclusions.

This PhD thesis is divided into three main parts: Literature overview (introduction and background of the study), Experimental part (microbial activities and their community structure in relation to repository relevant condition), and Conclusions. It is worthy to mention that part of the results obtained within this PhD thesis were conducted within the European Project MIND (www.mind15.eu) supported by Euratom Program.

The Literature review include 4 subchapters dealing with 1) the use of radionuclides in the generation of electricity within Nuclear Power Plants and the worldwide impact of this kind of energy. In addition, the PhD student summarize with updated information about the actual situation and future perspective of nuclear industry; 2) description of the DGR multibarrier systems used for future radioactive wastes disposal and the actual situation in Europe on the building of such disposal systems (e.g. Sweden, Finland, France, Czech Republic; 3) impact of biochemical processes driven by DGR natural microorganisms in the safety of future disposal systems; and 4) effect of different physicochemical parameters of the future DGR (temperature, radiation etc.) in the diversity and activity of microbial populations.
The literature review section is very well written and include all aspects of the Microbiology of Deep geological disposal or radioactive wastes.

The experimental section of this PhD thesis includes the results obtained and is structured in 4 parts. The most positive aspect of this section that theses chapters are already published in high impact journal like and other are in submission/review process.

The first chapter characterized the structure and composition of microbial populations in groundwater and bentonite sources in the Czech Republic using molecular biological tools including qPCR and sequencing. The obtained data would help to predict the impact of the natural microbial population in the safety of future DGR.
The objectives of the study are well defined, the methodology used is suitable to achieve the objectives of the work.

The second chapter described the impact of physicochemical parameters (radiation, bioavailability of nutrients) on the microbial diversity and activity of bentonite under repository relevant conditions. The abundance of nitrate and sulfate reducing bacteria in

irradiated bentonites were quantified using qPCR. In the addition, different nutrients were added to bentonite to investigate their effect in the natural microbial populations which in turn would help to predict the impact of microbial populations in the safety of future DGR.

The third chapter investigated impact of groundwater natural microbial processes in the corrosion of carbon steel as reference material for future DGR metal canister using a multidisciplinary approach combining spectroscopy, microscopy and molecular Biology tools. The results obtained clearly indicate that microbial activity impact on steel corrosion is higher than that caused by abiotic processes. In addition, this work identified SRB and NRB as main bacterial groups involved in the metal corrosion processes.
The results obtained are of great scientific and industrial impact in the field radioactive waste disposal in Czech Republic since it provides new insights in the identification of microbial populations involved in the metal corrosion and in the quantification of metal corrosion rate.
This chapter is well written highlighting the use of a multidisciplinary approach to tackle the address the impact of microbial activity on metal container corrosion for future DGR. In addition, the results of this chapter are published in 2 scientific journals.

The fourth chapter reported the impact of aged cementitious material in suspension on the structure and composition of microbial populations in presence of bentonite under repository relevant conditions. The results of this chapter are included in a manuscript for its publication in Environmental Microbiology Journal.

Conclusion section which include the main results obtained within this PhD Thesis**.**

The PhD thesis is very well written and structured. It addresses different issues related to the impact of microbial processes in the safety of different compartments of future DGR including, bentonite, metal container, concrete. The results obtained will be of great interest for scientific community working in this field and also for nuclear industry.

THEREFORE, I would like to recommend the defense of this PhD thesis in its actual state.

Sincerley,

Mohamed L. Merroun