TECHNICAL UNIVERSITY OF LIBEREC
Faculty of Mechatronics, Informatics
and Interdisciplinary Studies

Thesis topic: Microbiology in relation to nuclear waste repository safety

Study program: P3901 / Applied sciences in engineering

Field of study: 3901V055 Applied sciences in engineering

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Abstract

The globally accepted strategy for the management and treatment of high level and long-lived radioactive waste is to dispose the waste in a deep and stable geological formation. The physicochemical aspects have been carefully studied to ensure the long-term safety of the repository, while the influence of microorganisms was until recently rather underestimated, although it is well known that microorganisms can survive and propagate under environmental conditions expected in nuclear waste repositories. Anaerobic microorganisms with diverse types of metabolism present in the groundwater or buffer material may influence and compromise the long-term safety performance of the repository. This thesis, therefore, intends to improve the knowledge about the influence of microbial processes on radioactive waste disposal. Particularly microbial activity and survivability under different repository relevant conditions were studied with a focus on 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. Moreover, microbially influenced corrosion of carbon steel was studied under anaerobic conditions. All the experiments except the radiation one were carried out under a strictly anaerobic atmosphere in an argon-purged glove box with gaseous oxygen concentration lower than 1 ppm. The results were obtained employing a multidisciplinary approach combining advanced microscopy methods such as electron microscopy or electrochemical impedance spectroscopy analysis with molecular biology-based methods such as NGS and qPCR. Chemical analyses were performed using ion-chromatography or spectroscopy methods. Anaerobic microorganisms including sulfate, iron, and nitrate-reducing bacteria were mostly detected in the samples. Application of 19,656 Gy total absorbed dose of Gama radiation at the constant dose rate of 13 Gy/hr did not completely eradicate bacteria present in bentonite. Bacteria also strongly influenced the corrosion rate of carbon steel comparing to samples in sterile conditions. Particularly, abundance of

Methyloversatilis population positively correlated with corrosion rates. The presence of mackinawite, a corrosion product usually attributed to the activity of sulfate-reducing bacteria, was confirmed by Raman spectroscopy. Furthermore, 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. All these effects might have a negative impact on repository safety and should be further studied in following laboratory experiments and in-situ conditions in underground research laboratories.

Keywords: Microbial activity, Microbially influenced corrosion, Radioactive waste, Geological repository, Groundwater, Bentonite, Concrete

Abstrakt

V současnosti je všeobecně přijímaná strategie managementu a ukládání radioaktivního odpadu v úložišti hluboko v geologickém masivu. Zatímco fyzikálně-chemické aspekty úložiště jsou již desetiletí pečlivě studované s cílem zajistit jeho dlouhodobou bezpečnost, vliv mikroorganizmů byl ještě nedávno podceňovaný, i když je známo, že mikroorganizmy dokáží přežít a rozmnožovat se i v podmínkách úložiště. Metabolicky různorodé anaerobní mikroorganizmy, které jsou přítomné v podzemní vodě i bentonitech, mohou negativně ovlivňovat dlouhodobou bezpečnost úložiště. Tato disertace je proto zaměřená na studium vlivu mikrobiálních procesů v úložišti radioaktivních odpadů. Konkrétně je zaměřená na mikrobiální aktivitu a životaschopnost v simulovaných podmínkám, které mohou nastat v úložišti. Byl studován vliv různých dávek radioaktivního záření, vývoj mikrobiálního společenstva při různých koncentracích živin a interakce mikroorganizmů s bentonitem a betonem. Dále byla studovaná mikrobiálně ovlivněná koroze uhlíkové oceli v anaerobních podmínkách. Všechny experimenty, s výjimkou ozařovacího, byly provedené v anaerobním boxu s koncentrací plynného kyslíku do 1 ppm. Výsledky byly získány pomocí multidisciplinárního přístupu kombinujícího elektronovou mikroskopii, elektrochemickou impedanční spektroskopii s molekulárně biologickými metodami NGS sekvenování a kvantitativní PCR. Chemické analýzy byly provedené pomocí iontové chromatografie a spektroskopie. Nejčastěji byly detekovány anaerobní mikroorganizmy zahrnující sírany, železo a dusičnany redukující bakterie. Gama záření o celkové dávce 19656 Gy a konstantním dávkovém příkonu 13 Gy/h, nedokázalo úplně zničit bakterie v bentonitu. Bakterie také značně ovlivnily rychlost koroze uhlíkové oceli v porovnání se vzorky, které byly inkubované ve sterilních podmínkách. Například hustota populace bakterie rodu *Methyloversatilis* pozitivně korelovala s rychlostí koroze. Byla také potvrzena přítomnost mackinawitu, pravděpodobného produktu koroze indukované síran redukujícími bakteriemi. Dále bylo ukázáno, že přítomnost betonu, ačkoli obsahuje bohatou přirozenou mikroflóru, významným způsobem snižovala celkové početnosti přirozených bentonitových bakterií ve studovaných vzorcích a obzvláště potlačovala růst síran redukujících bakterií. Všechny tyto jevy mohou mít negativní efekt na bezpečnost úložiště a měly by proto být dále studovány in-situ v podzemních výzkumných laboratořích.

Klíčová slova: mikrobiální aktivita, mikrobiálně ovlivněná koroze, radioaktivní odpad, geologické úložiště, podzemní voda, bentonit, beton

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Introduction

1 Nuclear energy and spent fuel deposition

The availability of sustainable, reliable, and affordable sources of energy is important for economic growth and stability. Over the past 50 years, nuclear reactors have been established as reliable and secure sources for generating clean and economical electrical energy (Zinkle and Was, 2013). Radioactive metals such as uranium-235 and plutonium-239 are used as a nuclear fuel in nuclear power plants to produce energy. More than 441 nuclear reactors are in operation worldwide, currently providing 10.5% of electrical power generating 390 GW_e of electricity ("Reactor Database Global Dashboard - World Nuclear Association," n.d.). Nuclear energy is an alternative energy to fossil fuels so it helps to reduce greenhouse gas emissions and therefore, is viewed as an attempt to deal with global warming (Menyah and Wolde-Rufael, 2010). Beside affordable electricity, nuclear energy assists in many medical applications including nuclear magnetic resonance imaging technology (Ruppert et al., 2004) and nuclear medicine (Jankowski et al., 2003).

However, as a result of nuclear energy generation, a highly radioactive waste known as spent nuclear fuel (SNF) is produced. Radioactive waste should be managed securely and responsibly to ensure safety to the public, protection to the environment, and security from any accidental event to avoid contamination in the biosphere. Apart from NPPs, medicine and hospitals, scientific research work, industry, and defense military work represent other sources of radioactive waste. High and long-lived waste (HLLW) has a finite radiotoxic lifetime and it decays progressively in a natural way. Consequently, it should be disposed of in such a way that it does not further require any continued institutional control. Many countries around the globe have accepted the strategy of disposal of waste in deep geological formations. This thesis is focused on the situation in European countries with special attention paid to the planned deep geological repository (DGR) in the Czech

Republic. The major purpose of the DGR is to separate the SNF or radioactive waste material from the environment to avert environmental contamination. This strategy implicates the waste material enclosed in a metal container surrounded by highly compacted bentonite buffer embedded in stable host rock at the depth of about 500 m (Masurat et al., 2010). Bentonite, a clay mineral, is planned to be used by many countries as a part of an engineered barrier system for the disposal of HLW in deep geological formation (Stroes-Gascoyne et al., 2010). Bentonite provides mechanical protection to the waste container (reduced the effect in the case of rock displacement) (Masurat et al., 2010) and serves as a natural barrier for the migration of radionuclides to the environment. It should also prevent microbial activity at the canister surface. Crushed rock, concrete, and bentonite pellets are used for the backfill and sealing of the HLW repository. The granite has been selected as host rock by countries like Sweden, Finland (Pettersson and Loennerberg, 2008) and also the Czech Republic while clay formations will be used as natural barriers in countries like Belgium, France, and Switzerland (Delage et al., 2010)

2 Deep subsurface ecosystem

A deep geological environment is dark and anaerobic. In such an environment, microorganisms employ an anaerobic respiratory process that uses nitrate, manganese, iron, and sulfate as terminal electron acceptors instead of oxygen for energy generation. The electrons necessary for the reduction of electron acceptors in respiratory pathways are taken from oxidized substances known as electron donors. Various organic substances or molecular hydrogen are the two most important electron donors in deep subsurface anaerobic ecosystems (Madigan et al., 2015). Additionally, bentonite is not a sterile material. It comprises diverse microbial communities including spore-forming microorganisms. In the same way, porous rock such as granite, limestone, and gravel present deep down in the ground possess innumerable small spaces that can hold water and host-microbial consortia.

3 Effect of microbial processes on the deep geological repository

Microorganisms could cause the failure of an effective disposal system leading to the release and transportation of radionuclides to the environment. Microbial processes might result in numerous problems such as dissolution, mineralization, of bentonite buffer, microbially influenced corrosion (MIC) of waste containers, gas production, pressure change, and sorption, and migration of radionuclides (Mulligan et al., 2009; Stroes-Gascoyne, 2010). Only an intact metal waste container is the absolute barrier of radionuclides transportation in the disposal system because both the bentonite buffer and host rock are water-conducting is (Masurat et al., 2010b). However, corrosion of waste containers can be accelerated by the activity of sulfate-reducing bacteria (SRB) and hence, referred to as MIC (Zhou, 2012).

4 Effect of geological repository conditions on microbial processes

The environment of DGR is extreme in terms of temperature, pressure, density, radiation, redox conditions and pH. Microorganisms are selected according to their metabolic abilities and only those who possess the ability to survive will survive. Bentonite has high water affinity and swells when come in contact with the groundwater. The swelling pressure of bentonite is related to its density if the swelling is space-restricted because of a mechanical hindrance (Masurat et al., 2010b) and it determines the amount of water that bentonite will take for being saturated (Karnland, 1997). Conversely, an increase in swelling pressure corresponds to the decrease in water activity (a_w), which can be together with the space restriction and diffusion limitation, a limiting factor for the microbial processes (Masurat et al., 2010b; Motamedi et al., 1996). Radioactive waste can generate heat and radiation even after the fission process has stopped. Ionizing radiation induces various changes in cells directly or indirectly leading to cell death (Benetti, 2015). Likewise, the high alkaline pH of the concrete (used as backfilling and sealing of the repository) creates a relatively non-hostile environment for the microbial activity.

Thesis Aims

The overarching aim of this thesis was to improve and develop safety case knowledge about the influence of microbial processes on radioactive waste disposal with the implication for the safe performance of the waste disposal system.

The first objective was to characterize the microbial communities present in different underground water 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.

The second objective was to investigate the microbial activities and its community structure in relation to the 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.

Methods

For the strict anaerobic condition, the Argon-purged glove box (gaseous oxygen concentration < 1 ppm volume) (Jacomex GP, France) was used to carry out the experimental part in collaboration with Centrum výzkumu Řež s. r. o.,(CVŘ) Prague. Microbial analysis was carried by me in the Technical university of Liberec (TUL).

Any water samples collected from underground sources or from the experiment were concentrated by filtration through 0.22 µm GV Durapore® filter membrane (Germany). Surface biofilms were collected by sterile swab sticks, FLOQSwabs (COPAN Diagnostics Inc, USA) . When processing bentonite samples, 50 ml of bentonite suspension was centrifuged at 11500 × g for 15 minutes. The pellet was weighed and used for DNA extraction. The membrane filter containing bacterial biomass after filtration and the bentonite pellet after centrifugation were stored under -80 °C until the period of DNA extraction

Bacterial DNA from water samples orbiofilms were isolated according to the manufacturer instruction using a commercial kit, PowerWater® DNA Isolation Kit, from MO BIO. Similarly, DNeasy® PowerMax® Soil Kit from QIAGEN was used for extraction of DNA from bentonite pellets according to manufacturer's protocol. The isolated DNA for bentonite samples was further purified and concentrated by the Zymo research kit. Quantification of extracted genomic DNA was performed by using a Qubit 2.0 fluorometer (Life Technologies, MA, USA),

Quantitative Polymerase chain reaction (qPCR) was used to describe the relative changes in bacterial abundance in the samples. The 16S rRNA gene was used as a molecular marker for the identification of total bacterial biomass. Similarly, functional markers such as *apsA* and *dsrA* gene were used for identifying sulfate-reducing bacteria (SRB) while *nirK, nirS* and *nos_z* genes were used for detecting denitrifying bacteria. In addition, specific region of 16S rRNA gene was amplified for iron-reducing bacteria (IRB). All qPCR reactions were performed on LightCycler ® 480 Instrument (Roche Biochemicals, USA). The purity of the amplified fragment was determined through observation of a single melting peak. Crossing point (Cq) values were obtained using the ‘second derivative maximum’ method included in the Light Cycler® 480 Software. Relative quantification of each parameter was expressed as a fold change between two states (the sample at given sampling time and a sampling time of zero) by using the delta Cq method. The measured Cq values were normalized by the sample mass used for DNA extraction prior to calculations.

The next-generation sequencing (NGS) was used to study microbial diversity in groundwater and bentonite samples. Two consecutive PCR reactions per sample were performed during library preparation to amplify DNA from the V4 region of 16S rDNA. The PCR products were purified using the Agencourt Ampure XP system (Beckman Coulter, Brea, USA), and the concentration of the purified PCR products was measured with a Qubit 2.0 fluorometer (Life Technologies, USA). The barcode-tagged amplicons from different samples were then mixed in equimolar concentrations. Sequencing of the amplicons was performed on an Ion Torrent PGM (Thermo Fisher Scientific, USA) using the Ion PGM Hi-Q Sequencing Kit with the Ion 314 Chip following the manufacturer's instructions (Thermo Fisher Scientific).

Raw reads were split into particular samples by Mothur software (Schloss et al., 2009). The split samples were subsequently processed by the DADA2 software package (Callahan et al., 2016). Low quality and short reads were removed as well as chimeric sequences. Taxonomy classification by the DADA2 package used SILVA database (version 13, www.arb-silva.de). DADA2 output was transformed into a Phyloseq object in R and subsequent bioinformatics analyses were performed in the R software using the Phyloseq library (McMurdie and Holmes, 2013).

Surface analyses of various samples were performed using a LYRA3 scanning electron microscope (Tescan, Czech Republic) at CVŘ. The SEM was equipped with secondary electron detectors (SE and In-beam SE mode) and back-scattered electrons (In-beam BSE mode) at 5 kV accelerating voltage. Likewise, Raman analysis for determination of corrosion product was performed at CVŘ using a Thermo Scientific DXR2xi spectrometer with a 532 nm laser line coupled with an optical microscope using a 10x magnification objective lens.

The pH of the sample was measured by SenTix 980 combined IDS electrode with liquid electrolyte (WTW, Czech Republic). Redox potential (Eh) was measured by SenTix ORP-T 900 Pt – Ag/AgCl IDS redox electrode with liquid electrolyte (WTW, Czech Republic), and the values were recalculated and reported versus the potential of the standard hydrogen electrode.

For the chemical analysis, supernatants of bentonite suspension and filtrates of water samples were analyzed by ion spectroscopy to determine the concentrations of sulfate, nitrate, and dissolved organic carbon (DOC). The concentration of each compound was determined using Dionex ICS 90 chromatograph (ThermoFisher Scientific, USA) with 8 mM K2CO3 a 1 mM KHCO3 as the mobile phase in a Dionex IonPac AS14A column. The flow rate of the mobile phase was 1 ml/min and 10 µl of the sample was always injected. The calcium content from the dry mass of bentonite, concrete, and their mixture was measured by inductively coupled plasma optical emission spectrometry (ICP-OES). The sample was dissolved in nitric acid and diluted to the final volume in deionized water before the measurement.

I. Characterization of microbial communities present in groundwater sources and bentonite in the Czech Republic by molecular biological tools.

Microbial characterization of two different groundwater sources from Bukov Underground research facility (Bukov URF), and Josef Underground research Centre (Josef URC) (see *Figure 1*) and Czech bentonites (see *Figure 2*) were performed using molecular biology. This study intended to investigate the microbial community present in different groundwater sources in the Czech Republic and select the best source that reciprocates the microbial community present in a repository type environment for further study. In addition to groundwater, biofilms near water sources were also studied. This study also aimed to assess the microbial diversity naturally occurring in the Czech bentonite samples and to study differences in the microbial consortia between raw and homogenized bentonite to understand how the process of homogenization influences the structure of the microbial community.

Figure 1: Images A and B represents Bukov URF while C and D represent Josef URC. A) Water source from BK06 B) Biofilm near-source BK23, C) Underground tunnel D) Main tap of source VITA water under 2.5 bar from Josef URC

Water from Bukov URF was collected from seven different sources together with two biofilms. The result demonstrated a strong anthropogenic impact in almost all of these sources. Further, two sources were analyzed from Josef URC where one of the water sources named VITA was dominated by anaerobic microorganisms, especially sulfate –reducing bacteria(SRB) such as *Desulfobulbaceae*, *Desulfomicrobium*, *Desulfovibrio* and *Desulfovibrio* that is expected to exist typically in the environments similar to DGR and may accelerate corrosion of the metal container. Therefore, VITA underground water source was selected to be used as an inoculum for subsequent ex-situ laboratory studies of microbial processes in DGR stimulated conditions.

Figure 2: Homogenized bentonite on the left and raw (unhomogenized) bentonite on the right.

The microbial communities present in homogenized and raw (unhomogenized) bentonite samples from Černý vrch were very similar in terms of their OTU compositions, but the detected OTUs varied in quantity. The similarity of the microbial communities obtained from two bentonite samples suggests that the structure of the bacterial community was not much affected by the homogenization process. Microorganisms such as *Thiobacillus, Gallionella, Acidobcateria,* and *Nitrosomonas* capable of utilizing sulfur, iron, and nitrite as electron donors and *Rhodobacteraceae, Brevundimonas* and *Novosphingobium* capable of utilizing nitrate as electron acceptor were present in both bentonite samples. The species of these genera can enhance gas production as a part of their metabolic activity resulting in pressure generation and eventually, crack or fracture of the host rock. These results suggested that the mixing of underground water and bentonite may influence the development of different microbial communities.

II. Survival of indigenous microorganisms in bentonite subjected to radiation and effect of anaerobic condition on the evolution of microbial ecosystem in bentonite

After the closure of the repository, harsh and extreme environment will gradually qevolve. High compaction, desiccation, temperature, and radiation are expected to prevail in the DGR. However, some microorganisms show extreme adaptability to various unpleasant environmental conditions, and the conditions in the early stages post-deposition thus do not need to have so devastating effect on microbial survivability as previously expected. Ionizing radiation has a significant effect on microorganisms as it induces various changes in cells directly or indirectly.

This study intended to improve the knowledge about the effect of Gama radiation on the indigenous microbial community in bentonite and groundwater under conditions similar to the repository and the evolution of the microbial ecosystem in bentonite under anaerobic conditions. To stimulate the condition which is expected in the repository in reasonably long experimental time, the radiation of 19,656 Gy absorbed dose at the constant dose rate of 13 Gy/hr was used. Contrary to expectation, we were unable to maintain the anaerobic atmosphere during the irradiation and the irradiated samples were influenced by the presence of oxygen. Nevertheless, the application of 19,656 Gy absorbed dose of Gama radiation at the constant dose rate 13 Gy/hr did not manage to completely eradicate present bacteria, but it caused the visible decline in total microbial biomass in time (*Figure 3*) and caused slight changes in the microbial community structure. However, both of these effects could be also caused by the presence of oxygen and resulting limitation by the available electron donors. On the other in non-irradiated samples kept in anaerobic box it was observed that anaerobic conditions enhanced the microbial activity of indigenous microorganisms in BaM bentonite. Gradual changes in microbial community composition and their metabolic profile were observed mirroring the prevailing conditions in the samples. Anaerobic indigenous microbial community in bentonite generally evolved from nitrate reducers through iron reducers to the sulfate reducers. No effect of added nutrients on microbial composition within studied samples was observed, but the overall microbial abundance was higher in samples with added nutrients. The results further showed that iron and sulfate reduction are important processes under anaerobic conditions which can possibly affect performance and safety of the repository causing illitization of bentonite buffer and corrosion of waste metal containers. Interestingly, gram-negative non-spore-forming microorganisms dominated the aerobic irradiated samples although spore-formers are generally supposed to be more radiation-resistant whereas anaerobic samples were dominated by Gram-positive spore-forming bacteria generally more resistant to radiation.

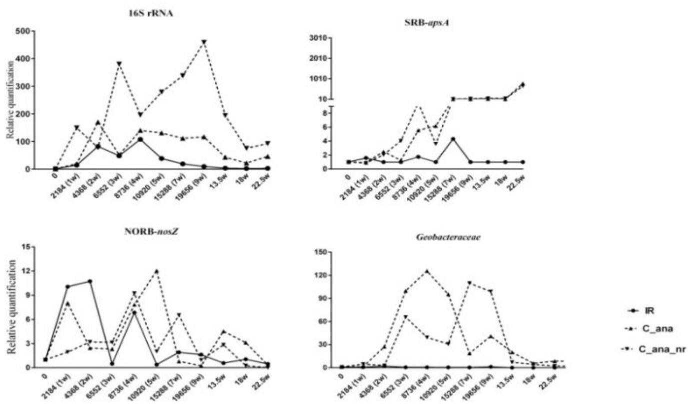

Figure 3: Relative quantification of changes in microbial abundance in irradiated and anaerobic samples. IR- Irradiated samples, C_ana – Anaerobic sample without nutrient, C_anocy_nr – Anaerobic sample with nutrient and w – week.

For a better understanding of the effect of irradiation on microbial community in bentonite under repository relevant conditions, irradiation experiments performed under a strictly anaerobic condition with even higher total absorbed dose are needed**.**

III. Microbially influenced corrosion of carbon steel under repository relevant condition

An intact metal waste container is the absolute barrier for radionuclide transportation in the waste repository is because both the bentonite buffer and host rock are water-conducting. Carbon steel is considered as a candidate container material in the Czech Republic as well as in several other European countries. The safe performance of carbon steel containers may be, however, influenced by corrosion accelerated by the microorganism. The formation of biofilm on the surface of a metal container is the initial step of MIC. Two major mechanisms have been involved in weakening waste container durability i.e., by direct uptake of electrons from the metal surface by microbial cells or by the production of corrosive metabolites. Effective uptake of electrons from the metal can trigger a cathodic reaction, and thus corrosion while corrosive metabolites are oxidants, such as protons, organic acids, and sulfides, which can attack metal and stimulate cathodic reactions. The effects of biofilm formation on a metal surface may range from the acceleration of corrosion to complete inhibition of corrosion. Microorganisms may accelerate or slow down corrosion by changing the nature or kinetics of rate-controlling reactions or processes.

Two different studies were conducted to describe MIC of carbon steel under repository relevant conditions in order to determine and understand the contribution of biocorrosion to overall corrosion processes, and to investigate microbial community composition responsible for corrosion and formation of biofilm. The first 8-month experiment was about MIC of carbon steel in the presence of anaerobic SRB naturally present in VITA groundwater from Josef URC while the second 26-month experiment comprised VITA groundwater in SBPOW in 1:10 ratio.

In both experiments, the steel corrosion rates were found higher in biotic (non-sterile) samples than abiotic (sterile control) samples indicating corrosion caused by microbial activity. Under strictly anaerobic conditions, exposure of carbon steel to natural VITA groundwater and with inoculation of VITA groundwater into SBPOW resulted in the formation of a biofilm and corrosion product layers. However, the microbial communities responsible to carry out corrosion of carbon steel were different. Molecular biological analysis of both water and biofilm indicated the dominance of *Desulfomicrobium* and *Desulfovibrio* spp. (both SRB) in the experiment with only VITA water (*Figure* ***4***) whereas the experiment with inoculation of VITA water in SBPOW demonstrated the dominance of different populations of nitrate reducers such as *Methyloversatilis, Brevundimonas,* and *Pseudomonas* *(Figure* ***5****).* The dominance of NRB was caused by the chemical composition of SBPOW which mimics the Czech BaM bentonite leachate, rich in nitrates that are thermodynamically favorable terminal electron acceptors to NRB. The formation of a biofilm on the carbon steel surface accelerated the corrosion process. .

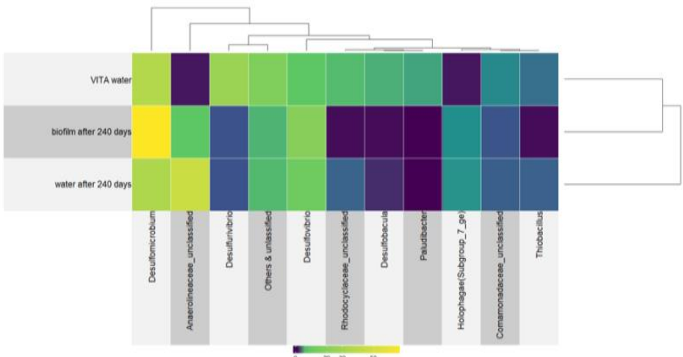

Figure 4: Heat map showing the results of next-generation amplicon sequencing (only taxa with abundance over 1% visualized).

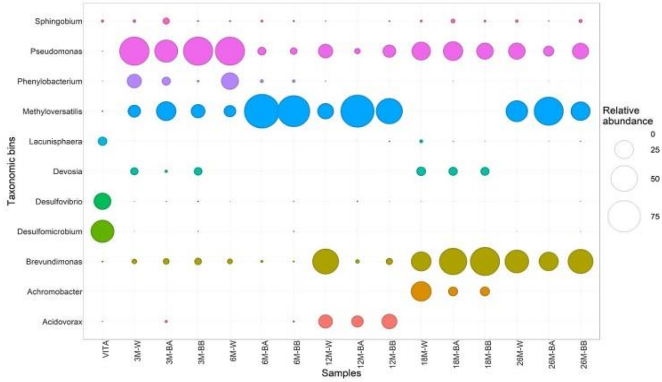

Figure 5: Result of 16S rRNA sequencing showing genera with the mean of relative abundance over 1%. W- Represents water sample (SBPOW inoculated with VITA), B – is biofilm where A and B are replicates.

Moreover, the presence of mackinawite, a corrosion product usually attributed to SRB activity was confirmed by Raman spectroscopy in both experiments. Detection of sulfur compounds by SEM/EDS in the first experiment provided evidence of the reduction of sulfates to sulfides by SRB metabolic activity. The carbon steel polarization resistance decreased by a factor of 2 after 8 months in the presence of SRB, indicating a higher corrosion rate when compared with the sterile sample. Similarly, weight loss measurement determined in the second experiment with SBPOW showed that the average corrosion rate of carbon steel in the sterile control sample and the sample with microorganisms was 1.28 µm/yr and 3.81 µm/yr, respectively. Interestingly, a high abundance of *Methyloversatilis* positively correlates well with the changes in corrosion rates.

These results are relevant for the Czech radioactive waste disposal concept and show the necessity to consider NRB in addition to SRB as a potential threat for bio-corrosion of the waste container since the surrounding environment might contain high concentrations of nitrates due to presence of bentonite buffer. Future studies should concentrate on this phenomenon.

IV. Effect of concrete on microbial ecosystem under repository relevant conditions

Concrete (cementitious material) is used not only for the encapsulation of low and intermediate level waste but will be an important part of engineering barriers of different HLW concepts. In both cases, the concrete will come into contact both with the bentonite or other clays and the underground water. The pH of the concrete is high due to high gypsum and soluble alkali content, which makes it a relatively unhostile environment for the microbial activity. Nevertheless, there are many alkaliphilic microorganisms capable of surviving in high pH environments. Moreover, over the period of waste disposal, the pH of the alkaline concrete is expected to decline gradually by the carbonation and by neutralization with the microbially produced mineral or organic acids consequently resulting in biodegradation of concrete. However, the microbial activity might have not only a detrimental effect but also a beneficial one (self-sealing and healing of crack) on concrete stability. Although the knowledge about the microbial effect on the concrete properties is relatively broad, little is known about the effect of concrete environment on the indigenous microorganisms in the surrounding DGR environment although it represents a natural source for the future microbial activity influencing the concrete. Our study aimed to develop knowledge about the effect of concrete on indigenous microbial ecosystems under repository relevant conditions.

This study was conducted under the strictly anaerobic conditions for two months using samples prepared from aged concrete, Czech BaM bentonite, and anaerobic VITA ground water from Josef URC (Czech Republic) and including several controls. Bentonite samples with concrete had a higher pH than the bentonite samples without concrete. Chemical analysis revealed that available nitrate was consumed fast by the microbial metabolism in all the samples and sulfate, which was especially rich in concrete containing samples, has not been used as a preferential electron acceptor yet. The results also suggested that the growth of SRB might be limited in the concrete environment, although longer experimental times would be needed to address this particular question. Moreover, the presence of concrete has strongly reduced the relative abundance of bacteria detected by the qPCR compared to the bentonite control samples (see *Figure 6*). Hence, the presence of concrete generally had a negative effect on overall microbial activity in studied experiemental system.

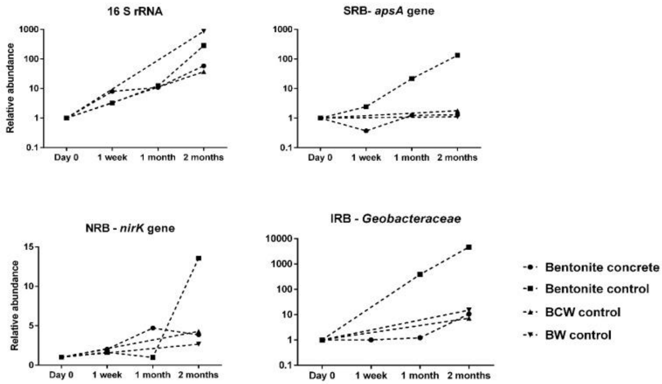

Figure 6: Relative quantification of changes in microbial abundance Bentonite concrete – concrete, bentonite and VITA water, bentonite control – bentonite and vita water without concrete. BCW control - bentonite, concrete and sterile water, and BW control - bentonite and sterile water. Only the Cq values above the detection limit were used for the calculations. Only those genes which have a remarkable change in relative value are shown in the figure. Relative values are presented in the log scale except for the nirK gene.

Nevertheless, several bacterial genera such as *Bacillus, Dethiobacter, Anaerosolibacter Promicromonospora, Pseudonocardia, Pedobacter, Paeniglutamicibacter, Devosia, Sphingobium, Pseudarthrobacter* or *Nocardioides* were able to proliferate in the concrete environment and were even specialized in this environment. On the other hand, genera like *Massilia, Citrifermentans (Geobacter), Paenobacillus,* or *Lacunisphaera* were probably limited by alkaline pH and were dominant in bentonite control samples. Interestingly, some genera like *Thermincola* and *Pseudomonas* were found to successfully proliferate in both environmental conditions.

Most of the bacteria detected in our samples might have a negative impact on repository safety. They can accelerate canister corrosion (thiosulfate and sulfate reducers), mineralization and dissolution of bentonite (iron reducers), acid production enhancing MID of concrete or they can generate gasses (e.g. nitrate reducers) in the repository environment which may result in the fracture of concrete or host rock leading to release of radionucleotide in the event of waste container failure. Nevertheless, some species of *Bacillus* (nitrate reducer) are capable of hydrolysis of urea and precipitate $CaCO_3$ that heals and seals the crack on the concrete biologically. Although this genus was common in our concrete containing samples and several other NRB genera should be also able to precipitate $CaCO_3$ as the byproduct of their metabolic activity, we have not detected any signs of the ongoing biomineralization processes in our samples. Therefore, further research is necessary to estimate the possible biomineralization or biodegradation activity of indigenous microorganisms in cementitious materials that might be important for repository safety.

THESIS CONCLUSIONS

The problematics of microbial activity in the geological repositories of radioactive waste is a relatively new scientific topic in the Czech Republic. Institute for Nanomaterials, Advanced Technologies and Innovation, which is the only one dealing with this topic in the Czech Republic, has just started to participate on the repository research when I began my Ph.D. studies. The results summarized in my thesis thus demonstrate gradual knowledge development since that time and all of them are relevant to the Czech waste disposal concept. I have 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. The main benefits of my thesis, therefore, are in improving the knowledge necessary for the safety assessment of the future repository program in terms of expected microbial processes, which might help to establish guidelines for the long-term safety of the HLW repository in the future.

Concerning microbial characterization, it was found that:

- VITA groundwater source from Josef URC was selected to be the most suitable for the studies on microbial activity at repository relevant conditions, because this source was dominated by anaerobic microorganisms, primarily sulfate reducers such as *Desulfobulbaceae, Desulfomicrobium, Desulfovibrio* and *Desulfovibrio.* These genera are expected to exist in the reducing conditions in repository environment and may accelerate the corrosion of waste containers. VITA water source was also rich in available water quantity compared to other sources.
- Water from Bukov URC was collected from seven different sources plus two biofilms and the results demonstrated a strong anthropogenic impact in almost all the sources.

- Microbial communities present in homogenized and raw (unhomogenized) bentonite samples from Černý vrch were very similar in terms of their OTU compositions, but the detected OTUs varied in quantity. Microorganisms such as *Thiobacillus, Gallionella, Acidobcateria,* and *Nitrosomonas* capable of utilizing sulfur, iron, and nitrite as electron donors and *Rhodobacteraceae, Brevundimonas* and *Novosphingobium* capable of utilizing nitrate as electron acceptor were present in both bentonite samples.
- The similarity of the microbial communities obtained from two bentonite samples suggest that the structure of the bacterial community was not much affected by the commercial homogenization process.

Concerning microbial activities and survivability under repository relevant conditions, it was found that:

- Anaerobic condition enhanced the microbial activity of indigenous microorganisms in Czech BaM bentonite. Gradual change in microbial community composition of bentonite and VITA water determined by the prevailing conditions was observed. Indigenous anaerobic microbial community in bentonite generally evolved from the nitrate reducers through the iron reducers to the sulfate reducers. Iron and sulfate reduction are important processes relevant for DGR long-term stability because they can change hydraulic conductivity and alter permeability and porosity of bentonite (due to illitization caused by IRB) or they can promote corrosion of waste metal container (caused by SRB or NRB) which can enhance the release the radionuclides to the biosphere.
- Aerobic application of 19,656 Gy total absorbed dose of Gama radiation at the constant dose rate 13 Gy/hr did not completely eradicate bacteria present in bentonite, but it caused the decline in total microbial biomass in time.
- Gram-negative non-spore-forming microorganisms dominated the aerobic irradiated samples, although spore-formers are generally supposed to be more radiation-resistant. Anaerobic samples were dominated by Gram-positive spore-forming bacteria.

Concerning the effect of microorganisms on the waste container corrosion, it was observed that:

- Corrosion rates were found higher in biotic samples than abiotic samples signifying the corrosion caused by microbial activity.
- Exposure of carbon steel with (i) only natural VITA groundwater and (ii) VITA groundwater in SBPOW (in 1:9 ratio) resulted in both cases in the formation of a biofilm and corrosion product layers indicating MIC. Biofilm on the steel surface-enhanced and localized the corrosion process. The dominance of SRB (*Desulfomicrobium* and *Desulfovibrio* spp.) in the corrosion experiment was detected only with VITA groundwater and the dominance of NRBs *(Methyloversatilis, Brevundimonas,* and *Pseudomonas*) with inoculation of VITA water in SBPOW was observed.
- Presence of mackinawite, a corrosion product usually attributed to SRB activity was confirmed by Raman spectroscopy in both experiments.
- In the experiment run in only VITA water, the carbon steel polarization resistance decreased by a factor of 2 indicating a higher corrosion rate than the sterile control sample. Likewise, the weight loss measurement technique in SBPOW inoculated by VITA water (9:1) revealed that the average corrosion rate on carbon steel for the sterile control sample and the sample with microorganisms was 1.28 µm/yr and 3.81µm/yr, respectively.
- In experiment with SBPOW inoculated by VITA water, a high abundance of *Methyloversatilis* positively correlated with the corrosion rates. This corrosion experiment confirmed that NRB in addition to SRB represent a potential threat for bio-corrosion of the waste container.

Concerning the effect of concrete on the bentonite and indigenous groundwater microflora, it was shown:

- The presence of concrete had a negative effect on bacterial activity and strongly reduced relative abundance of bacteria in all studied samples.

- The growth of SRB might be limited in the concrete environment, although longer experimental times would be needed to address this phenomenon
- Genera such as *Bacillus, Dethiobacter,* or *Anaerosolibacter* were able to proliferate in the concrete environment and were even specialized in this environment while the genera like *Massilia, Citrifermentans (Geobacter)* or *Lacunisphaera* were probably suppressed by concrete, but were dominant in bentonite control samples. Interestingly, some genera like *Thermincola* and *Pseudomonas* were found to successfully proliferate in both conditions. Most of these bacteria might have a negative impact on repository safety causing MIC of a metal container, MID of reinforced concrete containers, alteration of bentonite structure or gas production.

All my experiments revealed, that the microbes might play a very important role in the DGR-like environment under certain conditions, and microbiology in relation to the nuclear waste repository safety is thus highly relevant topic. Most of my research was conducted under repository-simulating laboratory conditions, which was necessary to gather basic knowledge and also laboratory skills with this very demanding field of expertise. Our future research should rely on these preliminary results and extend them in the following laboratory and in-situ projects.

Further laboratory research is necessary to estimate the possible effect of microorganisms on the alteration of bentonite or biomineralization or biodegradation activity of indigenous microorganisms in cementitious materials that might be important for DGR stability. On the other hand, long-term *in-situ* studies are needed to better understand the fundamental mechanisms of MIC in deep geological environments and to offer a realistic assessment of the contribution of MIC to the overall corrosion of metal containers.

The list of publications

Published papers:

- Černoušek, T., **Shrestha, R.,** Kovářová, H., Špánek, R., Ševců, A., Sihelská, K., Kokinda, J., Stoulil, J., Steinová, J., 2019. Microbially influenced corrosion of carbon steel in the presence of anaerobic sulfate-reducing bacteria. Corrosion Engineering, Science and Technology, 55, 127–137, https://doi.org/10.1080/1478422X.2019.1700642

- **Shrestha, R.,** Steinová, J., Ševců, A., Černoušek, T., Kovářová, H.,Jakub, K., Romana, H., 2018.The effect of Caesium ions on a natural anaerobic microbial community, Waste Forum, Prague, Czech Environment Management Center, 1, p. 140-145, ISSN: 18040195

- Polívka, P., Váňová, H., Černoušek, T., Hrabák, P., Steinová, J., **Shrestha, R.,** 2017. Influence of microorganisms on degradation products of gamma-irradiated organic radioactive wastes–preliminary results. Waste Forum, Prague, Czech Environment Management Center, ISSN: 18040195

Book chapter:

- Černoušek, T., Ševců, A., **Shrestha, R.,** Steinová, J., Kokinda, J., Vizelková, K., 2020. “Microbially influenced corrosion of container material.” In: The Microbiology of Nuclear Waste Disposal, 1st edition. Edited by Lloyd, J, and Cherkouk, A. Elsevier. ISBN: 9780128186954

Project reports:

- Tomas, C., Jakub, K., **Shrestha**, **R.,** Sihelská, K., Ševců, A., 2019. Anaerobic microbial corrosion of canister material, 62 pages, Deliverable report. ISSN: 1478-422X

- Ševců, A., **Shrestha, R.,** Černík, M., Špaček, P., Stoulil, J., Steinová, J., Dobrev, D., 2015. Microbial corrosion under deep storage conditions for the concept of steel UOS - compacted bentonite, Research report

Extended abstract in conference proceeding:

- **Shrestha, R.,** Steinová, J., Falteisek, L., Vlková, D., Ševců, A., 2016. Characterization of microbial communities in raw and homogenized bentonite samples. Proceedings of the 2nd Petrus-OPERA Ph.D. and early-stage researcher conference, Delft University of Technology, 75-77, ISBN/EAN: 978-94-6186-669-1

Manuscript in review:

- **Shrestha, R**., Černá, K., Kokinda, J., Špánek, R., Bartak, D., Černoušek, T., Ševců, A., 2020. Effect of concrete on microbial ecosystem under repository relevant condition. Environmental microbiology.

- Povedano-Priego, C., Jroundi1, F., Lopez-Fernandez, M., **Shrestha, R.,** Spanek, R., Martin-Sánchez, I., Villar, MV., Ševců, A., Dopson, M., Merroun, ML., 2020.. Deciphering indigenous bacteria in compacted bentonite through a novel and efficient DNA extraction method: insights into biogeochemical processes within Deep Geological Disposal of nuclear waste concept. Journal of Hazardous Materials.

Manuscripts under preparation:

- **Shrestha, R.,** Černoušek, T., Kokinda, J., Vizelková, K., Špánek, R., Ševců, A., Stoulil, J., Steinová, J., 2020. Anaerobic microbial influenced corrosion of carbon steel in synthetic bentonite pore water. The manuscript will be submitted to the Biofouling journal.

Invited lecture

Presented a lecture on a topic " Microbial activities in bentonite" at MIND Advanced training course - Geomicrobiology in radioactive waste disposal, 8 – 11 October 2018, SCK•CEN Academy, Mol, Belgium

Active participation on international conferences and project meetings

- **Shrestha, R.,** Černoušek, T., Kovářová, H., Špánek, R., Ševců, A., Sihelská, K., Kokinda, J., Stoulil, J., Steinová, J. Microbially influenced corrosion of carbon steel in the presence of anaerobic sulfate-reducing bacteria. Topical Day | Aquatic microbiota in or near nuclear facilities: insights, discoveries and solutions, SCK•CEN Academy, 12 September 2019, Brussels, Belgium

- **Shrestha, R.,** Černá, K., Kokinda, J., Špánek, K., Bartak, D., Černoušek, T., Ševců, A. Effects of concrete on a microbial community under repository relevant condition. FISA 2019 and EURADWASTE '19 Conferences, 4 – 7 June 2019, Pitesti, Romania

- **Shrestha, R.,** Ševců, A., Černoušek, T., Kokinda, J., Špánek, R., Katerina, V., Steinová. Anaerobic microbially influenced corrosion of carbon steel in synthetic bentonite pore water inoculated by granite pore water: a 26-month study. FISA 2019 and EURADWASTE ’19 Conferences, 4 – 7 June 2019, Pitesti, Romania

- **Shrestha, R.,** Ševců, A., Steinová, J., Polívka, P. Effect of irradiation and pressure on microbial activity in bentonite in relation to the safety of the radioactive waste repository. Goldschmidt Conference, 12 – 18 August 2018, Boston, USA

- Černoušek, T., Váňová, H., Steinová, J., **Shrestha, R.,** Ševců, A., Polívka, P. Long-term experiment on corrosion of carbon steel in artificial bentonite pore water inoculated with a natural consortium of SRB. European Corrosion Congress 2017, 20th International Corrosion Congress & Process Safety Congress 2017 conference, 3 – 7 September 2017, Prague, Czech Republic

- **Shrestha, R.,** Steinová, J., Špánek, R., and Ševců, A. Microbial Diversity in Czech Bentonite. Goldschmidt Conference, 13 – 18. August 2017, Paris, France.

- Černoušek, T., SteinovJ., **Shrestha, R.,** Kovářová. H., Ševců. A., Kokinda. J., Polívka.P. Microbially induced corrosion of carbon steel in a natural groundwater and a synthetic bentonite pore water. MIND Project Meeting, 3 – 5 May 2017. Prague, Czech Republic

- **Shrestha, R.,** Steinová, J., Falteisek, L., Ševců, A., Characterisation of microbial communities in raw and homogenized bentonite sample. PETRUS-OPERA on Radioactive Waste Management and Geological Disposal, 27 June – 1 July 2016, Delft, Netherlands.

- **Shrestha, R.,** Steinová, J., Falteisek, L., Ševců, A., Molecular Analysis of Microorganisms in Czech Bentonite. MIND Project Meeting, 2 – 5 May 2016, Granada, Spain

- **Shrestha, R.,** Steinová, J., Falteisek, L., Ševců, A., Deep Ground Water Sources In the Czech Republic: Characterization Of Microbial Diversity. MIND Project Meeting, 2 – 5 May 2016, Granada, Spain

- Černoušek, T., Polívka, P., Váňová, H., **Shrestha, R.** Microbially induced corrosion of stainless steel 316L under anaerobic conditions. MIND Project Meeting, Granada, 2 – 5 May 2016, Granada, Spain

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