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Faculty of Mechatronics, Informatics and Interdisciplinary Studies

Microbiology in relation to nuclear waste repository safety

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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

Thesis structure

This 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.

The literature overview is divided into four subchapters (Nuclear energy and spent fuel deposition, Deep subsurface ecosystem, Effect of microbial processes on deep geological repository and Effect of deep geological repository conditions on microbial processes). 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. Likewise, the third subchapter is about the possible effect of microbial processes on deep geological repository 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.

The first chapter focuses on the characterization of 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 to be used as inoculum for further studies. Differences in microbial community structure between raw and commercial homogenized bentonite were also determined. The outcomes of this study were published in (Shrestha et al., 2016).

The second chapter explores the survival of indigenous microorganisms in bentonite subjected to ionizing radiation (total absorbed dose was 19,656 Gy). Moreover, an effect of added nutrients on microbial metabolism and microbial community in bentonite is described under anaerobic conditions. This chapter is partially based on the Euratom/Horizon2020 MIND project deliverable report 2.10.

The third chapter focuses on the corrosion of carbon steel (a candidate canister material) influenced by microorganisms present in groundwater. **This chapter is further divided into two**

parts: 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 part on corrosion in groundwater is based on our published article (Černoušek et al., 2019) and a book chapter (Černoušek et al., 2020) while the corrosion in synthetic water is partially based on our MIND deliverable 2.13 and a manuscript (Shrestha et al. 2020) in preparation.

The **fourth chapter** describes the effect of aged cementitious material in suspension on the development of microbial communities under repository relevant conditions. The experiment was performed with concrete from EU 7th FP DOPAS project on plugs and seals for geological disposal facilities. A manuscript (Shrestha et al., 2020) is submitted to Environmental Microbiology journal.

The last part, **Conclusions,** summarizes the most important findings of my thesis.

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Abbreviations

16S rRNA	16S ribosomal Ribonucleic acid
apsA	Encode adenylyl-sulfate reductase alfa-subunit
DGR	Deep geological repository
DNA	Deoxyribonucleic acid
dsrA	Encode dissimilatory sulfite reductase subunit A gene
DOC	Dissolved organic carbon
EDS	Energy dispersive X-ray spectroscopy
EIS	Electrochemical impedance spectroscopy
E-MIC	Electrical microbially influenced corrosion
EPS	Extracellular polymeric substance
HLLW	High and long-lived waste
HLW	High-level waste
IAEA	International Atomic Energy Agency
ILW	Intermediate-level waste
IOB	Iron-oxidizing bacteria
IRB	Iron-reducing bacteria
LLW	Low-level waste
MIC	Microbially influenced corrosion
MID	Microbially induced deterioration
M-MIC	Metabolite microbially influenced corrosion
NGS	Next-generation sequencing
nirK	Encode nitrite reductase gene
nirS	Encode nitrite reductase gene
NOB	Nitrite-oxidizing bacteria
nosZ	Encode nitrous oxide reductase
NPP	Nuclear power plant
NRB	Nitrate-reducing bacteria
OTU	Operational taxonomic unit
PCoA	Principal coordinates analysis

PCR	Polymerase chain reaction
qPCR	Quantitative polymerase chain reaction
RCR	Research Centre Řež
SBPOW	Synthetic bentonite pore water
SEM	Scanning electron microscope
SNF	Spent nuclear fuel
SRB	Sulfate-reducing bacteria
SRP	Sulfate-reducing prokaryotes
SÚRAO	Radioactive Waste Repository Authority in the Czech Republic
URC	Underground research centre
URF	Underground research facility
VITA	Groundwater source from Josef Underground Research Centre

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 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.

The second objective was to investigate 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.

LITERATURE OVERVIEW

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). The energy comes from the fission of atoms in a reactor to heat the water into steam to turn a turbine and produce electricity in the nuclear power plant (NPP, *Figure 1*). Radioactive metals such as uranium-235 and plutonium-239 are used as a nuclear fuel in NPP 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 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).

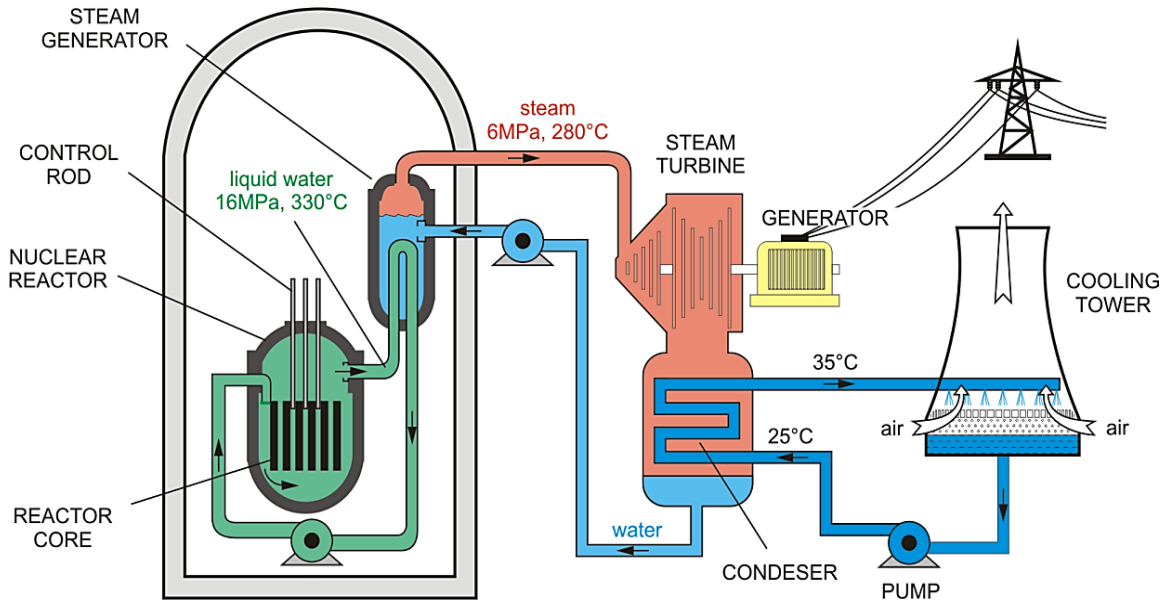

Figure 1: Diagram of Nuclear energy power plant.
(https://glossary.periodni.com/glossary.php?en=nuklearni+reaktor)

In the European Union, 13 out of 27 member states run NPPs contributing 28% to the European electricity mix by operating 128 nuclear reactors ("Nuclear energy statistics - Statistics Explained," n.d.), *Figure 2*. Nevertheless, the shares of nuclear energy among the member states vary widely. France is the largest nuclear power generating country as it has 58 nuclear reactors that contribute 71.7% to the national electricity while the Netherlands has only one nuclear reactor contributing 3% as in the year 2018 ("Nuclear shares of electricity generation - World Nuclear Association," n.d.). It has been reported that many NPPs in Europe have increased their energy generating capacity, e.g. in Belgium, Sweden, Switzerland, Finland, and Spain. The construction of new NPPs is ongoing in the member states including Finland, France, and Slovakia. As indicated by the World Nuclear Association, expansion in energy generating capacity to existing NPPs has been proposed or planned in Bulgaria, the Czech Republic, Finland, France, Hungary, Lithuania, Poland, and the United Kingdom by the end of 2030. However, according to The International Atomic Energy Agency (IAEA) the net nuclear capacity in Europe has been declining since 2000 as the priority has been given to more renewable energy (Welle (www.dw.com), n.d.).

On 26th April 1986, a disaster occurred in reactor number four in Chernobyl NPP near the city of Pripyat in the north of Ukraine. This catastrophe was the turning point for nuclear power in Europe along with the whole world, with only about 40 nuclear reactors built ever since ("Nuclear Power Today | Nuclear Energy - World Nuclear Association," n.d.). On 11th March 2011, an accident occurred at the Fukushima Daiichi NPP in Ōkuma, Fukushima Prefecture, Japan. This accident convinced many nations around the globe to phase-out nuclear power. Furthermore, the social attitude to nuclear energy production has been changed in the entire world by this incident. As an impact of these accidents, countries like Germany have decided not to build new reactors. Germany puts out of operation 8 of its 17 reactors permanently and is determined to phase-out its remaining nuclear reactors by 2022 (Rehner and McCauley, 2016). A year after the tragic incident of Chernobyl, Italy commenced nuclear phase-out after a referendum. Belgium and Spain faced public pressure to close the existing NPPs though these countries had the long-term nuclear phase-out policy. In contrast, countries like France and the UK decided to continue the production of nuclear energy (Kiyar and Wittneben, 2012). In the Czech Republic, in March 2009 about 70% of Czech citizens expressed their support for building a new nuclear reactor in the country (Polanecký et al., 2010).

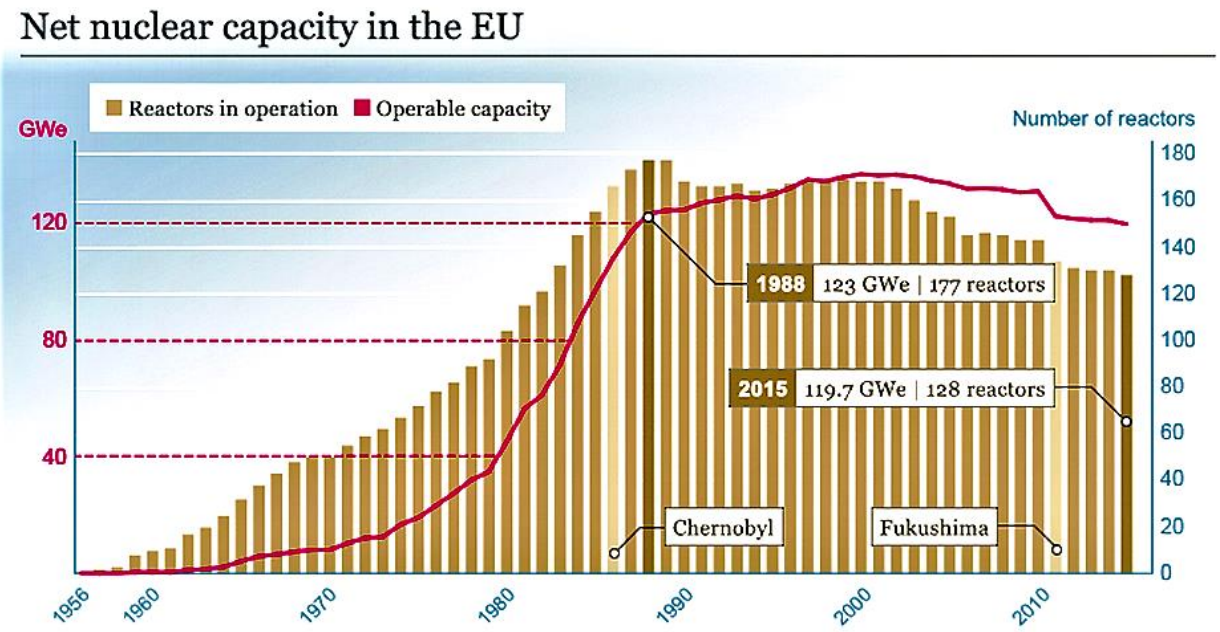

Figure 2: NucleNuclear capacity and number of the nuclear reactor in Europe (Welle (www.dw.com), n.d.)

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, other sources of radioactive waste are medicine and hospitals, scientific research work, industry, and defense military work. IEAE has categorized the radioactive waste depending on levels of exclusion and exemption for every single radionuclide. The generally referred categories of radioactive waste are: (i) Low-level waste (LLW) with a limited sum of long-lived radionuclides include items that have become contaminated with radioactive material or have become radioactive through exposure to neutron radiation. but are above exclusion level, (ii) Intermediate-level waste (ILW) with higher activity level than LLW and life span and (iii) High-level waste (HLW) containing the most concentrated radioactive material with higher quantities of long-lived radionuclides and the highest level of activity (Freiesleben, 2013), as depicted in *Figure 3*. HLW represents only about 3% of the total volume of radioactive waste is SNF but contains 95% of radioactivity ("What is nuclear waste and what do we do with it? - World Nuclear Association," n.d.). The waste can be either in solid, liquid, or in gas form. To ensure safe disposal of the waste, liquid, and gas waste

undergoes treatment processes of solidification (vitrification into glassy slags) (Tzeng et al., 1998).

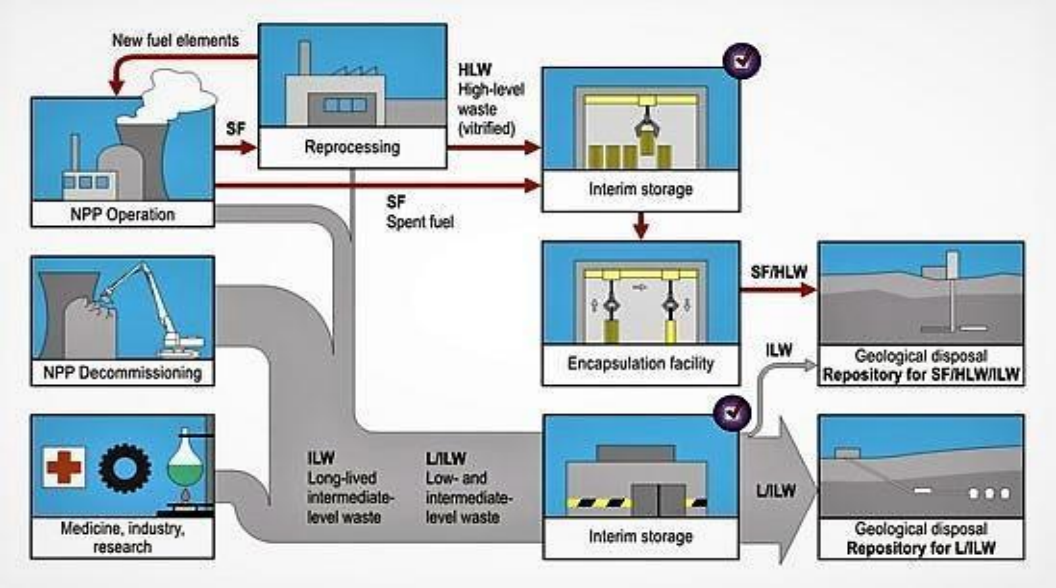

Figure 3: Types of radioactive waste, their intermediate storage, and disposal (Grimsel 2020).

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 ILW and HLW in deep stable 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. Briefly, a multi-barrier system including engineered barriers (metal, concrete), clay minerals, and natural barrier (host rock) work together to ensure the long-term confinement of ILW and HLW (Schütz et al., 2015). The major purpose of DGR is to separate the SNF or radioactive waste material 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), as illustrated in *Figure 4*. 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. Moreover, saturated bentonite limits microbial activity due to swelling pressure and limited nutrient availability (Pedersen et al., 2000). Crushed rock, concrete and bentonite pellets are used for the backfill and sealing of the HLW repository. Additionally, a waste management strategy for LLW and ILW is either the sub-surface repository or DGR, where the waste will be encapsulated in the metallic or concrete container (maybe reinforced) and where the gap between the waste package and the surrounding host rock will be filled by a backfill material such as unreinforced concrete and compacted bentonite (Koťátková et al., 2017). Clay formations play an important role in disposal systems as natural barriers in countries like Belgium, France and Switzerland (Delage et al., 2010) while the granite has been selected as host rock by countries like Sweden, Finland (Pettersson and Loennerberg, 2008) and the Czech Republic.

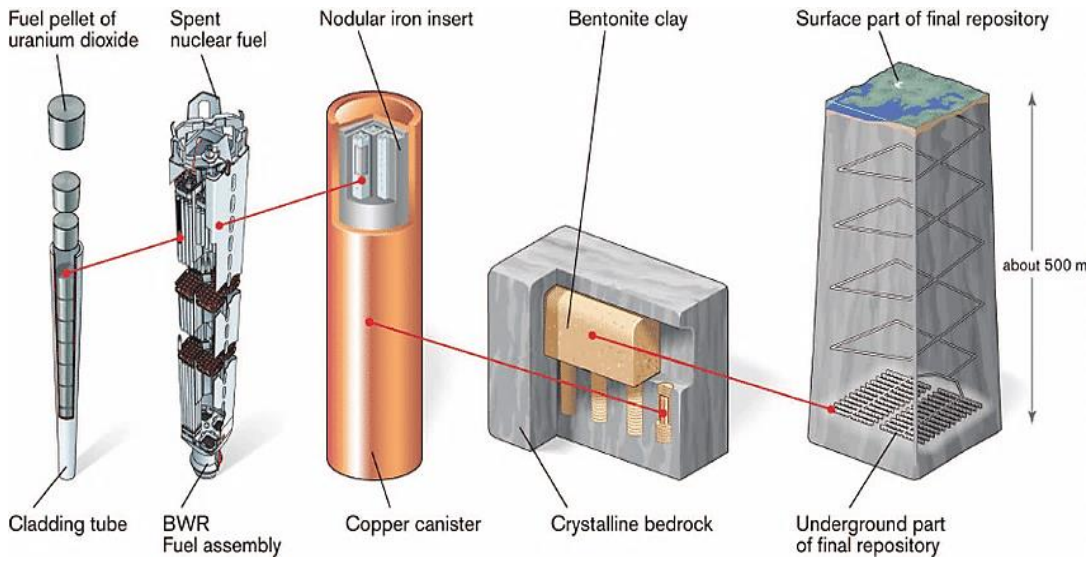

Figure 4: The KBS-3 concept for disposal of spent nuclear fuel by Svensk Kärnbränslehantering AB (SKB) (AB, 2011).

In terms of geological disposal plans for SNF, Sweden and Finland are considered to be the most advanced countries worldwide. Svensk Kärnbränslehantering AB known as SKB, a Swedish Nuclear Fuel and Waste Management Company, has introduced principles for the design of waste repository known as the KBS-3 concept, illustrated in *Figure 4* (AB, 2011). In brief, SNF should be protected by engineered and natural barriers, where the primary function is to hold the fuel within the container and in the case of breaching the barrier, the secondary

barrier should retard possible release of radionuclides. Furthermore, the principle suggests isolating the waste in a way that it is out of human intervention and not affected by any long-term climatic changes or any societal changes (AB, 2011). Furthermore, SKB together with POSIVA, Finnish Nuclear Waste Management Company, is investigating the concept of canister disposal in the vertical or horizontal position, generally named as KBS-3V or KBS-3H disposal concept, respectively. The disposal concept of KBS-3V was commenced in around 1980, while the KBS-3H concept started only around 2001. However, ongoing research work aims to bring the KBS-3H concept to the same maturity as KBS-3V (Pettersson and Loennerberg, 2008). Each barrier in the disposal system has a specific role to ensure the safety of the disposal system (see *Table 1*).

Table 1: Materials in the multi-barrier system and their role played in the repository. Adapted from (West et al., 2002).

Barrier system	**Roles**
Geological structure (Host rock/ Clays)	Ensure the stability of the repository and provides a natural sealing after closure to the repository.
Buffer material/ backfilling (Bentonite and Concrete)	Provides physical, chemical, and hydrological protection to the waste container and helps to limit the migration of radionuclides.
Container/ over pack (Steel/ Copper/ Iron)	Delivers physical isolation and shields waste matrix.
Waste matrix (Radioactive material)	Contains radioactive material (immobilized radionuclide) in a solid form

Site selection for DGR is a long-term process requiring comprehensive research on geological and technical aspects. Finland has selected the site for the repository construction at Eurajoki near Olkiluoto, approved by Parliament in 2001 and a construction license was issued in 2015. The operation of the DGR is expected to begin in 2023. POSIVA plans to apply for the operating license in 2020. In 2009, Sweden chose its disposal facility site at Söderviken close to the Forsmark NPP, north of Stockholm. SKB plans to start its construction work in early 2020 and commence operational work in 2030 (Litmanen et al., 2017). Sweden and Finland are followed by France. National Agency for Radioactive Waste Management, ANDRA is responsible for the construction of the geological repository in France. The Industrial Centre for

Geological Disposal (CIGEO) is located near the Bure village. Its license work is in progress with an expectation of commencing repository in 2025 (Labalette et al., 2013). In contrast, the waste disposal plan in Germany is based on the deep borehole disposal concept where the SNF will be disposed in extremely deep boreholes rather than in DGR. The site selection process seems to be a topic of political debate in Germany for decades though a site selection process was restarted by the Site Selection Act in 2017 and is expected to finalize site by 2031. The selection process has to be open to all potential host rocks like rock salt, crystalline rock, and claystone (Bracke et al., 2019).

In the Czech Republic, the general concept of DGR is based on the Swedish KBS-3 concept with certain modifications. Czech repository will be constructed in crystalline host rock using a steel-based disposal container (contrary to the copper-based canister in KBS-3 concept) and bentonite as a buffer material (Pospiskova et al., 2017). The waste container should be composed of two layers - carbon steel outer layer and stainless steel inner layer. The hermetically-sealed waste container will be disposed of horizontally in long boreholes as shown in *Figure 5*. Radioactive Waste Repository Authority, known as SÚRAO is the responsible state organization for the safe treatment and management of radioactive waste and SNF in the Czech Republic.

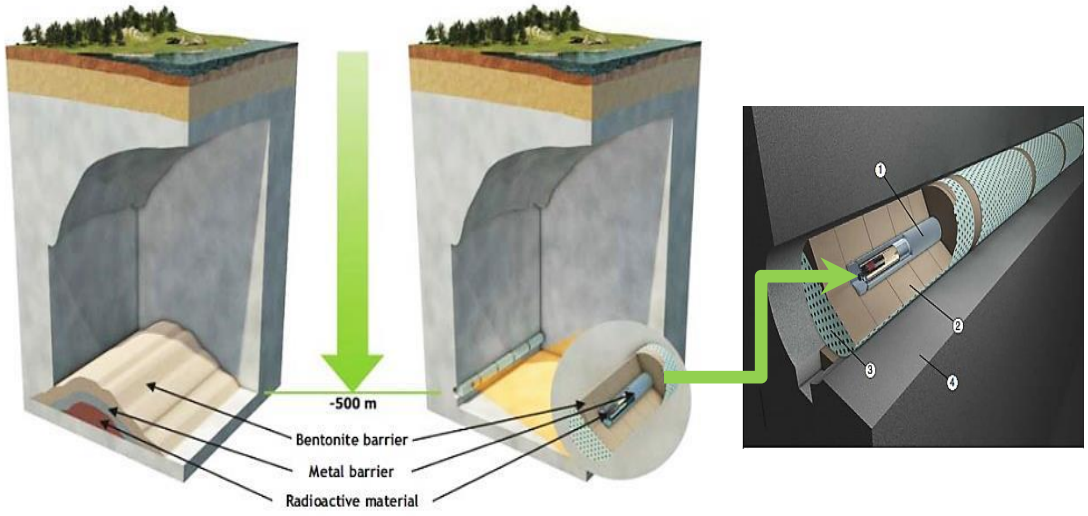

Figure 5: Concept of geological disposal of High level and long-lived waste (Left) and a super container in a disposal borehole where number 1 represents a container, 2– pre-cast bentonite elements, 3- external basket from perforated steel sheet and 4– host rock (Right) (SÚRAO 2016).

Currently, the Czech Republic operates two power plants in two different locations, Temelín and Dukovany with 6 nuclear reactors in total, where the SNF is cooled and stored until the repository is built (*Figure 6*). SÚRAO has initiated the implementation of DGR after approval from the Czech government in 2002. Four sites have been considered for the construction of DGR. The candidate sites are Březový Potok, Hrádek, Horka, and Janoch. It is expected to select one site as final by 2025. These sites are subjected to continual investigation and survey. The full operation of DGR should start in 2065 (SÚRAO 2019). LLW and ILW are disposed of in the near-surface repositories in Dukovany, in old mines Richard and Bratrství and in Hostím which is now closed. The concrete structure is used as a barrier or for the backfilling of these repositories ("Radioactive wastes and radioactive waste handling," 2009).

Figure 6: location of facilities and nuclear installations in the Czech Republic (OECD and Nuclear Energy Agency, 2006).

The disposal system is required to be safe for at least 100,000 years (Pedersen, 2010). However, various thermal, hydraulic, and mechanical aspects have a direct impact on the safety performance of host rock and may influence the long-term geo-disposal system (Rutqvist et al., 2005). Similarly, abiotic alterations in the physical and chemical properties of bentonite may take place during the expected life of the repository. Besides these abiotic factors, biotic processes can play a crucial role in the deterioration of this barrier system. Microbial activities in

bentonite buffers and groundwater can result in the compromising of the barrier system performance by the means of their metabolic processes and products (Mulligan et al., 2009). 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. Additionally, groundwater which naturally comprises microbial consortia also regularly supplies energy and nutrients required for the growth of bacteria that may come in contact with bentonite buffers through a rock fracture (Pedersen, 2010) and thus alter the environment of the DGR.

Corrosion of SNF/HLW container has been a primary concern for the safe repository establishment of Metal containers with radioactive waste is expected to remain intact for tens of thousands of years to prevent the direct release of radionuclides into the repository. However, metal containers are susceptible to corrosion. Corrosion is a direct result of electrochemical reactions on the metal surface that results in the deterioration of the metal. It can be influenced by different physicochemical conditions, such as pH, temperature, ionic strength, oxygen concentration, redox potential, and conductivity, or by microbial activity in the vicinity of a given metal's surface. Microbially influenced corrosion (MIC) can take place, where conditions are suitable for the microbial growth, including the presence of water and essential nutrients, and will depend on the particular metal and structure of microbial consortia. Most often is the corrosion rate accelerated in the presence of microorganisms.

Microorganisms deep down the ground level belong to small viable communities that mostly exist in the inactive (dormant) state due to very limited availability of water and space. However, construction and excavation work of the repository may assist the proliferation of microbial communities in different ways mainly, (i) growth of indigenous microorganism from the host rock due to the rock disturbances that offered favorable space, water, and nutrient which allow the microorganism to resuscitate from dormant form, (ii) introduction of non-indigenous microorganism by anthropogenic activities during excavation and operation of DGR. Some natural analogs study has demonstrated that microorganisms can be active in high alkaline conditions (pH up to 12-13) and anaerobic geochemical environments that are expected to be similar in DGR (Bertron, 2014).

2 Deep subsurface ecosystem

A deep geological environment is dark and anaerobic. It has been calculated that oxygen will disappear within the first 300 years of repository closure of the repository (Wersin et al., 1994). In such environment, microorganisms employ an anaerobic respiratory process that uses nitrate, manganese, iron, and sulfate as terminal electron acceptors instead of oxygen for energy generation (*Figure 7*). Furthermore, autotrophs like methanogens and acetogens can also actively perform their metabolic activity in this environment by reducing carbon dioxide. 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).

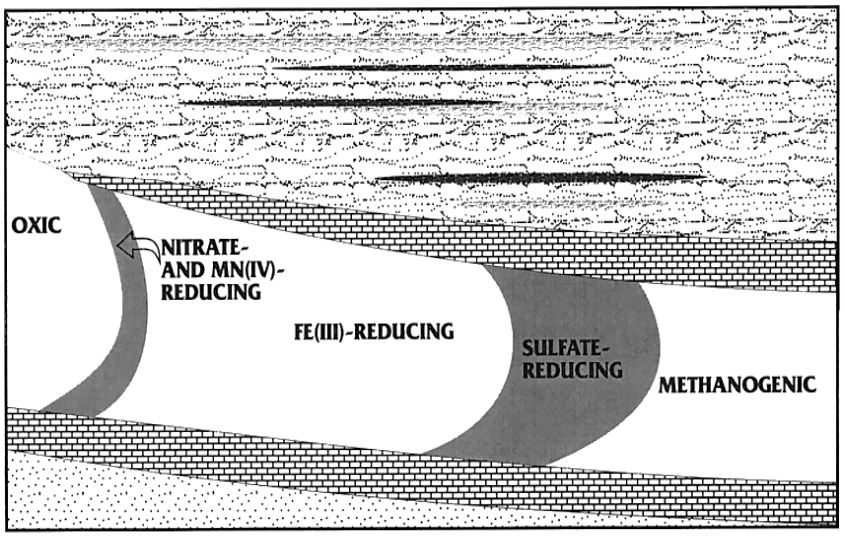

Figure 7: Distribution of major terminal electron accepting process in deep aquifers (Lovley and Chapelle, 1995).

Deep biosphere is a well-developed ecosystem containing various electron acceptors and donors that differs in redox potential. Hence, deep biosphere harbors active microorganisms (Anderson et al., 2011). The energy available by redox reactions of terminal electron acceptors can be described by a redox ladder, where the system changes from oxidizing to reducing condition with a decrease in redox potential (*Figure 8*). A decrease in redox potential subsequently changes the anaerobic respirations to low energy-yielding processes. The type of

terminal electron acceptors present in an environment defines the ecological niches for specific microorganisms (Sikora et al., 2017).

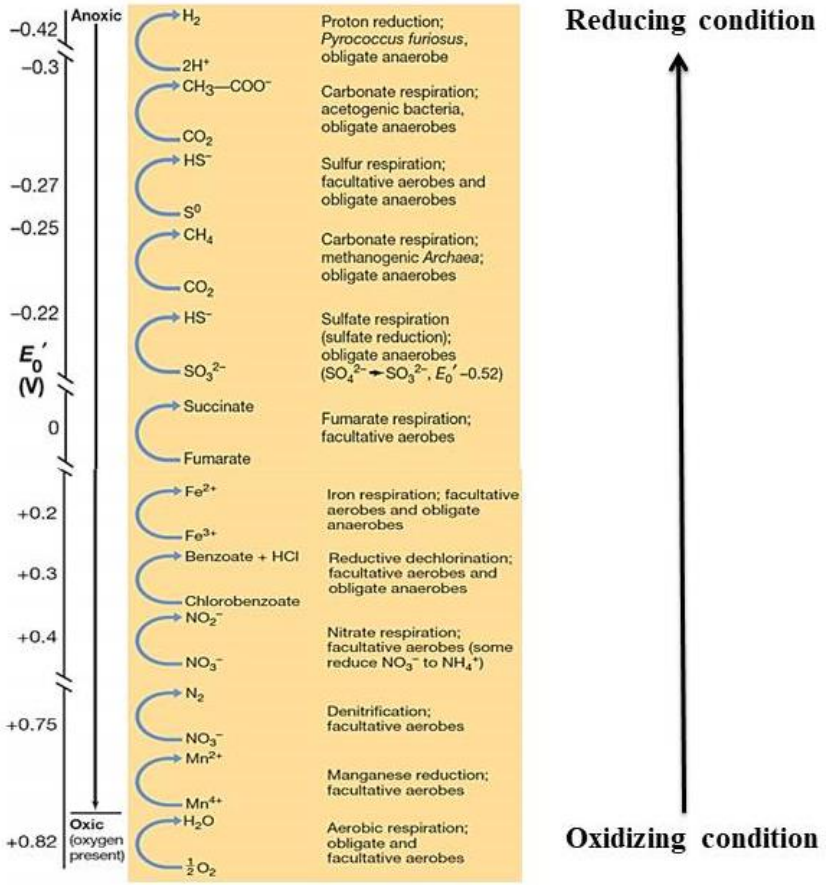

Figure 8: Redox potential with the strongest energy electron acceptors at the bottom and lowest at the top (Madigan et al., 2015).

Although the availability of electron acceptors determines the community composition, their usage is limited by the availability of suitable electron donors. Reduced organic substances represent energetically most favorable electron donors and organics can be also used as a substrate for non-respiratory fermentation processes. Organic compounds can be present both in groundwater as well as in the host rock. Although microorganisms generally prefer using small organic molecules as electron donors, even macromolecular organic matter present in small quantities could potentially break down into smaller bioavailable compounds that favor the growth of microorganisms. In addition to low molecular weight compounds like acetate, microorganisms can also use complex forms of organic compounds like aromatic substances or

aliphatic chains. Depending on the oxidative ability of organic compounds, microorganisms can be divided into two kinds, *Figure 9*. Some genera of microorganism are capable to completely oxidize organic carbon source to carbon dioxide whereas other do not possess the mechanism of acetyl-CoA oxidation hence, could perform only incomplete oxidation of organic carbon which result in the production of hydrogen (Muyzer and Stams, 2008).

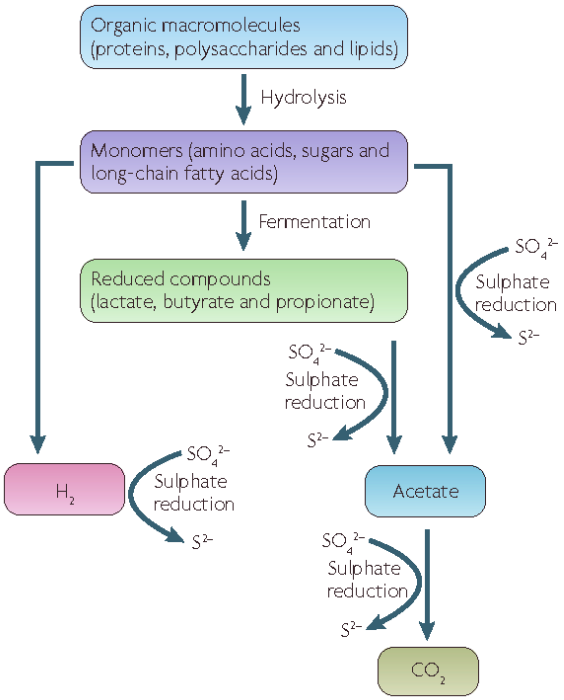

Figure 9: Degradation of the complex organic compound under anoxic environments by sulfate reducing microbes (Muyzer and Stams, 2008).

Besides hydrogen and organic compounds, methane produced from abiotic or biotic processes also serves as an electron donor (Costa et al., 2000). Because available organics as a preferred electron donor is rapidly consumed by the microorganisms in the deep subsurface, the main reason for the existence of active microbial life in deep intra-terrestrial ecosystems is the accessibility of hydrogen produced from diverse geological sources such as minerals reaction, radiolysis, volcanic activities or anaerobic chemical metal corrosion which serves as the source of energy and electron donor sustaining the growth of autotrophic microorganisms over the time even when the organic substances became unavailable (Pedersen, 1999).

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, microbially influenced corrosion (MIC) of waste container, alteration of bentonite, gas production, pressure change, and sorption, and migration of radionuclides (see *Figure 10*) (Mulligan et al., 2009; Stroes-Gascoyne, 2010). Although the primary function of the bentonite layer in the repository is to seal the canister from the environment and protect both the canister and the environment, compacted bentonite cannot fully protect the system from microbial activities. Bentonite itself is rich in indigenous microflora well adapted to this environment and colonization by bacteria was observed up to a density of approximately 2000 kg/m^3 on interaction with groundwater containing indigenous microorganisms after 5 years (Fru and Athar, 2008).

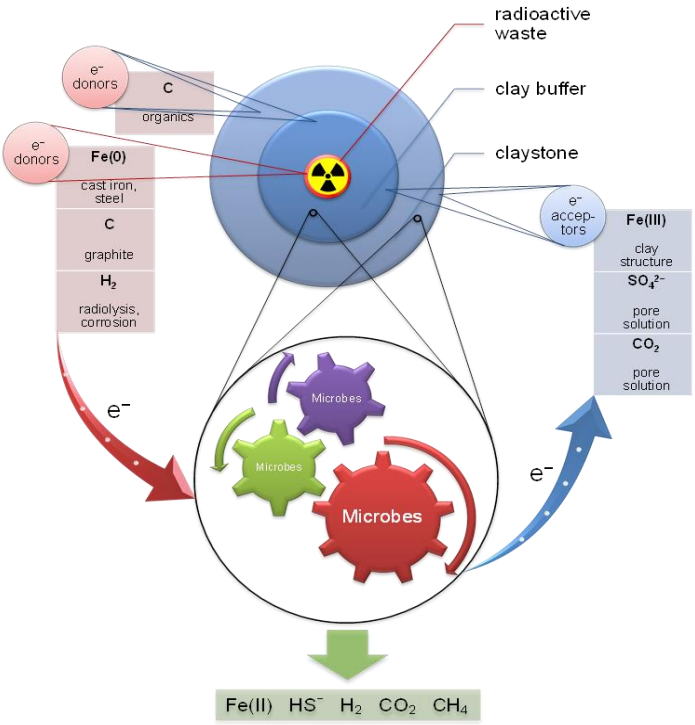

Figure 10: Microbial processes in the DGR environment presented with a summary of electron donors and acceptors (Meleshyn, 2014).

3.1 Dissolution and mineralization of bentonite buffer

The structure of the bentonite buffer comprises two basic building blocks: aluminum octahedral sheets and silica tetrahedral sheets. A single unit of bentonite cell is made up of one aluminum hydroxide octahedral sheet sandwiched between two silica tetrahedral sheets. The silica layers have a slightly negative charge which is compensated by exchangeable cations (Na^{+}, $Mg^{2+,}$ or Ca^{2+} ions) in the intermediate layers (Ross and Shannon, 1926). Furthermore, at the intermediate layer between two successive units, the water molecules are present where other polar molecules can enter. Bentonite clays mostly comprise of the mineral called montmorillonite. The montmorillonite clays consist of silica and aluminum sheets that are not tightly bound (*Figure 11*). Therefore, water can enter, causing the clay to swell which is an important feature for the radioactive waste repository. In contrast, illite clays are similar to montmorillonite, but the space between the sheets is occupied by poorly hydrated potassium cations that are responsible for the absence of swelling (Ehrlich et al., 2015).

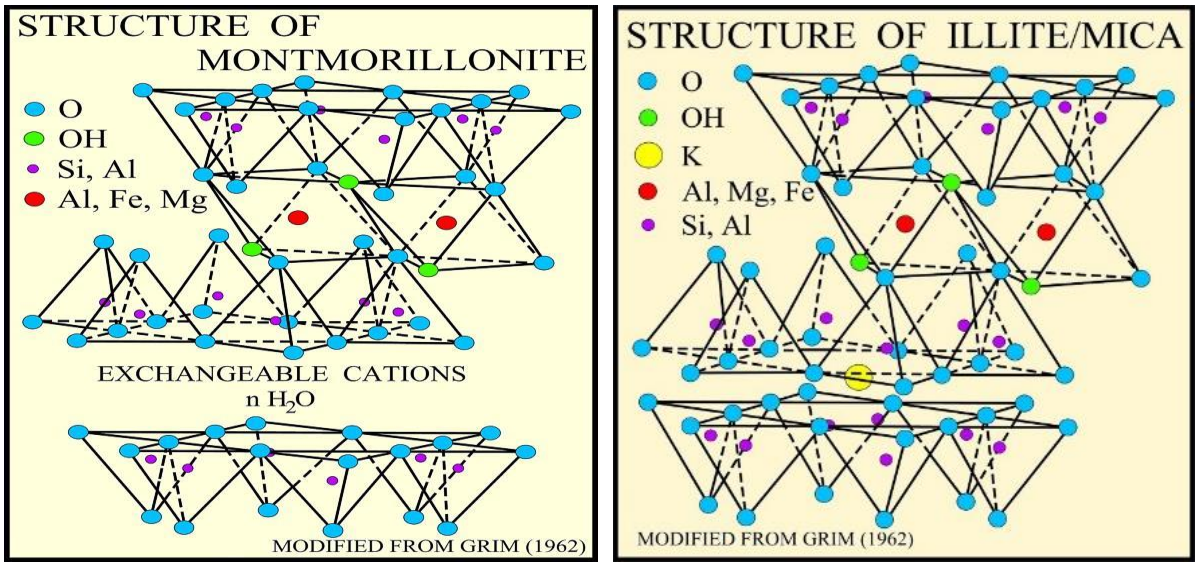

Figure 11: Schematic presentation of Montmorillonite and Illite (Grim, 1962).

Dissolution of montmorillonite results in the formation of illite. Dissolution can occur by the reduction of structural Fe^{3+} to Fe^{2+} and subsequent irreversible conversion of montmorillonite to illite, *Figure 11*. In absence of microbial activity, the process of conversion may take longer, but in the presence of microorganisms, especially iron reducing bacteria (IRB), the process might

be accelerated (Meleshyn, 2014). Microorganisms enhanced the dissolution of bentonite by reducing structural Fe^{3+} in a period of 2 weeks at room temperature with pressure 101 kPa. This process would otherwise need temperature from 300 to 350 °C with a pressure of 100 MPa and a period of 4 to 5 months without microbial activity (Kim et al., 2004). Similarly, an experimental interaction between bentonites (MX-80 and nontronite, dry density of 1300 kg/m^3) and the facultative anaerobic bacteria (*Shewanella putrefaciens*) under anaerobic condition revealed that the presence of bacteria in MX-80 bentonite had noticeably increased water content and available pore space while the dissolution of minerals was noticed in nontronite owing to the bacterial activity (Julia N. Perdrial et al., 2009). The process of illitization has a great impact on the porosity of the buffer by altering the buffer's properties in terms of hydraulic conductivity (Mulligan et al., 2009). Mineral - bacterial interactions was studied to understand the formation and dissolution of minerals in bentonite by Dai et al. (2014). In this study, gram-negative *Bacillus* strain isolated from soil was subjected to interaction with bentonite buffer of different content. In the presence of bacteria, the release of Ca^{2+} and Mg^{2+} was detected and the tendency of dissolution of these cations was elevated with the increase of bentonite content. As a result of active microbial metabolism, the interlayer space of bentonite was found to be increased approximately by 0.283 – 0.534 nm corresponding to the decrease of mineral content. Beside this, accumulation of mixture constituent of nanoparticles was also successfully detected that may be defined by the release of Si^{4+} and Al^{3+} from the buffer material (Dai et al., 2014).

Generally, mineralization of the bentonite buffer in DGR can be affected by physical, chemical, and biological factors, specifically activities of microorganisms present in it. Microbial interaction with minerals can affect biogeochemical processes and thus, support the formation or dissolution of minerals (Dai et al., 2014). Alteration of minerals caused by microbial activities is a process of biomineralization, *Figure 12*. Biomineralization may result in increased permeability owing to decreased solid content or in coagulation of pores owing to precipitation. For instance, the size of the pores of the buffer material may increase under anaerobic environment by the reduction of Mn and Fe oxides. Alike, carbonate that is one of the constituents of commercial bentonite can be either dissolved or precipitated as a function of microbial process compromising the properties of bentonite for long-term geological disposal concept (Mulligan et al., 2009).

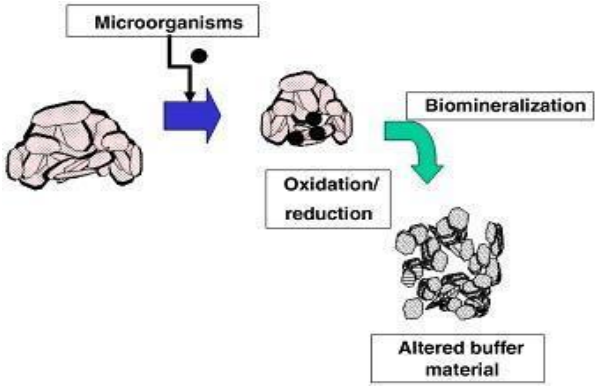

Figure 12: Biomineralization of clay buffer (Mulligan et al., 2009).

3.2 Formation of biofilms

Microorganisms have an ability to assemble and attach on a surface by the production of extracellular polymeric substance (EPS) forming a biological film known as a biofilm. Biofilm accumulation is the net result of cell attachment, growth, and detachment. The major role in biofilm formation, maturation, and maintenance is played by EPS that is composed of polysaccharides, nucleic acids, and proteins. The process of biofilm formation follows a sequence of steps that are initiated by the adsorption of macromolecules (e.g. polysaccharides, nucleic acids) and micromolecules (fatty acids, lipids) onto solid surfaces. A film is formed from the adsorbed molecules that can change the physiochemical condition of the environment including hydrophobicity and electrical charge. Diffusive transport owing to the Brownian motion, convective transport due to liquid flow, and active movement of motile bacteria near the interface are the reasons behind transport and attachment of microorganisms to an interface (Little and Lee, 2007). After attachment, EPS is produced by microorganisms that provide the matrix to hold bacteria together allowing the formation of microcolonies and eventually, the formation of a mature biofilm. Dispersion is the final step of biofilm formation, where the microorganisms are detached and dispersed by the process of sloughing (rapid and massive removal of the biofilm), erosion (continuous removal of small portions of the biofilm) and abrasion detachment due to collision of particles from the bulk fluid with the biofilm) (Donlan, 2002). Hence, motile microorganisms are dispersed while some remain as sessile (*Figure 13*).

Biofilm formation under repository conditions leads to the poor performance of the disposal system because the film provides good protection and shelter to the microbes against harsh environmental conditions including physical, chemical, and biological stresses and further supports their survival under such unfavorable conditions (Meleshyn, 2014). Moreover, the formation of biofilm can influence the cation and anion sorption capacities of the underlying mineral surface; however, it depends upon the nature of the component in the environment. It has been reported that a decrease in adsorption capacity of Co (II), Th (IV) and Np (V) and an increase in adsorption capacity or no significant change on Pm (III) and Am (III) on the granitic rock surface has been observed by the formation of biofilm (Anderson et al., 2007; Meleshyn, 2014). Likewise, it can also affect the chemical condition of the bulk solution. Biofilm formation was found to be responsible for the reduction of pH in the confined pore space within two weeks of the experiment (Barker et al., 1998; Meleshyn, 2014). Subsequently, it can enhance the phenomenon of reduction and dissolution of clay minerals (Meleshyn, 2014).

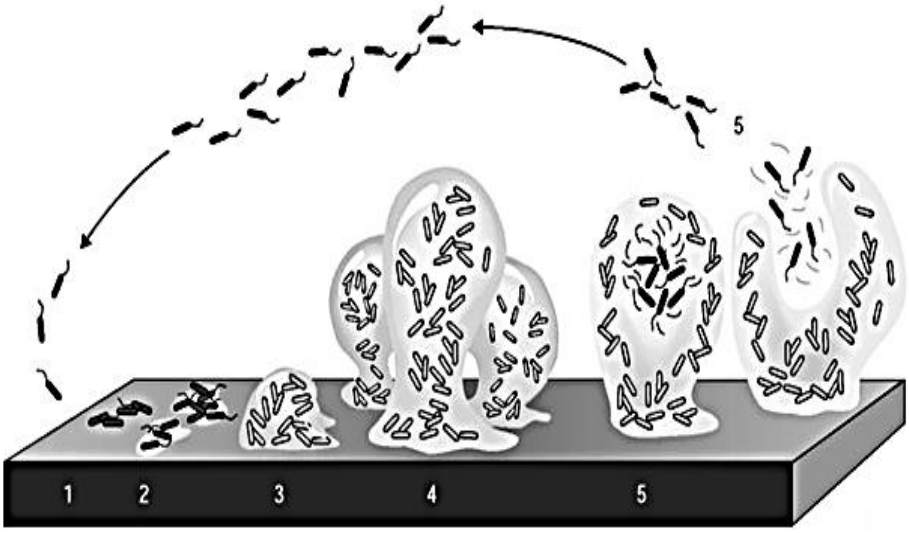

Figure 13: Formation of biofilm. Stage 1 is an initial reversible attachment of bacterial cells to the surface. Stage 2 is an irreversible attachment of the cells facilitated mainly by exopolymeric substances where they lose flagella-driven motility. At stage 3, the proliferation of cells starts where the first maturation phase is reached. The second maturation phase is reached at stage 4 with a fully mature biofilm (complex biofilm architecture). Eventually, stage 5 is the dispersion stage where single motile cells (dark cells in the figure) disperse from the microcolonies while some remain as sessile (Stoodley et al., 2002).

In contrast, biofilm can affect the mass transport and hydrodynamics of buffer material by reducing the porosity and permeability of the adjacent pore space. The availability of pore

space is an essential factor for the growth of microorganisms (Meleshyn, 2014). A report about the crushed granitic rock from Äspö Hard Rock Laboratory confirmed that the packed column of crushed rock became impermeable due to the formation of biofilm by Fe (III) reducing bacteria within 2 days (Meleshyn, 2014; Tuck et al., 2006). Similarly, biofilm formation of SRB on a container surface or intensive growth in bentonite close to the container is considered the worst scenario for the disposal system as it can highly influence and boost up the process of corrosion (Masurat et al., 2010b). Nonetheless, biofilm may also have a passivation effect initially against corrosion and radionuclide transportation forming a protective layer and by sorption of radionuclide, respectively, however, by the time goes, biofilm get porous, loose, weak and easy to break down (Paula et al., 2016). The formation of biofilm under DGR environment has thus much more adverse impact on safety-relevant processes than lack of biofilm formation because of their bulk effect.

3.3 Microbially influenced corrosion of the waste container

The absolute barrier of radionuclides transportation in the designed disposal system is only an intact metal waste container because both the bentonite buffer and host rock are water-conducting (Masurat et al., 2010b). Corrosion is the result of electrochemical reactions on the surface of the metal caused by the physiochemical condition. Additionally, corrosion can be accelerated by the activity of microorganisms and hence, referred to as MIC (Zhou, 2012). MIC may occur either by indirect utilization of hydrogen or organic compounds or even by direct uptake of electrons from the metal surface. Any local or general corrosion in the metal container could lead to the migration of radionuclide and subsequently, results in the failure of the disposal system.

3.3.1 Microorganism involved in MIC

The formation of biofilm on the surface of a metal container is the initial step of MIC. In both natural and engineered environments, microorganisms often exist as a biofilm, a central factor for the occurrence of biodegradation of barrier systems (Beech and Sunner, 2004; Dall'Agnol et al., 2014). Microorganisms can cause pitting corrosion, general, and localized corrosion (Rajala et al., 2015). Generally speaking, oxygen introduced into a repository during its excavation and operational phases creates an oxidizing environment for the first few hundred years and

gradually disappears establishing a reducing environment after the closure of a repository (Pedersen, 2013). Under such conditions, diverse groups of microorganisms are able to promote MIC on metal containers (see *Table 2*). Biofilms on metal surfaces may be formed by bacteria, archaea, and eukaryotes, although bacteria tend to be most responsible for MIC.

Table 2: Examples of bacteria involved in MIC and their effects.

Microorganisms	Characteristics	Effects	References
Sulfate-reducing prokaryotes *Desulfobacterium corrodens* *Desulfovibrio alkalitolerans* *Desulfovibrio ferrophilus* *Desulfomonas* spp. *Desulfonatronovibrio hydrogenovorans* *Thermodesulfovibrio* *Thermodesulfobacterium*	Anaerobic; Use H_2 to reduce SO_4^{2-}, SO_3^{2-}, and $S_2O_3^{2-}$ to S^{2-}; iron may serve as an electron donor under organic carbon limitation ($Fe \rightarrow Fe^{2+} + 2e^-$)	Cathodic depolarization by hydrogen uptake; anodic depolarization by corrosive iron sulfides; precipitation of H_2S and FeS	(Dinh et al., 2004; Enning et al., 2012; Gittel et al., 2008; Rabus, 2006; Rao et al., 2000; Venzlaff et al., 2013; Wikieł et al., 2014)
Metal-oxidizing bacteria *Gallionella* spp. *Leptothrix* spp. *Mariprofundus* spp. *Methanococcus maripaludis* *Sulfobacillus thermosulfidooxidans* *Sulfobacillus acidophilus* *Acidithiobacillus ferrooxidans*	Aerobic and anaerobic; oxidize Fe^{2+} to Fe^{3+} and Mn^{2+} to Mn^{3+}	Deposition of cathodically reactive ferric and manganic oxides	(Lee et al., 2013; Linhardt, 2010; Norris et al., 1996; Rao et al., 2000; Uchiyama et al., 2010; Wang et al., 2014)
Metal-reducing bacteria *Carboxydothermus ferrireducens* *Carboxydothermus hydrogenoformans* *Desulfitobacterium hafniense* *Geobacter metallireducens* *Geobacter sulfurreducens* *Geothermobacter* spp. *Shewanella* spp. *Thermincola potens*	Aerobic and anaerobic; reduce Fe^{3+} to Fe^{2+}	Reduction of iron and manganese oxides	(Finneran et al., 2002; Lee et al., 2013; Nevin and Lovley, 2000; Rao et al., 2000)
Acid-producing bacteria *Acetobacter* spp. *Acidithiobacillus caldus*		Acids corrode metal, dissolve iron, and chelate copper, zinc and iron	(Dong et al., 2018; Xu et al., 2016)

Nitrate-reducing bacteria *Bacillus licheniformis* *Pseudomonas aeruginosa*	Anaerobic; reduce NO_3^- to N_2 or NH_4^+; iron may serve as an electron donor	Iron oxidation, formation of iron nitride	(Jia et al., 2017a; Xu et al., 2013)

Prokaryotes involved in MIC are usually categorized into a few main groups consisting of taxonomically diverse organisms varying considerably in their metabolic capabilities. Sulfate-reducing prokaryotes (SRP), metal-oxidizing bacteria, metal-reducing bacteria, methanogens, acid-producing bacteria, nitrate-reducing bacteria (NRB), nitrite-oxidizing bacteria (NOB), and fermentative hydrogen sulfide producing bacteria are the typical culprits involved in corrosion, *Table 2***.** These organisms typically coexist in naturally occurring biofilms and form complex consortia on corroding metal surfaces (Chapman et al., 1987). Under anaerobic environmental conditions, MIC is mainly influenced by the activities of Sulfate-reducing bacteria (SRB) though other microbial populations may play a significant role in the complex corrosion processes, depending on local physicochemical conditions. The most severe deterioration of metal was reported when the biofilm is composed of different species due to the interaction between multispecies (Kip and van Veen, 2015). Besides SRB, iron-oxidizing bacteria (IOB) are also responsible for the deterioration of metal containers by the anaerobic oxidation of metal (Liu et al., 2015).

3.3.2 Sulfate-reducing microorganisms

Sulfate-reducing microorganisms are highly specialized and can use sulfite, thiosulfate, and sulfur as a terminal electron acceptor for their energy metabolism. The sulfide produced is a potentially corrosive element that can affect the integrity of metal containers in deep repositories. Generally speaking, dissimilatory sulfate reducers are considered more important in MIC than assimilatory reducers. Of all the SRB genera, *Desulfovibrio* spp., *Desulfotomaculum* sp., and *Desulfomicrobium* sp. are often identified as the main culprits responsible for corrosion (Chang et al., 2014; Lee and Characklis, 1993; Vigneron et al., 2016; Xu and Gu, 2014), while several extremely thermophilic sulfate-reducing archaea, e.g., *Archaeoglobus fulgidus*, *Archaeoglobus profundus*, *Methanococcus maripaludis* and *Ferroglobus placidus* have been described as possible agents accelerating corrosion of ferrous metal pipelines in an anaerobic environment (Hafenbradl et al., 1996; Slobodkin, 2005; Uchiyama et al., 2010). To date, most studies on anaerobic corrosion have focused on corrosion by SRB.

Phylogenetically and metabolically, SRB are the most diverse bacterial group and are considered unique due to their role in the biogeochemistry of the environments they inhabit (Rabus et al., 2015). SRB are widely distributed in terrestrial, sub-terrestrial, and marine ecosystems and are capable of producing sulfide under a wide range of environmental conditions. In general, most SRB are heterotrophic prokaryotes that require anaerobic conditions for growth. However, recent studies have shown that a few SRB species are capable of carrying out micro-aerobic respiration and may also be autotrophic and lithoautotrophic. Low molecular weight organic compounds, such as lactate, acetate, propionate, and amino acids, act as the main sources of carbon and electron donors, while hydrogen gas may serve as both a source of energy and as an electron donor for SRB metabolism (Barton and Fauque, 2009). Consumption of hydrogen gas by SRB, leading to decrease in undesired pressure expected in the deep geological repository, could be beneficial if the bacteria will be mostly present far from the container, e.g., within the engineered higher-permeability zone between host rock and compacted bentonite (Bagnoud et al., 2016). Some of the best-known SRB isolated from extreme environments include *Desulfotomaculum*, *Desulfovibri*o, and *Desulfomicrobium*. *Desulfotomaculum* is an endospore-forming thermophilic species that can exist in a dormant phase for many years while awaiting favorable conditions (Aullo et al., 2013). This genus was also reported from a long-term corrosion study on carbon steel in compacted bentonite in the Mont Terri Underground Research Laboratory, along with two other SRB *Desulfurispora* and *Desulfosporosinus* that dominated natural Opalinus Clay pore water (Smart et al., 2017). Notably, SRB numbers were higher in the internal sections of the compacted bentonite implying that SRB originated mostly from the bentonite and minor part came from natural pore water. Several other studies have also reported activity of SRB in bentonite buffers and groundwater in relation to deep geological repositories for radioactive waste (Bengtsson and Pedersen, 2016; Masurat et al., 2010; Pedersen, 2013; Pedersen et al., 2017; Stroes-Gascoyne et al., 2010). Microbial analysis of groundwater showed a higher number of SRB and biofilm formation on the surface of the copper container (Hallbeck et al., 2012). The occurrence of microbes was proved on copper and titanium rod embedded in compacted bentonite buffer incubated with underground water for 9 weeks to densities in between 1750 to 2000 kg/m^3 in the increment of 50 kg/m^3 (Persson et al., 2011). Several species of microorganisms including the genus *Desulfosporosinus* (anaerobic endospore-forming sulfate reducers), *Pseudomonas stutzeri* (denitrifying bacteria), and *Clostridiisalibacter paucivorans*

(halophilic bacteria) were accumulated on the metal rods embedded on a bentonite buffer. Amazingly, it was also noted that SRB could maintain viability on embedded copper rods despite water saturation and high compaction processes.

3.3.3 Mechanism of MIC by SRB

The most important mechanisms resulting in MIC are:

Cathodic Depolarization (CDP) Theory

The corrosion of metal by SRB was initially proposed in 1934 by Von Wolzogen Kuehr and Van der Vlugt (von Wolzogen Kühr and Van der Vlugt, 1964). A simplified version of this theory is presented in *Table 3***.** This mechanism follows a theory of cathodic depolarization where corrosion by SRB is induced due to depolarization by the oxidation of cathodic hydrogen. A metal becomes polarized when it reacts with water resulting in an anodic reaction by losing positive metal ions. In the absence of oxygen, the free electron reduces the proton of water to produce hydrogen following a cathodic reaction. This hydrogen is expected to be consumed by SRB as a good source of energy and electron using hydrogenase enzymes.

Table 3: Cathodic depolarization theory by SRB on the metal corrosion mechanism.

Anodic reaction (1)	$4\,Fe \rightarrow 4Fe^{2+} + 8e^-$
Water dissociation (2)	$8H_2O \rightarrow 8H^+ + 8OH^-$
Cathodic reaction (3)	$8H^+ + 8e^- \rightarrow 8H + 4H_2$
Hydrogen oxidation (4)	$SO_4^{2-} + 4H_2 \rightarrow H_2S + 2H_2O + 2OH^-$
Precipitation (5)	$Fe^{2+} + H_2S \rightarrow Fes + 2H^+$
Precipitation (6)	$3Fe^{2+} + 6OH^- \rightarrow 3Fe(OH)_2$
Total Reaction:	$4Fe + SO_4^{2-} + 4H_2O \rightarrow FeS + 3Fe(OH)_2 + 2OH^-$

Cathodic depolarization is achieved through the metabolic oxidation of hydrogen from the metal surface by (though not necessarily exclusively) SRB (*Figure 14*). Other bacterial populations that might influence cathodic depolarization are listed in *Table 2*. The mechanism of cathodic depolarization accelerates the anodic reaction resulting in anodic metal dissolution and subsequently, the formation of corrosion products such as FeS and $Fe(OH)_2$ (Kakooei et al.,

2012). Today, it is widely accepted that this mechanism is not the only mechanism that plays a role in local corrosion of iron.

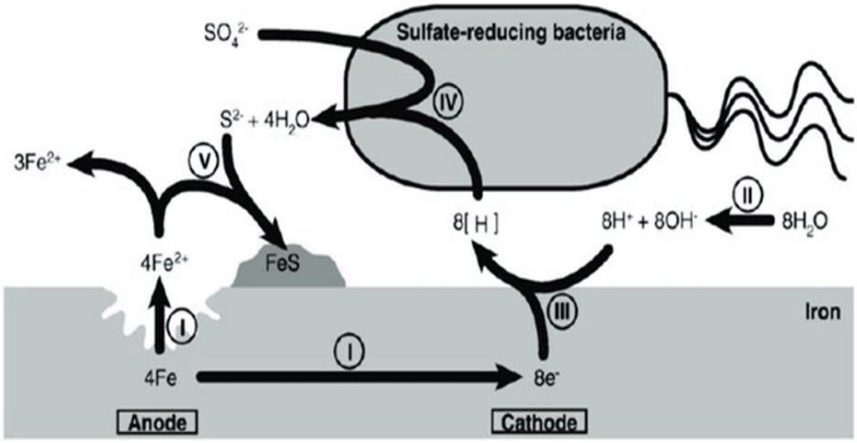

Figure 14: Scheme of iron surface corrosion induced by SRB based on the process postulated by the cathodic depolarization theory following 5 major steps starting with the dissolution of iron (I), dissociation of water(II), proton reduction(III), sulfate reduction (IV) and finally, sulfide precipitation (V) (Mori et al., 2010).

Iron sulfides (King's Mechanism)

In 1971 King and Miller suggested that solid FeS formed on the surface of the metal acts as an absorber of molecular hydrogen and then induces in the reproduction of iron sulfide. Here, part of the iron below the biofilm becomes anode and the other part covered by iron sulfide behaves as cathode. Thus, the rate of corrosion will remain high. Schematic illustration of this mechanism has been presented in *Figure 15* where no film of sulfide are formed after the formation of plenty of FeS for commencing a galvanic cell between FeS and Fe while a high corrosion was recorded due to galvanic corrosion (Kakooei et al., 2012).

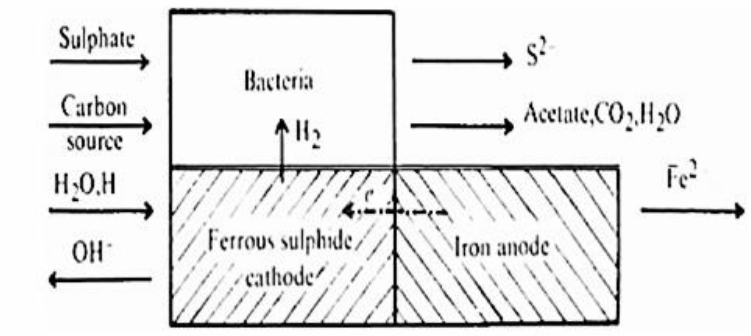

Figure 15: Corrosion of metal by SRB proposed by King'S Mechanism (King and Miller, 1971).

Fe- Binding exopolymers

Microorganisms are capable of producing EPS, resulting in the formation of biofilm and the major purpose of biofilm is protection and shelters to microorganisms. The EPS produced by SRB has a unique ability to bind with metal ions and speed up the process of corrosion. SRB with different composition of EPS was observed to have different rates of corrosion (Kakooei et al., 2012). Furthermore, Chan et al., (2002) showed that EPS alone can be a metal corrosion agent. They studied the metal corrosion in two test solutions, one with 1% EPS and other without any EPS and the result demonstrated that EPS enhanced corrosion rates (Chan et al., 2002).

Biocatalytic cathodic sulfate reduction (BCSR)

The BCRS theory proposes that oxidation of insoluble iron occurs outside the SRB cells, while the sulfate reduction occurs within cells. The electrons released through elemental iron oxidation are transferred from outside the cells into the cytoplasm, where sulfate reduction then takes place. When organic carbon sources, such as lactate, are available, oxidation occurs within the SRB cytoplasm. As such, the electrons released do not need to be transported across the SRB cell wall. If the environment has insufficient electron donors due to the biofilm barrier, the sessile SRB may attack the iron to obtain electrons for BCSR (Gu and Xu, 2010) resulting in electrical MIC. The electron transfer into the sessile cells could be direct, through conductive pili or using endogenous mediators excreted by the cells themselves (Zhang et al., 2015).

According to the theory, electrons released by the dissolution of iron at the anode are used to reduce sulfate at the cathode, resulting in MIC with the help of SRB that is attracted to the metal surface. Owing to the presence of biocatalysis, the following reactions occur on the surface of the iron:

$$anodic: 4Fe \rightarrow 4Fe^{2+} + 8e^{-} \ (Iron\ dissolution)$$

$$cathodic: SO_4^{2-} + 8H^{+} + 8e^{-} \rightarrow 6HS^{-} + OH^{-} + 3H_2O$$

MIC of the candidate metal to use as a SNF container has been demonstrated in the chapter III of the experimental part under different condition.

3.4 Gas production and pressure change

Gas production by microorganisms is the result of their respiration. Under anoxic conditions, gases like dinitrogen, hydrogen sulfide, carbon dioxide, and methane are produced by nitrate-reducing bacteria, SRB, and methanogens. Additionally, hydrogen gas can be generated in a deep biosphere at repository conditions by processes such as water radiolysis and chemical corrosion besides the fermentation. Gas production is strongly possible in buffer and backfill material and would depend on the geochemical and redox condition of the environment (Stroes-Gascoyne, 2010). As a consequence, gas pressure could be built up due to disparity occurring between the gas diffusion and gas production which can lead to crack or fracture of the host rock. However, as discussed earlier, hydrogen gas can be oxidized as an electron donor by bacterial hydrogenase reducing the risk of overpressure (Meleshyn, 2014).

Gas production could have an adverse effect on hydro-mechanical properties of the bentonite buffer as it can increase the permeability of buffer materials by disrupting the mechanical structure of the buffer (Mulligan et al., 2009). Production of carbon dioxide by the degradation of organic waste can also play a vital role in increasing solubility of radionuclide and consequently their transportation. Moreover, it can influence the solubility by persuading chemical gradients in addition to micro-environmental nutrients (Hersman, 1997; Mulligan et al., 2009).

3.5 Microbial interactions with radionuclides

In the case of release of radionuclide from the waste repository by container failure, microorganisms existing in the groundwater will play a significant role in sorption and migration of radionuclides (Francis, 1990; Pedersen, 1999). Presence of microorganisms in deep groundwater can affect the migration of radionuclides from the repository in various ways. Interestingly, freely moving microbes with a mobile suspended particle possess a higher capacity of radionuclide sorption in comparison to the microbes surrounded by the buffer or host rock (Pedersen, 1999; Pedersen and Albinsson, 1991). They are believed to enhance and speed up the process of migration of escaped radionuclides (Pedersen, 1999). Moreover, the active metabolic function of various microbes can greatly influence radionuclides and their mobility. Depending

upon microbial activities and their state, immobilization or mobilization of radionuclides may take place under DGR as illustrated in *Figure 16* (Pedersen, 2005).

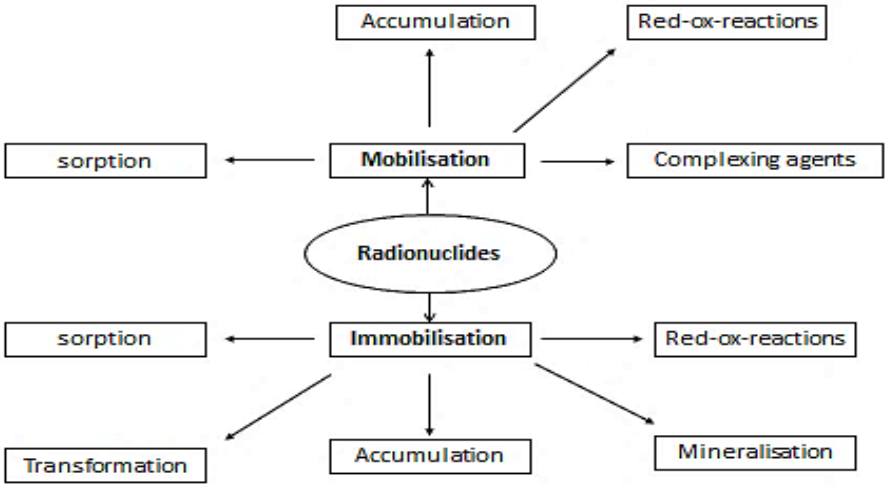

Figure 16: Schematic view of microbial interaction with their surroundings and their effect on radionuclide mobility from geological HLW repositories (Pedersen, 2005).

3.5.1 Biosorption of radionuclides

Among all microbial processes, biosorption is the process which does not necessarily need any active energy-driven function (Pedersen, 2005). It is the result of electrostatic attraction between the negatively charged cell surface and the nuclide cations. Biosorption can take place directly through the interaction of anionic cell wall and nuclide cation or indirectly by either EPS, S-layer, or by capsule (Shukla et al., 2017). Hence, radionuclide sorption on cells is the metabolism-independent sorption of radionuclides onto microbial cells. The sorption could be intracellular or extracellular and both the living and dead biomass have the capability of bio-sorption (Lloyd and Macaskie, 2002; Pedersen, 1999).

3.5.2 Bioaccumulation, biotransformation and biomineralization of radionuclides

Bioaccumulation is the metabolic process where the microorganisms have developed an energy-dependent uptake system for the physiologically important metal. The size and charge of the element may influence the bioaccumulation mechanism (Pedersen, 2005). As for an example, radionuclides like Cs^+ can be taken up by microorganisms through incorporation via the K^+ transport system due to its chemical similarity to K^+ (Kato et al., 2016).

The metabolic process includes an electron donor and an acceptor which combine in redox couples to generate energy for microorganism. Redox reaction has a strong ability to alter the solubility of the radionuclide as their mobility relies on their oxidation state (Pedersen, 2005). The reduction of U^{6+} from a toxic and soluble form to insoluble and less toxic U^{4+} is suggested mechanism for avoidance of migration of this radionuclide through groundwater (Merroun and Selenska-Pobell, 2008). The microbial metabolic processes lead to the formation of oxides, coprecipitates, ionic, organic, or inorganic complexes of radionuclide through transformation and mineralization. Microorganisms can consume oxides in the form of radionuclides and metals such as Tc, Cr as a terminal electron acceptor (Francis and Dodge, 2009; Shukla et al., 2017)

The capability of SRB and IRB to utilize uranium as a substitute for sulfate (Lovley and Phillips, 1992; Pedersen, 2005) and ferric iron (Lovley, 2000; Pedersen, 2005), respectively as terminal electron acceptors have been widely studied to understand this process in relation to DGR. However, it is still not fully understood if this process has any significant importance. Normally, the concentration of sulfate and ferric iron will be much higher than the possible occurrence of U^{6+} hence; escaping of radionuclide from repository and biotransformation may not be significant. However, it has to be verified under in situ conditions (Pedersen, 2005; Shukla et al., 2017). Moreover, microorganisms with the generation of ligands such as phosphate, carbonate, or sulfide are capable of precipitating radionuclides. In the access of ligands, the radionuclide of metal ions should be removed from the solution. Iron oxidizers such as *Gallionella* spp oxidize ferrous to ferric iron and these biological iron oxides may have a retardation effect on radionuclide due to their stalks and sheaths which increases the volume of the iron oxides and helps to trace the radionuclide (Pedersen, 2005).

3.5.3 Formation of chelating agent

Like multicellular organisms, microorganisms also need metal for their metabolism. To access the required element, various kinds of chelating agents are produced by microorganisms. Nevertheless, the produced ligands may not always be specific and may potentially mobilize elements such as heavy metals and radionuclides to the environment (Pedersen, 2005).

4 Effect of deep geological repository conditions on microbial processes

The environmental condition of DGR is extreme in terms of temperature, pressure, density, radiation or salinity, and pH. Microorganisms are selected according to their metabolic abilities and only those who possess the ability to survive will survive (see *Table 4* for extremophiles). Organic or inorganic compounds serve as a source of energy as described in (2 Deep subsurface ecosystem) whereas the source of carbon is fulfilled either by organic carbon (in case of heterotrophs) or by carbon dioxide (autotrophs). Numerous studies have investigated microbial activity under the DGR relevant conditions (Bengtsson and Pedersen, 2017; Fru and Athar, 2008; Masurat et al., 2010b; Pedersen, 2010). These studies demonstrate the response of microorganisms to relatively high radiation, heat, pressure, and redox conditions.

Table 4: Examples of microorganisms living under extreme conditions.

Conditions	Microorganism	Growth limit	Presence	Reference
High temperature	*Bacillus stearothennophilus*	155 °C	Soil, hot springs, ocean sediment	(David and Merson, 1990)
Low temperature	*Psychromonas ingrahamii*	-12 °C	Sea ice	(Riley et al., 2008)
High pH	*Bacillus pseudofirmus*	11.4	Soil/alkaline soda lake	(Janto et al., 2011)
Low pH	*Picrophilus torridus*	0	Solfataric locations	(Schleper et al., 1995)
Radiation	*Deinococcus radiodurans*	60 Gy/ hr	Marine, lakes, and deserts	(Daly, 2006)
Salinity	*Haloarcula marismortui*	5 M NaCl	Sea	(Müller-Santos et al., 2009)
Pressure	*Colwellia marinimaniae*	140 MPa	Challenger deep	(Kusube et al., 2017)
Water activity	*Lactobacillus plantarum* (Vegetative cells)	0.2	Gastrointestinal tracts and food product	(Laroche et al., 2005)

Interestingly, some microorganisms were always able to adapt and survive (Libert et al., 2014), partially because some microbes are capable of forming spores. Bacterial endospores have the unique ability to survive under adverse conditions of high desiccation, low water content, heat, and radiation. These spores can exist in a dormant state for long periods resisting unfavorable environmental conditions and have the capacity to revive back to active life when the conditions change. For example, a bacterial spore of 10^5 years old was recovered from an environmental sample (Ratto and Itavaara, 2012).

4.1 Host rock

Clay formations, granite, or crystalline rock serve as the host rock in the disposal system. For the safety assessment of DGR, understanding far-field geochemistry and hydrology is equally important as the near-field from a geomicrobiological point of view as well. Depending upon a rock type, a wide range of groundwater chemistry (dissolved inorganic and organic minerals) has been documented. In crystalline rock, at depth of about 500 m down the ground, the concentration of sulfate and chlorine increases while the concentration of bicarbonate decreases due to precipitation of minerals (calcite). Moreover, alteration in rock-water interaction increases pH and decrease of redox potential (Frape et al., 2003). Nevertheless, the salinity of the surrounding environment can influence microbial processes. Candidate host rocks such as granite and claystone tend to elevate the salinity of the surrounding (Stroes-Gascoyne et al., 2011). The salinity of host rock in the countries located near the coastal area is higher compared to the country that lies far from the cost. It can especially favor sulfur-reducing halophiles such as *Desulfohalobium, Desulfohalobiaceae,* and *Desulfobacteraceae* (Kjeldsen et al., 2007).

4.2 Bentonite buffer

Bentonite consists predominantly of smectite minerals, typically montmorillonite. Commercial high-quality bentonite contains over 80% of montmorillonite. It is selected as a part of the engineered barrier system because of its distinctive properties like low permeability, low hydraulic conductivity, high swelling pressure and high adsorption capacity (Perdrial et al., 2009). Moreover, the buffer material is expected to sorb accidentally released radionuclides through its porous matrix and thus retard the mobility of radionuclide from DGR. However, the process of retardation can be adversely affected if the permeability of the surrounding buffer is

increased and if sorption of radionuclides by the microorganisms is higher than that of buffer and host rock (Pedersen, 1999; Pedersen and Albinsson, 1991).

The chemical composition and structure of bentonite was described in *3.1 Dissolution and mineralization of bentonite buffer* of this chapter. Various kinds of bentonite differing in their respective dominant element are expected to be used by different countries as a buffer material. Besides the primary elemental composition, the amount of other accessory minerals such as calcite, cristobalite, feldspars, gypsum, pyrite, and quartz also vary in different bentonites. Different exchangeable interlayer cations in clay buffers resulted in variation in different bacterial populations (Perdrial et al., 2009). *S. putrefaciens* showed better growth in nontronite than in MX-80 clay and the reason behind this was the presence of Ca cation of nontronite interlayers. Ca serves as an ionic bridge between negatively charged bacteria and negatively charged clay particles. Divalent cations are known to ease bacterial adhesion which further helps to access nutrients for the bacteria at the mineral surface (Perdrial et al., 2009; Simoni et al., 2000). On the other hand, montmorillonite of MX-80 comprises more Na than Ca in the interlayers and thus, the degree of divalent bridging is decreased. On the same hand, high osmotic swelling pressure of MX-80 generally creates a limitation for the bacterial access of minerals that can reduce the growth of bacteria. It is by the formation of gel because of extensive hydration of monovalent Na that prevents the survivability and mobility of bacteria while the bentonite containing Ca as the dominant element at the interlayer do not form a gel (Perdrial et al., 2009).

Normally, the condition in the repository is expected to inhibit the microbial processes because of insufficient nutrient, water, and restricted pore space (Pedersen, 2000; Pedersen et al., 2000; Perdrial et al., 2009) however; a certain group of microorganisms with a developed metabolic ability are anticipated to survive. Especially, spore former are likely to exist over the period in the repository (Fru and Athar, 2008; Perdrial et al., 2009). Bacteria are present to the pore spaces and attached to the mineral surfaces especially during the early stages of hydration. Accessory minerals like calcite and pyrite are also present in the aggregates. The micro-pores get closed and the pressure increases due to swelling but to survive this environment, as a protection mechanism bacteria produces EPS. Simultaneously, new pore space is provided by the pyrite oxidation and calcite hydrolysis. After the death of bacteria due to compaction and dissolution of

calcite, additional pore space is created where extra swelling can take place and at the same time, remaining EPS is also bound to aggregates. It is suggested that in the vicinity of EPS, aggregates serves as nucleation sites (Perdrial et al., 2009). Männik et al. (2009) have reported bacterial growth and motility in sub-micron constrictions. It has been demonstrated that *E. coli* and *B. subtilis* are motile in microfabricated channels with an only marginal difference in the width of the channel being surpass than their diameter. At the smaller width of the channel (<0.8 μm), the mobility disappeared but *E. coli* still managed to penetrate the channel with a width smaller than its diameter by a factor of approximately 2 by growth and division. Under such constriction, bacteria are significantly squeezed but still possess the ability to grow and divide. Unexpectedly, after leaving the channel, a variety of anomalous cell shapes was acquired by *E. coli*. However, *B. subtilis* was not observed passing through a channel with a smaller width than its diameter. This result show that sub-micron size pores and cavities are surprisingly prolific where bacteria can still be present and undergoes morphological adaptations (Männik et al., 2009). The pore size of the bentonite buffer is usually 100 to 1000 times smaller than the average size of most microbes which inhibits the migration of microbes inside the buffer. Yet, likelihood of microbial transportation is still there as some natural microbes are more tolerant (Mulligan et al., 2009; Pedersen et al., 2000) and could adapt to small size by starvation (Ratto and Itavaara, 2012). Moreover, fracture and faults in bentonite enables reactivation of indigenous bentonite microorganisms as well as introduction of groundwater bacteria to the barrier system. The viable microbial biomass in Fennoscandian shield groundwater from the depth up to 1000 m has been determined. This work showed that microorganisms in deep groundwater could vary expressively in size and metabolic responses are a function of the prevailing condition of the environment (Eydal and Pedersen, 2007). Masurat et al, (2010b) investigated sulfide production in bentonite supplied with groundwater from the borehole of Äspö Hard Rock Laboratory. SRB from both, groundwater and bentonite was concluded to be responsible for sulfide production (Masurat et al., 2010b). This study showed that the groundwater has an ability to migrate within the buffer material.

4.2.1 Swelling pressure and water activity

Bentonite clays have high water affinity and swell when they 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). Furthermore, the compaction of bentonite and finite volume in the rock 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). Therefore, a_w can be a limiting factor for the microbial processes (Masurat et al., 2010b; Motamedi et al., 1996). Normally, a_w above 0.6 to 0.7 is required for the microbes to maintain their life. If not, they can survive as spores in a dormant phase (McCabe, 1990). However, (Laroche et al., 2005) has demonstrated that thermophilic *Lactobacillus plantarum* vegetative cells can still survive at a_w of 0.2.

The effect of the physical properties of highly compacted bentonite on the culturability of indigenous microorganisms was studied by (Stroes-Gascoyne et al., 2010). Wyoming MX-80, commercially available bentonite, was compacted to different dry densities ranging from 800 to 2000 kg/m^3. Bentonite plugs were saturated with the distilled deionized water comprising 0 to 200 g/l of NaCl in incremental of 50 and with granitic water with some dissolved solids (0.7 g/l from 240 m level and 89 g/l from 420 m level) from Atomic Energy of Canada Limited‘s Underground Research Laboratory (URL) for 40-90 days. It was concluded that a_w less than 0.96 and swelling pressure more than 2 MPa could suppress microbial culturability below the background level. Yet, to achieve this condition under actual repository situations, dry density must not be below 1600 kg/m^3. Additionally, it was also claimed that high salinity of porewater, >100 g/l could be a major cause to keep water activity <0.96 and culturability of aerobic microbes under background level. Nevertheless, under such tough environmental conditions microorganisms have a strong ability to survive as inactive spores.

4.2.2 Density of compacted bentonite

High compaction of bentonite does not favor microbial activities as it results in high swelling pressure, low porosity, and low water viability. It has been confirmed that high compaction of bentonite can suppress the growth of microorganisms only if the dry density of bentonite is ≥ 1600 kg/m^3 because higher compaction only deactivates microorganism to a significant level but not necessarily eliminate or kill them (Masurat et al., 2010b; Stroes-Gascoyne et al., 2011). Masurat et al., (2010b) conducted a study under repository relevant conditions to measure the microbial sulfide generation in compacted bentonite at densities of 1500, 1800, and 2000 kg/m^3. The results disclosed that the bentonite density negatively correlated with the sulfide production

rate and the sulfide production rate at 2000 kg/m^3 was a hundred to thousand times lower than the rate needed to corrode copper material over 100000 years (Masurat et al., 2010b). Likewise, (Stroes-Gascoyne et al., 2011) experimentally reduced dry density of compacted bentonite from 1600 kg/m^3 to 1000 kg/m^3 and the result showed the recovery of cultivability of microorganisms. It was concluded that a reduction in dry density stimulated the growth and cultivability of indigenous microorganisms. In a repository, reduction of bentonite dry density should be effectively minimized by implication of well-designed highly compacted bentonite.

4.2.3 Thermo-hydro mechanical effect of bentonite on microorganism

Understanding the effects of thermo-hydro-mechanical (T-H-M) behavior of bentonite buffer on the survivability of microorganisms is principally important for the assessment of engineered barrier systems development and related estimation of the safety performance of DGR (Aoki et al., 2010). Encapsulated waste is a considerable source of higher thermal energy which induces heat convection forming a complex hydrologic system and thermal stress altering the mechanical properties of the buffer. Coupled T-H-M phenomenon results in change in hydraulic conductivity, alternation in permeability and porosity of bentonite buffer, the occurrence of fissure or fracture of buffer or host rock, and change in water pressure (Hudson et al., 2001). All these factors have a strong potential to influence the microbial process in the DGR.

Many studies (Collin et al., 2002; Hudson et al., 2001; Plötze et al., 2007; Tsang et al., 2012; Villar and Lloret, 2004) have been focused on coupled T-H-M phenomenon though a very few studies were published on microbial analysis in association with T-H-M. Aoki et al., (2010) has investigated the activity of microorganisms in compacted (1653 kg/m^3) OT-9607 bentonite as an impact of T-H-M phenomenon at the Kamaishi Mine, northeast Japan. Deep groundwater from host rock containing heterotrophic bacteria including viable SRB, NRB, and denitrifying bacteria was used for the experiment. The T-H-M experiment was conducted with a heater up to a temperature of 100 °C for 260 days and gradually cooled to room temperature for 180 days. Also, water content and dry density of the plugs were 15% and 1656 kg/m^3, respectively. After the termination of the experiment, bentonite samples were analyzed for the detection of survival of naturally occurring microorganisms. The result disclosed the existence of viable aerobic heterotrophic bacteria in the bentonite samples. Nonetheless, they were reduced in bentonite samples bearing low water content (<12%). At the same time, the water content of the buffer

increased with decreasing temperature owing to the distance from the heater. The water content in the buffer material is the crucial factor for the survival and activity of a viable microbial population in bentonite. This study suggests that because of compaction, heat, desiccation, and lower water content, microbial processes are strictly limited near the waste container, until the conditions remain undisturbed (Aoki et al., 2010).

4.3 Temperature

Radioactive waste can generate radiation and heat even after the fission process has stopped. Consequently, the temperature of buffer material is higher due to radioactive decay heat released from the container (Ye et al., 2014). Ideally, the amount of SNF in the container and the distance between containers in the DGR is selected in such a way that the surrounding temperature will reach around 80 °C at the warmest location (Pedersen, 1999). However, in some concepts, the temperature is expected to be about 90-100 °C. The high heat emissions from the container together with the resulting desiccation of the surrounding bentonite buffer are two major factors that should avoid microbial activity (Bennett and Gens, 2008). In agreement with this, (Pedersen et al., 2000) presented a report where the spore-forming SRB was the only surviving bacteria at 80 °C after a 28-week experiment in compacted MX-80 bentonite. Nevertheless, other research has shown a lower effect of heat on microbes. Sulfide producing activity of SRB was detected in bentonite that was heated to 120 °C for 15 hours though the rate of sulfide production was lower by 1.3 to 16 times than in control-treated at 25 °C (Masurat et al., 2010b). Similarly, heat treatment of the MX-80 bentonite at 110°C for 170 hours failed to eradicate sulfate producing bacteria (SPB). Instead, intensive sulfide-producing activity and large numbers of cultivable SPB were observed (Bengtsson and Pedersen, 2017). Greater resistance to wet heat treatment appears to be a distinctive character of spores that can withstand harsh exposure of temperatures higher than 100°C for long periods (Setlow, 2006). It has been reported that mechanisms involved in killing of spores are different for wet and dry heat. Wet heat damages the core proteins of the spores, whereas dry heat is responsible for the damaging of the DNA (Setlow, 2014).

4.4 Radiation

The alpha decay of actinides like ^{235}U, ^{237}Np, ^{239}Pu, ^{241}Am, and ^{244}Cm and the beta decay of fission products like ^{90}Sr and ^{137}Cs are the main sources of radiation in HLW repository (Ewing et al., 1995). During the first 500 years of DGR, the beta decay of fission products will dominates the radiation flux because of a shorter lifetime, and then the alpha decay of actinides will be the dominant source, because of their much longer half-lives. After 10^3 years, the total absorbed dose of beta or gamma radiation is estimated to be 600 MGy that gives an average dose rate of 68.5 Gy per hour and for alpha radiation, it would be 90 MGy giving an average dose rate of 10 Gy per hour radiation. Further, after 10^6 years, the total dose absorbed of beta or gamma radiation is predicted to be 1 GGy with an average dose rate of 0.11 Gy per hour while it is estimated to be 800 MGy with an average dose rate of 0.09 Gy per hour for alpha radiation. (Brown, 2014) These values only give an indication of doses in the vitrified waste-forms themselves; alpha radiation will be absorbed by the waste and its container whereas gamma doses away from the container surface will decrease significantly with distance (Brown et al., 2017).

Research on radiation dose in a simulated HLW container carried in Boom clay demonstrated that the dose rate can be as high as 400 Gy per hour at the interface between the container and clay, in decreasing order to 25 Gy per hour at 20 cm distance (Noynaert et al., 1998). A different dose rate of radiation at the outer surface of SNF container is expected by different countries. To illustrate, Finland expected to have a dose rate of around 0.33 Gy/hr while the expected dose rate for Sweden and Switzerland is less than 0.1 Gy/hr (Bennett and Gens, 2008). It is suggested that the dose rate at the waste container surface should not exceed 1 Gy/hr to prevent the effect of radiolysis of water in saturated bentonite or to limit the occurrence of humid air before water saturation (Werme, 1998). Radiation in the form of alpha, beta, and gamma rays from the decay of radioactive material is defined as ionizing radiation. Ionizing radiation induces various changes in cells directly or indirectly leading to cell death (Benetti, 2015) as presented in *Figure 17*.

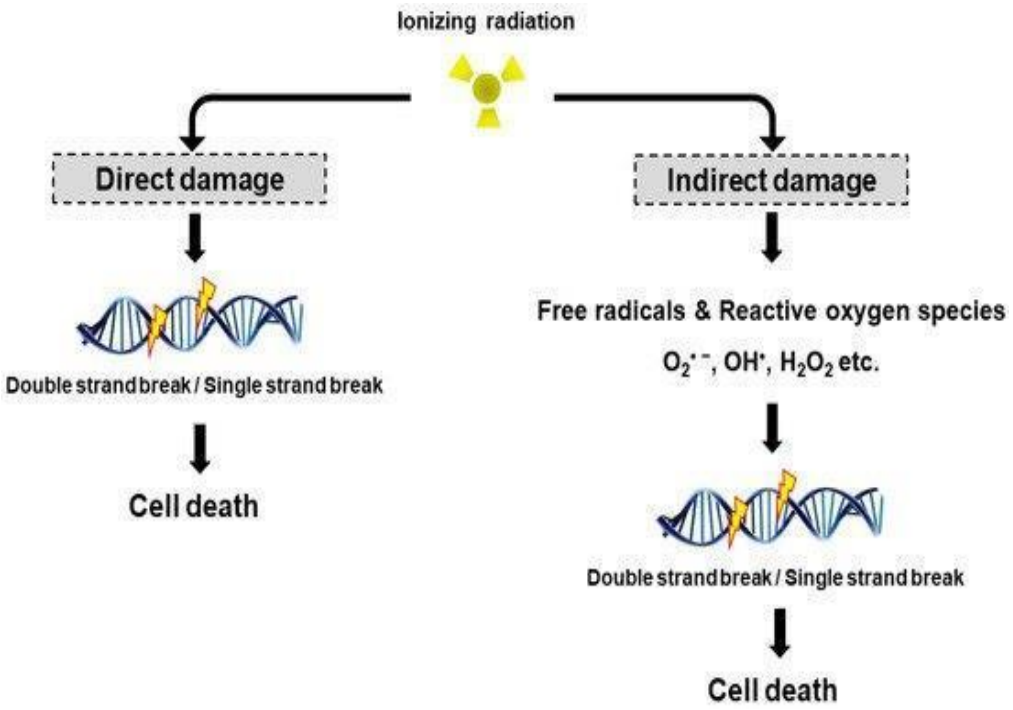

Figure 17: Effect of ionizing radiation on cells (Benetti, 2015).

Decimal reduction dose (D_{10}) is widely used to determine the microbial resistance to the radiation, which is stated as the dose of radiation (kGy) needed to decrease the number of microorganisms by one log. This means the dose should be enough to reduce 90% of the total number of microorganisms (van GERWEN et al., 1999). The radiation sensitivity of indigenous microorganisms in different bentonites was studied by (Stroes-Gascoyne et al., 1994). Depending upon the type of bentonite, the D_{10} dose was between 0.65 and 1.68 kGy at a dose rate of 100 Gy per minute for naturally occurring microorganisms. Survivability of microorganisms decreased with increasing total radiation dose.

Despite exposure to high radiation, some bacteria can still survive and are resistant to radiation. The bacterium that can grow under high chronic Gamma radiation (60 Gy per hour) or recover from acute doses greater than 15,000 Gy is *Deinococcus radiodurans* which is a non-spore-forming bacteria (Daly, 2006). The average D_{10} dose for this species is 10,4 kGy (van GERWEN et al., 1999). Resistance to desiccation and oxidative/hypertonic stress of *Deinococcus radiodurans* is also relatively high. The radiation resistance phenomena of this bacterium are probably connected to its high tolerance for desiccation as both effects cause very similar cellular damage (V Mattimore and Battista, 1996) are driven by two major mechanisms.

The first is the efficient repair of damaged DNA and the second is efficient cellular mechanisms, see *Figure 18* (Jin et al., 2019). The effect of irradiation on indigenous microorganisms from bentonite and groundwater is presented in chapter II of the experimental part.

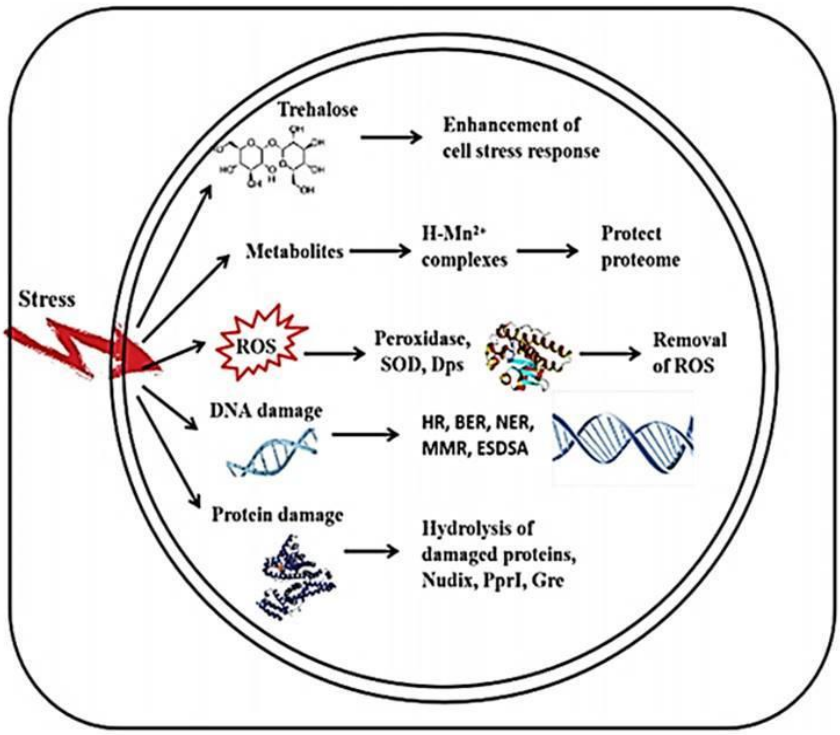

Figure 18: Radiation resistance mechanism of Deinococcus spp. (Jin et al., 2019).

4.5 Concrete barrier and high pH

Concrete, a cementitious material, plays a major role in engineered barrier systems. Concrete is used not only as a structural element in the waste repository for encapsulation of LLW or ILW but also for the backfilling and sealing as a plug of the repository. The cementitious material with high gypsum and soluble alkalic content causes an increase in pH in the surrounding environment (Williams et al., 2017). Therefore, pH values in the vicinity of concrete are expected to be as high as 13.5 (Bertron et al., 2013); moreover, the pH of bentonite is alkaline (Ye et al., 2014). The high alkaline pH of the concrete creates a relatively non-hostile environment for the microbial activity. Microbial communities at the interfaces between the cementitious material, bentonite buffer, and host rock can be severely affected by the presence of concrete. However, there are many alkaliphilic microorganisms capable of surviving in high pH environments. The

detail on alkaliphilic microorganisms has been provided in the chapter IV of this thesis along with the effect of concrete on indigenous microorganisms of bentonite and groundwater.

Alkaliphilus transvaalensis, an endospore forming SRB was isolated from a deep gold mine in South Africa. It can withstand a pH ranged from 8.5 to 12.5 and temperature from 20 to 50°C (Takai et al., 2001a). Similarly, a moderate thermophilic *Desulfotomaculum alkaliphilum* was found to reduce sulfate to sulfide under the condition with pH 8 to 9.15 and temperature 30 to 58 °C (Pikuta et al., 2000). Usually, high pH is favored by nitrate-reducing bacteria (NRB) because they are metabolically very active in alkaline conditions (Bertron, 2014). Nitrate reducers are responsible for creating a more reduced environment within the repository that favors the propagation of other anaerobic microorganisms (Bertron et al., 2013). Moreover, the metabolic products of nitrate and sulfate reducers (such as organic acids, mineral acids, or sulfur compounds) are chemically aggressive to cementitious material which results in a decrease in pH and then mineralogical and microstructural changes in cementitious surface (Bertron, 2014).

EXPERIMENTAL PART

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

1 Background

Microbial activities at DGR can compromise the safety of the buffer barrier. It is, therefore, very important to understand the possible influence of microorganisms before radioactive waste storing. To know the possible microbial effect, the characterization of the microbial community in both groundwater and bentonite is essential. Deep underground and bentonite represent a large and heterogenic (phylogenetically and metabolically) pool of microorganisms. Additionally, the diversity of microorganisms in long-term stored bentonite barriers is not driven by whether particular microbes will get there but depends on prevailing environmental conditions that will establish after the closure of the repository.

Microbial communities present in groundwater sources and bentonite in the Czech Republic have been poorly characterized. Moreover, microbial metabolic processes and effects related to Czech bentonite were not investigated before this study. In the Czech Republic, different types of bentonite are available such as BaM, BCV, B75, and S65. The elemental composition of particular montmorillonites varies a lot among different bentonites and there is also a variable amount of other accessory minerals in bentonites such as feldspars, quartz, cristobalite, gypsum, calcite, and pyrite (Karnland et al., 2006). BaM bentonite was proposed to be used in Czech research projects as candidate bentonite to be used in DGR. Different countries have proposed different clay as a barrier material. For instance, France has chosen clay of Callovo-Oxfordian age and has performed an intensive study on it. Similarly, Spain is performing a study on FEBEX bentonite in context to their repository. We cannot depend upon the results produced by other countries simply because each buffer type behaves differently. Therefore, it is important to study Czech bentonite for a better understanding of the nature of the material. Other reasons behind abiding by Czech sources are the availability of materials and lower cost.

The widely used method for the study of microbial diversity in bentonite is the determination of cultivable bacteria (Lopez-Fernandez et al., 2015; Persson et al., 2011). However, this technique can detect only a small proportion of bacterial populations present (*Figure 19*). The other approach is to use molecular biology tools, such as qPCR and sequencing. However, extraction of DNA from bentonite is very difficult, because DNA is absorbed onto bentonite and is protected against chemical or enzymatic degradation of the cell (Perdrial et al.,

2009). Investigation on the interrelation between DNA and clay has concluded that the interaction is influenced by several factors such as ionic strength, mineralogy of the sorbent, length of DNA and pH of the medium (Paget et al., 1992). Moreover, DNA could be immobilized in soil with higher clay contents and adsorption of DNA by the clay provides a good means for DNA to be protected against nuclease activity. The electrostatic interaction between the absorbed cations and phosphate anion is connected to the higher affinity of DNA to the surface in the case of a divalent cation charged montmorillonite. The phosphate anion has a strong binding with Ca^{2+} than Mg^{2+} (Paget et al., 1992). The presence of Ca^{2+} in the bentonite establishes an ionic bond between bentonite and negatively charged bacteria. Such divalent cation provides adhesion to bacteria and access to nutrients on the mineral surface (Julia N Perdrial et al., 2009). Thus, it makes the extraction process more difficult and complicated. Another reason for the poor DNA yield could also be because of low biomass present in the bentonite. Some researchers have isolated DNA from bentonite using detergent followed by the thermal lysis process (Lopez-Fernandez et al., 2015; Selenska-Pobell et al., 2001) while some researchers preferred commercial DNA isolation kit (Engel et al., 2019).

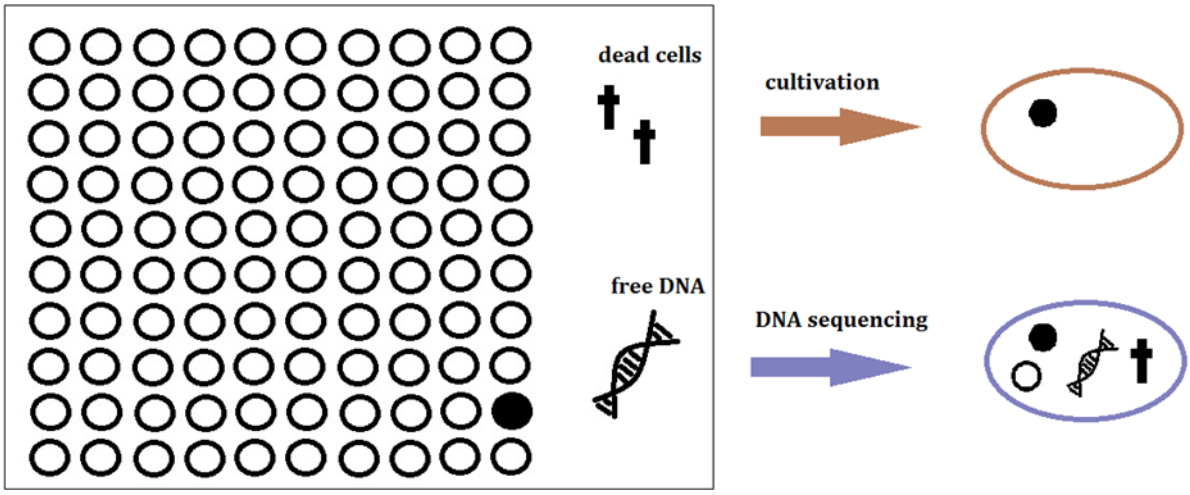

Figure 19: Image illustrating the microorganisms' content in groundwater or bentonites detectable by cultivation or DNA sequencing method. Circles represent viable cells. Filled and empty circles represent the cultivable (active) and uncultivable (dormant) cells respectively. The cross shows dead cells and the DNA molecule represents free DNA. Using cultivation methods only a few to less than one percent of viable cells can be analyzed. Using DNA sequencing information about cultivable, uncultivable microbes, dead cells and free DNA can be gained. (Ševců et al., 2018).

The first objective of this study was to characterize the microbial community present in deep groundwater and biofilm samples from two deep groundwater sources in the Czech Republic and select the most suitable groundwater source(s) to carry further experiments. The second objective was to analyze the microbial communities present in the homogenized and raw Czech bentonite from Černý vrch and describe their diversity using molecular biology tools.

2 Materials and method

2.*1* *Groundwater*

For the study of microbial activities in an environment similar to DGR in a laboratory, natural groundwater has been used as a source of microorganism. In this study, two deep groundwater sources 1) Bukov Underground research facility (Bukov URF) and 2) Josef Underground research Centre (Josef URC) in the Czech Republic (*Figure 20*) were studied.

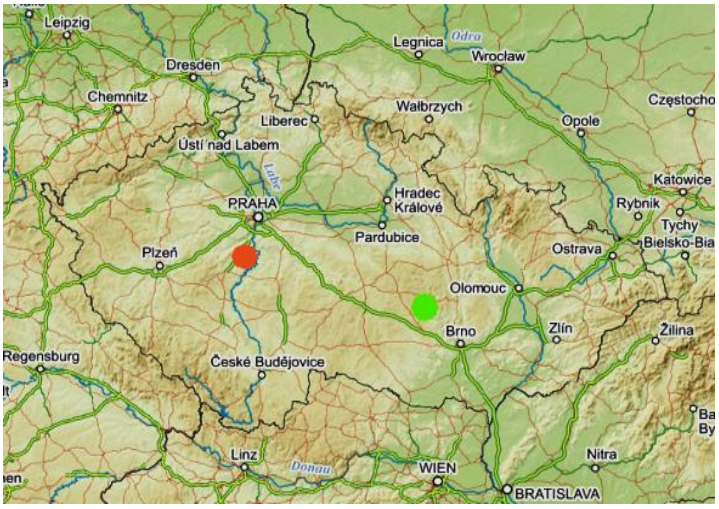

Figure 20: Map representing the location of underground sources. Josef URC spotted by orange color and Bukov URF spotted by green color.

Bukov underground facility is located near the village of Bukov, in the Žďár nad Sázavou district of the Vysočina region which lies in the south part of Rožná uranium mine. It is situated at the crystalline rock at approximately 520 m down the ground surface. To be precise, the geochemistry of this groundwater is of Ca–HCO_3 type, mostly presenting a low kind of mineralization (up to 0.3 g/l) (Havlová et al., 2015). From Bukov URF, water was collected from seven different sources together with two biofilm samples.

Figure 21: Images representing Bukov URF. A) Water source from BK06 B) Biofilm near-source BK23 C) Collection of water sample flowing from ceiling and D) Measurement of pH from BK18 Source.

Likewise, the Josef URC is located at Psí Hory gold-bearing district. The Josef gallery passes through Veselý hill across the rock and connects two gold-bearing deposits – Čelina deposit and Mokrsko deposit that is named after villages situated in their neighborhood (Pacovská et al., 2012).

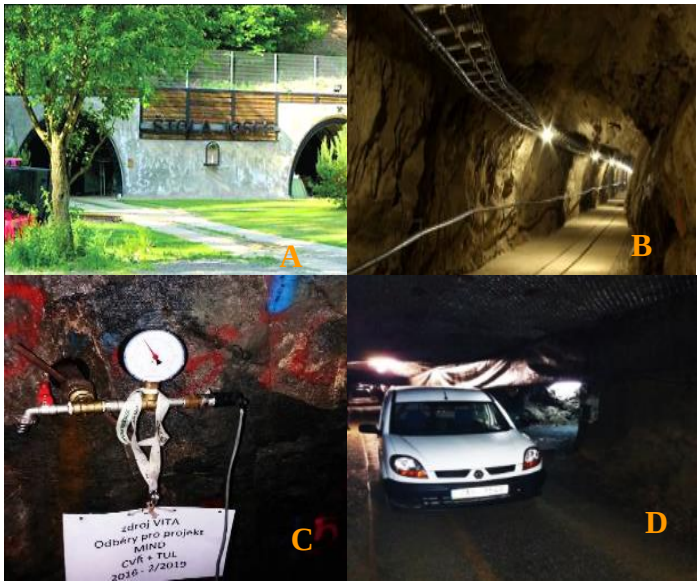

Figure 22: Images representing Josef URC. A) Entrance to Josef URC B) Underground tunnel C) Main tap of source VITA water under 2.5 bar and D) Transport of VITA water in the Josef URC tunnel.

This underground research center is located in granitic rock beds at approximately 125 m down the geological surface, however; the overburden height varies from 30 m (Čelina-West) to 180 m

(Mokrsko) (Pacovská et al., 2012). From Josef URC water was sampled at two sites: VITA and HV1 located relatively close to each other.

2.2 Czech Bentonite

BaM bentonite from Černý vrch (north-western region of the Czech Republic) was obtained from Keramost a. s. (the product is called Bentonite a montmorillonite "BaM'') to study microbial diversity. Two types of bentonites were analyzed 1) homogenized bentonite and 2) raw unhomogenized bentonite (*Figure 23).* It consists of 78.2% of montmorillonite and its natural water content is up to 8% by weight. BaM bentonite includes 7.59% of Fe, 2.48% of K, 2.46% of Mg, and 1.22% of Ca by weight (Matal et al., 2018).

Figure 23: Homogenized bentonite on the left and raw (unhomogenized) bentonite on the right.

2.3 Molecular biology analysis

2.3.1 Water sampling and filtration

Clean plastic water bottles were used for sampling after the exposure of empty bottles for 5 min under UV light. Bukov URF has a different form of water sources like flowing water, water falling from rock fractures, and even a tap fitted on a pipe and hence, were sampled simply by filling the bottles without the use of pressure pump. Conversely, for the Josef URC a pressure pump was used for sampling the water from the well. A water sample collected from underground sources was processed by filtration to concentrate the biomass underflow hood maintaining the sterile environment. Water samples were filtered using a metal filter apparatus, *Figure 24*. To avoid any kind of contamination, the filter apparatus was autoclaved for 1 hour at

170 °C. On the same hand, 0.22 µm GV Durapore® filter membrane (Germany) with a 47 mm diameter was used to filter the water samples. The membrane filter containing bacterial biomass after filtration was stored under -80 °C until the DNA extraction.

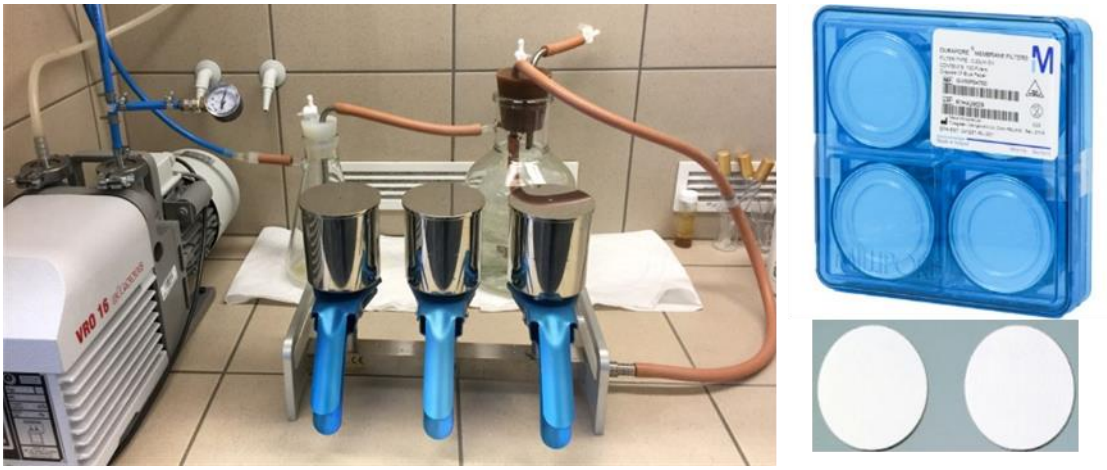

Figure 24: Filter apparatus for filtration of water (left), 0.22 µm GV Durapore® filter membrane (right).

2.3.2 Extraction of DNA from water samples

Bacterial DNA from water sample and biofilm were isolated according to the manufacturer's instruction using a commercial kit, PowerWater® DNA Isolation Kit, catalog number: 14900-50-NF from MO BIO (Carlsbad, CA, USA). The procedure involves chemical lysis of cells followed by mechanical lysis and precipitation of DNA using ethanol.

2.3.3 Extraction of DNA from Bentonite

To study the microbial diversity in the two Czech bentonites, DNA was extracted from 10 g of each bentonite. SDS was used to lyse the cells. Furthermore, lysis was combined with precipitation of extracted DNA with polyethylene glycol followed by the purification step using AXG-100 cartridges. This extraction technique followed a protocol used by (Lopez-Fernandez et al., 2015; Selenska-Pobell et al., 2001).

2.3.4 Quantification of genomic DNA

Quantification of extracted genomic DNA was performed using a Qubit 2.0 fluorometer (Life Technologies, MA, USA). The Qubit is a small fluorometer instrument used for quantification of DNA, RNA, and protein. It uses fluorescent dyes to determine the concentration of nucleic acids.

2.3.5 Library preparation and next-generation sequencing (NGS)

Library preparation is an initial step for the sequencing of genomic DNA. Primers 530F and 802R as shown in *Table 5* were used for amplification of variable V4 region of 16S rDNA gene for sequencing of amplicons. The size of the amplicon was kept below 400 bp to cover as much microbial diversity as possible by performing *In silico* analysis of primers (Němeček et al., 2017).

Two consecutive Polymerase chain reaction (PCR) reactions per sample were performed during library preparation. Primers 530F and 802R were used in the first PCR reaction and the PCR conditions were as follows: 95°C for 3 min; 15 cycles at 98°C for 20 s, 50°C for 15 s and 72°C for 45 s; and a final extension at 72°C for 1 min. Subsequently, we performed a second PCR reaction with tagged barcode fusion primers. We used 21 differently tagged bar code fusion primers in one library preparation which enabled us to sequence up to 20 samples (plus mockup) in one run. The second PCR was performed as follows: 95°C for 3 min; 35 cycles at 98°C for 20 s, 55°C for 15 s and 72°C for 45 s; with a final extension at 72°C for 1 min. The quality of the library product was checked by gel-electrophoresis technology. 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).

Table 5: Primers for amplicon sequencing of the 16S rRNA gene.

Primer	**Sequence 5´- 3´**	**Coverage**			**Reference**
		Archaea	**Bacteria**	**Eukaryotes**	
530F	GTGCCAGCMGCNGCGG	54.9	96.9	94.0	(Dowd et al., 2008)
802R	TACNVGGGTATCTAATCC	91.8	92.5	0.9	(Claesson et al., 2009)

Following this step, the barcode-tagged amplicons from different samples were 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).

2.3.6 NGS data processing

Sequence data were analyzed by the pipeline SEED v. 1.2.3 (Větrovský and Baldrian, 2013). Sequences of insufficient quality or mismatches in tags were removed from the dataset. All sequences with minimal read length 275 bp were clustered into operational taxonomic units (OTUs) and chimeric sequences were removed using UPARSE implementation in USEARCH 7.0.1090 (Edgar, 2013) with a 97% similarity threshold. The consensus from each OTU was constructed from a MAFFT alignment (Katoh et al., 2009) based on the most abundant nucleotide at each position. The OTUs were identified and their environmental requirements were assessed by the mega BLAST and BLASTn algorithms against GenBank nt/nr database.

3 Results and Discussion

Two deep groundwater sources 1) Bukov Underground research facility (Bukov URF) and 2) Josef Underground research center (Josef URC) in the Czech Republic were analyzed for the microbial characterization to select the most suitable water source(s) for further experiments. The results of the microbial characterization of the different water sources sites are shown in *Table 6*. In Bukov URF, the most important factor determining microbial diversity was the opportunity to oxidize reduced sulfur- and iron-containing compounds. BK23 was influenced by a higher concentration of iron which is in agreement with the biofilm composition at the BK23 source. The biofilm contained *Gallionella* and more iron-oxidizing autotrophs, *Ferriphaselus*. Heterotrophic bacteria detected in BK23 were different from those detected in all other sources and is most probably closely connected with biotic processes in thick biofilm. Overall, BK23 was very poor in terms of microbial abundance as well as of its diversity. Microbial diversity of other sources, especially BK6, 6B, 7, 15, and the ceiling, was homogenous, probably due to the anthropogenic impact. In BK18, anaerobic bacteria such as *Desulfobulbus* and ferric iron-reducing *Ferribacterium* were found to dominate. Some members of oxidizing bacteria and other

autotrophs were present as a probable sign of anthropogenic activity. Overall, the low abundance of microorganisms is most probably caused by the scarcity of energy sources. The relatively high diversity in this nutrient-limited environment was rather unexpected.

The water sources from Josef URC, VITA, and HV1 were closely located to each other, and despite this closeness, the microbial community composition was significantly different. The main reason for this is that VITA is anoxic whereas HV1 has the same water source but also has a free surface water level. The microbial diversity in the VITA sample was low due to the high selective pressure of the environmental conditions with typical SRB representatives such as *Desulfobulbaceae*, *Desulfomicrobium*, *Desulfovibrio* and *Desulfovibrio* which can accelerate corrosion of waste container and fermenting anaerobic bacteria like *Spirochaeta*. Sulfate reduction and oxidation of various organic compounds were the main metabolic processes detected in VITA. The microbial community in HV1 was poor and limited mainly by the scarcity of electron acceptors.

The microbial community structures of groundwater sources collected at Bukov URF and Josef URC were very different. A strong anthropogenic impact was observed in most of the water samples along with biofilm samples collected from Bukov URF. Surprisingly, only two water sources (VITA at Josef UCR and BK18 at Bukov URF) contained bacteria representing a typical anaerobic environmental condition. By the investigation of the microbial community present in these two groundwater sources, the source VITA from Josef URC was selected to be used as an inoculum for further experiments because it was mostly dominated by anaerobic microorganism including high number SRB than any other source of Bukov URF and was available in sufficient quantities.

Table 6: Results of the 16S rRNA amplicon analysis of groundwater sources: only selected (most common) OTUs with marked abundances are shown. The intensity of color represents the number of OTUs where dark red color indicates a higher number and green being less.

Locality	Bukov URF									Josef URC		Determination
Sample type	water	water	water	water	biofilm	water	water	water	biofilm	water	water	
OTU	BK06	BK6B	BK07	BK15	BK15 - biofilm	CEILING	BK18	BK23	BK23 - biofilm	VITA	HV1	
1	16	15	1387	2378	390	1753	23	55	48	3	0	*Thiobacillus*
2	312	297	2404	867	261	677	102	46	1	1	0	*Sulfuritalea*
3	846	775	199	617	130	1272	22	96	4	1	0	*Nitrospiraceae*
4	915	733	12	6	2	12	49	180	10	1850	0	*Desulfobulbaceae*
5	844	776	720	148	25	148	20	31	0	1	0	*Planktophila*
6	327	450	60	1384	612	763	7	161	3	0	0	*Sulfuricella denitrificans*
7	635	690	106	126	419	205	5	156	48	1	0	*Gallionella*
8	0	0	0	0	0	0	1	0	1	0	3405	unclass. Alphaproteobacterium
10	328	194	268	98	726	136	15	9	0	162	0	*Sulfuritalea hydrogenivorans*
11	32	3	6	2	1	15	52	108	0	0	53	*Hydrogenophaga*
16	138	98	42	23	1	287	1	414	3	1	0	unclass. Gammaproteobacterium
17	15	92	0	5	39	1171	0	0	8	0	0	unclass. Deltaproteobacterium
19	0	5	0	0	0	4	212	1	0	0	0	*Acinetobacter*
20	0	0	0	0	0	0	0	0	0	1282	0	*Desulfomicrobium*
21	0	0	0	0	0	0	1166	0	0	0	0	*Ferribacterium*
22	0	1	0	2	0	0	80	16	1	0	0	*Chromatiales*
23	0	262	0	0	0	500	102	0	0	0	1	*Massilia*
26	9	2	9	6	0	2	289	113	3	0	1	*Rhodobacteraceae*
28	0	124	0	0	0	0	0	0	0	0	0	*Arthrobacter*
30	203	46	3	28	40	5	410	0	1	0	0	*Chlorobi*
32	195	1	4	27	175	0	48	1	1	0	0	*Sphingomonas*
35	12	15	4	14	8	12	311	0	1	6	4	*Ralstonia*
38	0	3	4	0	0	0	71	3	0	0	0	*Novosphingobium*
47	1	0	0	2	0	0	105	10	0	450	23	*Desulfovibrio*
51	31	2	19	20	64	0	27	104	90	0	0	*Hyphomicrobium*
52	8	1	97	3	35	0	16	46	0	15	74	*Brevundimonas*
53	0	0	0	0	0	0	0	0	0	0	491	*Desulfobulbaceae*
54	105	118	1	0	0	1	1	10	3	138	0	*Desulfovibrio*
60	3	0	1	2	0	0	9	2	0	1	403	*Sulfurospirillum multivorans*
65	22	30	149	0	0	42	7	22	0	1	0	*Lysobacter*
66	0	4	0	1	7	0	0	216	149	0	0	*Ferriphaselus*

The homogenized bentonite (BaM) and the raw bentonite samples from Černý vrch were more similar than expected in terms of the microbial community structure based on the results from the operational taxonomical unit (OTU) analysis which is presented in *Table 7*. Most OTUs were shared between the two samples. Out of 126 shared OTUs with a frequency higher than 10, only 18 of them had a very asymmetric distribution (the ratio between the two samples 1:10 or 10:1). Beta- and Alphaproteobacteria dominated in both bentonites.

Table 7: Result of the 16S rRNA amplicon analysis of bentonites showing only selected OTUs.

OTU	BaM" homogenized bentonite	Raw bentonite	Determination
1	454	98	*Thiobacillus*
7	19	68	*Gallionella*
11	143	200	*Hydrogenophaga*
26	88	47	*Rhodobacteraceae*
28	112	57	*Arthrobacter*
32	87	12	*Sphingomonas*
34	1	772	*Phreatobacter*
35	59	252	*Ralstonia*
37	29	165	*Novosphingobium*
38	64	144	*Novosphingobium*
40	280	96	*Bradyrhizobium*
44	5	211	*Aquabacterium*
45	398	47	*Xanthomonadaceae*
52	115	42	*Brevundimonas*
62	288	36	*Arenimonas*
63	81	227	*Nitrosomonas*
65	71	10	*Lysobacter*
67	395	1	*Beijerinckiaceae*
68	315	44	*Lysobacter*
81	135	82	*Microbacteriaceae*
89	67	71	*Comamonadaceae*
95	23	72	*Acidobacteria*
98	62	13	unclassified
102	68	11	unclassified
105	142	16	*Luteimonas*
114	58	58	*Nocardioides*
122	1	211	unclassified
135	5	190	*Methylophilaceae*
157	67	10	*Bacteroidetes*
161	87	32	*Micrococcineae*
201	5	70	*Porphyrobacter*
203	66	38	*Curvibacter*
214	75	3	*Bradyrhizobiaceae*

Chemolithotrophic bacteria with a possible corrosion capability were present, though in lower abundances. Typical soil bacteria like *Bradyrhizobium, Lysobacter, Methylocapsa, Microbacteriacea,* and *Acidobacteria,* were present. These bacterial taxa are also known to inhabit oligotrophic environments. Interestingly, chemolithotrophs that could utilize Ammonia, Manganese, Ferrous, and sulfide as electron donors were present in relatively high abundances in both bentonite samples. Invariable are *Thiobacillus, Gallionella, Rhodobacteraceae,* and *Nitrosomonas.* Moreover, species of genus like *Rhodobacteraceae, Brevundimonas,* and *Novosphingobium* are capable of utilizing nitrate as a terminal electron acceptor. This could be explained by slow and long-term adsorption of reduced compounds onto bentonite from the upper layers of soil in the Černý vrch mine and the consequent establishment of oxidative conditions during mining. The species of nitrate-reducing genera can result in the formation of gas as a part of their metabolic activity. As a consequence, gas pressure could be built up due torast disparity occurred between the gas diffusion and gas production which can lead to crack or fracture of the host rock (Mulligan et al., 2009).

4 Summary

Microbial characterization of two different groundwater sources (Bukov URF and Josef URC) and Czech bentonites 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.

Water from Bukov URF was collected from seven different sources together with two biofilms despite which the result demonstrated a strong anthropogenic impact in almost all of the sources. Nonetheless, only two sources were analyzed from Josef URC where one of the water source named VITA was dominated by anaerobic microorganisms especially 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 groundwater source was selected to be used as an inoculum for ex-situ study of microbial processes in DGR stimulated conditions.

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 groundwater 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 community in bentonite

1 Background

The repository conditions in the near-field following high radioactive waste deposition generally evolve from initially warm and oxidizing to cool and anoxic in the long term and consists of four phases summarized by King et al. (King et al., 2017): (i) immediate post-placement, when the environment will be aerobic and the radiation dose rate will be at its highest level. The second stage is (ii) dry-out, whose duration will depend on the initial compacted bentonite moisture content. At the third stage, (iii) container re-wetting and saturation of buffer at the near-field environment takes place. The last stage (iv) is the long-term anoxic phase, which begins once the near-field reaches full saturation. It is the period of the continued cooling and anoxic conditions. Because of these extreme conditions, most early analyses considered repository to be either a completely sterile environment, or at least not seriously threatened by bacterial activity (Stroes-Gascoyne and West, 1997). Nevertheless, many microorganisms show extreme adaptability to various unpleasant environmental conditions. For this reason, the conditions in the early stages post-deposition do not need to have so devastating effect on microbial survivability as previously expected. One of the most important factors responsible for creating an extreme environment in the repository is ionizing radiation. Many studies (Bengtsson and Pedersen, 2017; Masurat et al., 2010b; Pedersen et al., 2000; Stroes-Gascoyne et al., 2010) have evaluated the effect of bentonite compaction, level of desiccation, and temperature on the bacterial survival in the bentonite under repository conditions. Nevertheless, these studies were performed without radiation. According to the Swedish KBS-3 HLW repository concept, the expected maximum dose outside the canister is less than 0.5 Gy/hr (Svensk Kärnbränslehantering AB, 2006). On the other hand, the Canadian HLW concept predicts the maximum dose rate of 52 Gy/hr at the surface of a Titan waste canister or dose rate 15 Gy/hr for the surface of the copper canister (Stroes-Gascoyne et al., 1995).

In the dry environment, microorganisms generally possess tolerance to higher radiation due to adaptation to desiccation, which causes similar kinds of cell damage as radiation (Mattimore and Battista, 1996). Conversely, spores' resistance to radiation was observed to rise with an increase in the moisture content of the environment. The dose of 25 kGy was required for the inactivation of *Bacillus atropheus* spores dry powder, while 35 kGy was required for the inactivation of *Bacillus atropheus* spores in liquid suspension (Hilsen et al., 2005). Cytoplasmic

water radiolysis resulting in the production of reactive oxygen species (ROS) - hydroxyl radical (HOo), ionized water (H_2O^+), hydrogen radical (Ho), and hydrated electrons (e^-) is the most significant change in cells caused by radiation. A successive chemical reaction produces another molecule which damage DNA (by destroying the bases or breaking double strands) or other vital biomolecules like RNA, proteins or lipids eventually resulting in the cell death (Azzam et al., 2012; Reisz et al., 2014; Riley, 1994).

This study aims to develop knowledge about the effect of Gama radiation on the indigenous microbial community in bentonite and study the evolution of microbial community under anaerobic conditions. Bentonite suspension was exposed to the constant dose rate of 13 Gy/hr up to 19,656 Gy total absorbed doses. Irradiation of samples in completely anaerobic conditions was not possible due to the aerobic installation of the irradiation chamber, but we attempted to minimize the oxygen concentration in the experimental system to achieve as much the repository relevant condition as possible. However, the presence of oxygen could not be fully controlled. Completely anaerobic non-irradiated samples were used to study the evolution of microbial community under anaerobic conditions.

2 Materials and Method

2.1 Bentonite and VITA water

Czech BaM bentonite produced commercially by the Keramost company, Obrnice plant, and natural groundwater (VITA) collected at the Josef URC, Czech Republic a day before performing of each experiment were used to study the effect of different level of radiation on indigenous microorganisms in bentonite and VITA water. The pH of the VITA water was ≈ 7.8. During the collection, the anaerobic VITA water was poured into sterile bottles and the contact time with the air was minimized during the handling. The VITA water was transported to the laboratory in Centrum výzkumu Řež s. r. o. (Research Centre Řež, RCR) and kept under anaerobic conditions inside the argon-purged anaerobic glove box (gaseous oxygen concentration < 1 ppm volume) (Jacomex GP, France) until the experiment began.

2.2 Sample preparation and Experimental design

The next day after VITA water collection, 64 samples in glass reagent bottles were prepared, each containing 60 g of deoxygenized BaM bentonite and 200 ml 1:1 mixture of VITA water and deoxygenized filter-sterilized tap water. The glass reagent bottles were used because they are fully resistant to radiation. All the preparations were performed in an anaerobic box (*Figure 25*). From these samples, 20 were used for irradiation, 22 as anaerobic samples, and the last 22 samples were used as anaerobic with additional nutrients (2.46 mM sulfate and 0.29 mM nitrate final concentration), *Table 8*. The glass reagent bottles were tightly sealed with several layers of parafilm membrane that was able to keep the oxygen level in the bottle below 0.4% in the oxic environment (measured by the oxygen sensor in an anaerobic box).

Figure 25: Preparation of samples for irradiation experiments in anaerobic box.

In the collaboration with RCR, the prepared anaerobic samples were kept in the argon-purged anaerobic glove box during the whole course of the experiment. The samples for irradiation were placed in the irradiation chamber Prazdroj situated in ÚJV Řež, a.s. Irradiation was conducted with a rod source ^{60}Co ϒ (cobalt 60), nominal activity 500 TBq. Samples were situated regularly in the circle around the cobalt source and Alanine/EPR spectroscopy (electron paramagnetic resonance) was used to measure the actual doses, *Figure 26*.

Table 8: Sampling schedule of irradiated and anaerobic samples and cumulative doses of Gama radiation. C_ana is anaerobic control and C_ana_nr is anaerobic control with nutrient.

Sampling time	Cumulative dose (KGy)	Irradiated samples (IR)	Anaerobic samples	
			C_ana	C_ana_nr
0 day	0	0	2	2
1 week	2.184	2	2	2
2 week	4.368	2	2	2
3 week	6.552	2	2	2
4 week	8.736	2	2	2
5 week	10.92	2	2	2
7 week	15.288	2	2	2
9 week	19.656	2	2	2
13.5 week	not*	2	2	2
18 week	not*	2	2	2
22 week	not*	2	2	2
Number of samples		**20**	**22**	**22**

*Irradiation stopped at 19.656 Gy

The samples in the irradiation chamber were irradiated up to 9 weeks by the dose rates of 13 Gy/hr resulting in the maximum total absorbed dose of 19,656 Gy. After reaching this dose, the remaining samples were stored up to three months to detect the microbial recovery after the irradiation. The experiment was sampled each week in duplicates based on the schedule in *Table 8*.

Figure 26: Samples inside the irradiation chamber, diameter about 100 cm.

2.3 Sample processing

To detect the microbial composition of VITA water, defined amounts (500-1000 ml) of VITA water samples were filtered through sterile 0.22 μm GV Durapore® filter membrane, and the filters were stored in a deep freezer and subsequently used for DNA extraction as described below.

At each sampling time, approximately 50 ml of each bentonite suspension sample was centrifuged at 11500 × g for 15 minutes. The supernatant (40 ml) was used for chemical analysis, the remaining pellet was used for DNA extraction. The supernatant and pellet were stored in a deep freezer (-80°C) until further processing.

2.4 Molecular biological analysis

2.4.1 DNA Extraction and measurement

The VITA water was filtered and the DNA was extracted as described in chapter I, section 2.3.1 and 2.3.2, respectively. For the extraction of DNA from bentonite pellets, DNeasy® PowerMax® Soil Kit from QIAGEN was used according to the manufacturer's protocol. Approximately 15 g of the bentonite pellet was used for the extraction from each sample. Concentration and purification of isolated DNA from bentonite was performed by Zymo Research kit following the manufacturer's protocol. The concentration of genomic DNA was subsequently measured by Qubit® 2.0 Fluorometer (Invitrogen, Life Technologies, USA) according to the manufacturer's protocol.

2.4.2 Quantitative PCR (qPCR)

Quantitative PCR was used to describe relative changes in selected bacterial groups in the bentonite samples. Because obtaining standards fully reliable to our environmental samples is very difficult and absolute quantification without a standardized calibration curve is not possible, we used relative quantification (RQ) using ΔCq (crossing point) calculation method. It estimates the magnitude of difference in Cq values between the sample zero state at the beginning of the experiment and the sample after treatment (or in time in no treatment samples) using the formula $RQ = effectivity^{(-\Delta Cq)}$. PCR effectivity for each marker was estimated beforehand by measuring the slope of curves constructed from a serial dilution of template DNA from five internal

environmental standards. The measured Cq values were normalized by the sample volume used for DNA extraction before calculations.

Specific markers for various bacterial groups (such as total Eubacteria, sulfate-reducing bacteria, nitrate-reducing bacteria, and *Geobacteraceae*) were used. These markers were amplified using primers described in *Table 9* on a LightCycler ® 480 Instrument (Roche Biochemicals, USA).

Table 9*: *qPCR primers. SRB – sulfur-reducing bacteria, NRB – nitrate-reducing bacteria, IRB – iron-reducing bacteria.

Primer	Sequence 5´- 3´	Specificity	Description	Reference	Annealing temp.
16SqPCR-F 16SqPCR-R	TCCTACGGGAGGCAGCAGT GGACTACCAGGGTATCTAATCCTGTT	All bacteria	Gen for 16S rRNA	(Clifford et al., 2012)	60°C
RH1-aps-F RH2-aps-R	CGCGAAGACCTKATCTTCGAC ATCATGATCTGCCAgCGgCCGGA	SRB	Functional bio-marker gene *apsA*	(Ben-Dov et al., 2007)	60°C
RH1-dsr-F RH3-dsr-R	GCCGTTACTGTGACCAGCC gGTGGAGCCGTGCATGTT	SRB	functional bio-marker gene *dsrA*		
nirK 1F nirK 5R	GGMATGGTKCCSTGGCA GCCTCGATCAGRTTRTGGTT	NRB (denitrifying bacteria)	*nirK* gene for nitrite reductase	(Geets et al., 2007)	60°C
nirS cd3AF nirS R3cd	GTSAACGTSAAGGARACSGG GASTTCGGRTGSGTCTTGA	NRB (denitrifying bacteria)	*nirS* gene for nitrite reductase		
nosZ-F nosZ 1622R	CGYTGTTCMTCGACAGCCAG CGSACCTTSTTGCCSTYGCG	Nitrous oxide reductase bacteria (NORB)	*NosZ* gene for N_2O reductase		
Geo494F Geo825R	AGGAAGCACCGGCTAACTCC TACCCGCRACACCTAGT	Geobacteraceae (IRB)	Amplifying specific region of 16S rRNA	(Wei and Finneran, 2011)	55°C

Reaction mixtures were prepared in 10 µl of reaction volume. The mixture contained 1 µl of DNA template, 5 µl KAPA SYBER FAST qPCR kit (Kapa Biosystems. Inc., MA, USA), 0.4 µl of µM forward and reverse primer mixtures (Generi Biotech, Czech Republic, IDT, US) and 2.6 µl ultra-pure water (Bioline, UK). For each DNA sample, qPCR reaction was performed in duplicate along with negative control where DNA template was replaced by nuclease-free water.

Reaction conditions consisted of initial 5 min incubation at 95°C, followed by 45 cycles of denaturation at 95°C for 10 s, annealing at 60°C for all primer except for Geobacteraceae which was 55°C for 15 s and extension 72 °C for 20 s with a final extension at 72°C for 3 min. Finally, a melting curve was set for 5 s at 95°C, 1 min at 65°C and final ranging from 60 to 98°C, with a temperature gradient of 4°C per 10 s. The purity of the amplified fragment was determined through observation of a single melting peak. Crossing point values were obtained using the 'second derivative maximum' method included in the LightCycler® 480 Software.

2.4.3 Library preparation and next-generation sequencing

The procedure of library preparation and sequencing followed the same method as described in section 2.3.5 of chapter I.

2.4.4 NGS data processing

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). The accuracy of classification was verified and evaluated against a predefined artificial MOCK community sample containing 4 bacterial strains (*Klebsiella pneumonia, Bacillus subtilis, Staphylococcus aureus* and *Enterococcus faecalis)* and yeast *(Saccharomyces cerevisiae*). DADA2 output was transformed to a Phyloseq object in R and subsequent bioinformatics analyses were performed in the R software using the Phyloseq library (McMurdie and Holmes, 2013). Rarefaction curves were created using the Phyloseq and vegan packages in the R software. The relative frequency of OTUs was visualized by a heat-map showing only OTUs with the mean of relative frequency higher than 1%.

Differential expression analysis on OTU table was done using Deseq2 library (Love et al., 2014). Deseq2 was used to determine taxa being mostly influenced by irradiation and anaerobic condition. We did use no filtering on taxa and padj < 0.05. Deseq2 library estimates variance-mean dependence in count data from sequencing assays and test for differential expression based on a model using the negative binomial distribution. Furthermore, in subsequent metabolic profile analysis, 50 most abundant OTUs were selected for each sample and from these most

frequent OTUs only OTUs successfully determined to particular genera were used for metabolic characterization of the microbial community within the samples. On average 84% of OTUs were used in the final metabolic analysis. For each OTU successfully determined to particularly known genus, we searched through the literature and listed the information about Gram stain, spore-forming, trophy, used electron acceptors, donors and oxygen requirement in this genus and we used these categories to characterize the bacterial community and compare it with prevailing Physico-chemical condition within the samples.

2.5 Chemical analysis

We measured pH by SenTix 980 combined IDS electrode with liquid electrolyte (WTW, Czech Republic). For chemical analysis, the supernatant of each sample was analyzed by ion spectroscopy to detect the concentrations of nitrate and sulfate in the samples. The anion concentration was determined using a Dionex ICS 90 chromatograph (ThermoFisher Scientific, USA) with 8 mM K_2CO_3 a 1 mM $KHCO_3$ as the mobile phase in a Dionex IonPac AS14A column. The flow rate of the mobile phase was 1ml/min and 10 µl of the sample was always injected.

3 Results and Discussion

We aimed to estimate the effect of irradiation on the indigenous microbial community in bentonite under anaerobic conditions, which would best resemble the conditions expected in the DGR post-closure. However, as we will demonstrate further, the parafilm lining of the bottles had strongly degraded during the irradiation and the residual oxygen in the irradiation chamber influenced the results. We are thus not able to precisely distinguish the effect of irradiation from the effect of oxygen presence. For the same reason, the completely anaerobic samples kept in the anaerobic glove box for the whole time are not fully comparable to the irradiated samples. Nevertheless, the anaerobic samples can still be very useful to see the evolution of bentonite microcosm under anaerobic conditions and to detect microorganisms (or the whole microbial communities), that can be important for the long-term safety of DGR.

3.1 Molecular biological analysis

3.1.1 Microbial abundance in the bentonite suspensions

The microbial abundances were estimated relatively (i.e. compared to the zero sample microbial abundances) by the qPCR (*Figure 27)*. Only the gene which has a change in relative abundance is shown in the figure. In the irradiated samples, the total biomass (estimated by 16S rRNA gene) was clearly lower than in both types of anaerobic samples during the whole experiment. Microbial biomass gradually decreased after the fourth week of irradiation in these samples. The observed decrease can have two possible explanations. It can be caused by the effect of radiation or/and by the presence of oxygen and resulting limitation by the available electron donors. At the end of the fourth week, the applied radiation dose reached approximately 8,736 KGy. The applied dose of 7.0 KGy was reported to have a lethal effect on *E. coli* (Hieke and Pillai, 2018). Similarly, about 99% of fungal spores were eliminated in a silt loam soil irradiated at 10 kGy (Johnson and Osborne, 1964). Alternatively, the decrease in the microbial abundance can be explained by the limitation in available electron donors in the closed reaction system.

Generally, microorganisms preferentially couple both aerobic and anaerobic respiration with the oxidation of various organic substrates that are energetically most favorable electron donors (Madigan et al., 2018). As a result, microorganisms in the closed microcosms can be very limited by the available electron donors (Matschiavelli et al., 2019) (i.e. the level of available organic substances), especially in aerobic conditions, where they cannot switch to another favored electron donor such as molecular hydrogen, that often occurs anaerobically (see below). Although we have not tested this hypothesis for BaM bentonite, we have observed a strong effect of electron donor availability in both aerobic and anaerobic microcosms without additional electron donors in subsequent microcosm experiments with another Czech bentonite BCV (unpublished results). We thus assume that both factors could simultaneously affect the microbial community abundance in irradiated samples and further experiments would be needed to reliably estimate the single effect of both.

Although the massive proliferation of sulfate (SRB) and iron reducers (IRB) in both types of anaerobic samples (see below) was observed, these microbial groups were almost not detected in irradiated samples. It is not very surprising because the IRB and SRB are obligatory anaerobes

or at least microaerophiles (Bergey et al., 2015). The absence of these microbial metabolic groups in the irradiated samples was probably caused by a prevailing aerobic condition in the samples rather than the effect of irradiation, because (Brown et al., 2015) showed, that both iron and sulfate reducers were able to survive in the irradiated bentonite microcosms and the iron reduction was even stimulated at the 0.5 Gy/hr radiation dose. The density of facultatively anaerobic nitrate reducers was rather similar to the densities detected in anaerobic suspensions. It was highest at the beginning of the experiment and subsequently gradually declined in time.

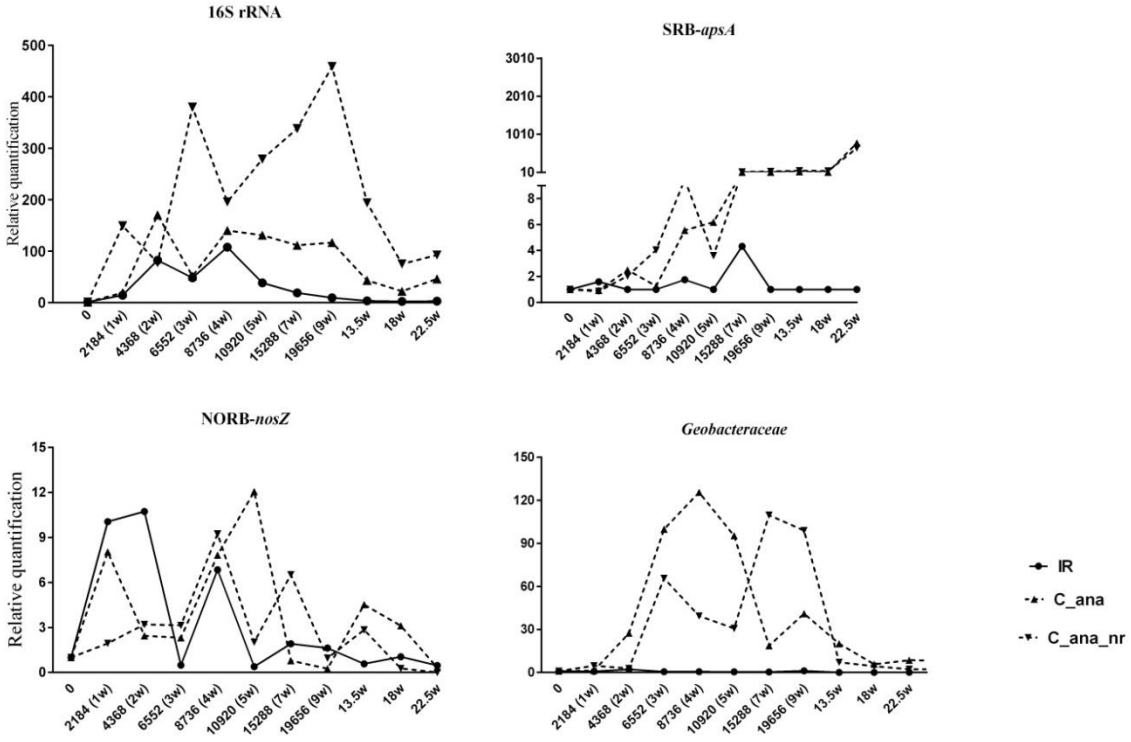

Figure 27: Relative quantification of changes in microbial abundance in irradiated and anaerobic samples. IR- Irradiated samples, C_ana – Anaerobic sample without nutrients, C_ana_nr – Anaerobic sample with nutrients and w – week.

Though the quantity of the total biomass in irradiated samples at the end of the experiment (19,656 Gy cumulative absorbed doses) was reduced and was only two to three times higher than detected in zero samples at the beginning of the experiment, the applied dose was not sufficient to completely eradicate all bacteria in the bentonite-VITA suspension. Moreover, a big proportion of the detected effect could be also caused by the nutrient limitation as described

above. Interestingly, we have not detected any sign of microbial recovery past irradiation in none of the studied markers. This result is contradictory to the postulation made by (Pitonzo et al., 1999a, 1999b) where they stated that irradiated bacteria (up to 9.34 kGy total dose) might be resuscitated to the completely cultivable state with time when environmental conditions become more favorable. However, it is highly probable that the recovery might not be possible due to nutrient limitation that was described above. In both types of anaerobic samples, a gradual increase in the overall microbial abundance was detected from the first week of the experiment until the approximately 14th week, when the density started to slowly decline. The microbial abundance in the samples with added nutrients was approximately two to three times higher than in samples without nutrients. This nicely demonstrates the positive effect of additional electron donors.

The use of specific qPCR markers helped us to distinguish between different microbial communities. The abundance of nitrate reducers (NRB) was generally rather low and highly variable during the experiment. The highest peak in their relative abundance was observed in around the fifth week in both types of anaerobic samples. The iron reducers (G*eobacteraceae*) proliferated gradually since the beginning of the experiment and had the highest abundance in the fourth week in case of anaerobic samples without nutrients and in approximately 7th week in case of anaerobic suspensions with nutrients. The addition of nutrients thus seems to have some effect on the evolution in the microbial ecosystem in studies suspension. Subsequently, the iron reducers seem to be partially replaced by another microbial population. We observed a rapid increase in the SRBs relative abundance in both types of anaerobic samples at the end of the experiment, although their density was slightly increasing also before. Such a result well corresponds to the detected rapid decline of sulfate concentration in these samples (see below).
Similar shifts in microbial community composition in closed microcosms under anaerobic conditions were also described by (Brown et al., 2015) or (Matschiavelli et al., 2018, 2017).

3.1.2 Microbial composition

Generally, the composition of VITA water was very different from the composition of BaM bentonite with only a few genera being present in both environments, such as *Pseudomonas* and *Massilia* (*Figure 28*). Based on the detected microbial profiles, we can generally state that most of the bacterial genera unique to VITA water (not present in BAM) were not able to adapt to the

new environment of bentonite suspension. The indigenous bacteria from BaM thus formed a crucial part of the microbial community in bentonite suspensions. High variability in microbial composition was also found in the first sampling points (1-3) of irradiated and anaerobic samples. We ascribe this stochastic pattern on one hand to the generally low DNA concentration of the zero point samples and on the other hand also to the rapid changes during this awaking phase. Subsequently, a gradual change in microbial community composition was observed in all the studied samples with clear difference between irradiated and anaerobic samples.

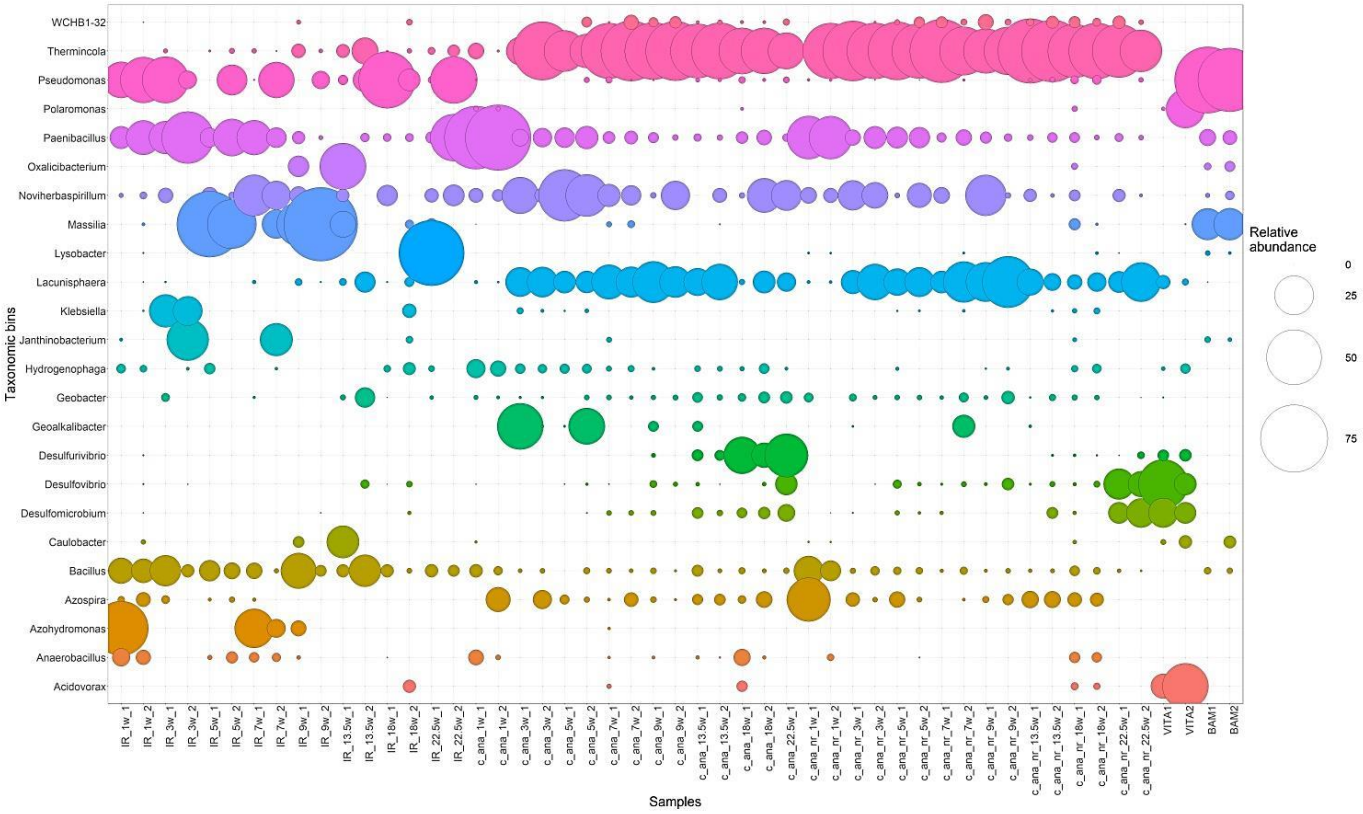

Figure 28: Relative abundance of the genera in VITA, BaM, and their suspension samples throughout the experiment. IR- Irradiated samples, C_ana – Anaerobic sample without nutrients, C_ana_nr – Anaerobic sample with nutrients and w – week.

The irradiated samples were dominated by a few genera such as *Massilia*, *Pseudomonas*, *Noviherbaspirillum*, *Paenibacillus*, and *Bacillus* most of the time. All these genera belong to facultative anaerobes that use organic material (or H_2 in the case of *Noviherbaspirillum*) as electron donors. On the other hand, the abundance of other facultatively anaerobic genera

Janthinobacterium and *Azohydromonas* common at the beginning declined noticeably in the irradiated sample in time and no sign of their recovery was detected in the later phases of the irradiation experiment. A similar trend was followed by two other genera, *Lysobacter* and *Acidovorax.* Both of these genera are heterotrophic facultative anaerobes and were detected only after 18 weeks though *Lysobacter* was observed profoundly. In general, we have not observed any sign of possible recovery of either the microbial community or any particular genus after the irradiation phase finished. However, all the genera dominating the irradiated samples can be considered as high radiation tolerant with the ability to survive at least 20 kGy total absorbed dose of Gama radiation. McNamara et al. showed that majority of soil bacteria need about 20 kGy doses of ionizing radiation to be eliminated, but a dose higher than 70 kGy may be required to kill certain radio-resistant bacteria, which is in accordance with the result of our experiment (McNamara et al., 2003).

The microbial composition of anaerobic samples was clearly different than in irradiated samples except for the few facultatively anaerobic genera such as *Paenibacillus, Noviherbaspirillum,* or *Bacillus,* that were common both in irradiated and also anaerobic samples. Anaerobic samples were clearly dominated by the anaerobic IRB genus *Thermincola* proliferating since the third week of the experiment and its presence well agrees with the qPCR detected peak of *Geobacteraceae*. Other most frequent genera are auto/heterotrophic facultatively anaerobic *Noviherbaspirillum*, facultatively anaerobic *Paenibacillus,* and aerobic heterotrophic *Lacunisphaera*. As the anaerobic samples were strictly anaerobic, we assume, that detected representative of the genus *Lacunisphaera* species must either be capable of anaerobic respiration or fermentation, which is an unknown feature of this new genus (Rast et al., 2017), or it can represent a contaminant species from lab environment or the DNA extraction kit (Salter et al., 2014). We have detected an increase in the abundance of SRB genera such as *Desulfovibrio, Desulfurivibrio,* and *Desulfomicrobium* in anaerobic samples which well agrees with the results of qPCR.

Based on the sequencing results, Deseq2 analysis was performed comparing the genera in irradiated and anaerobic samples (see *Figure 29*). Genera specifically enriched in the irradiated sample have positive value falling above zero levels in the analysis while the genera distinct to anaerobic condition fall below the zero levels with a negative value. The irradiated samples were

enriched especially in genera *Herpetosiphon* and *Azohydromonas.* Generally, most of the bacteria specifically enriched in irradiated samples belonged to the phylum Proteobacteria. Although we cannot distinguish between the effect of irradiation and oxygen presence, when we compare the irradiated samples with anaerobic ones, the genera enriched in the irradiated sample can be definitely considered as radiation tolerant as they could withstand irradiation dose of approximately 20 KGy. On the other hand, anaerobic samples were specifically enriched in many genera with the biggest effect visible for *Geoalkalibacter, Anaerosporomusa, Caenimonas,* or *Desulfurispora.*

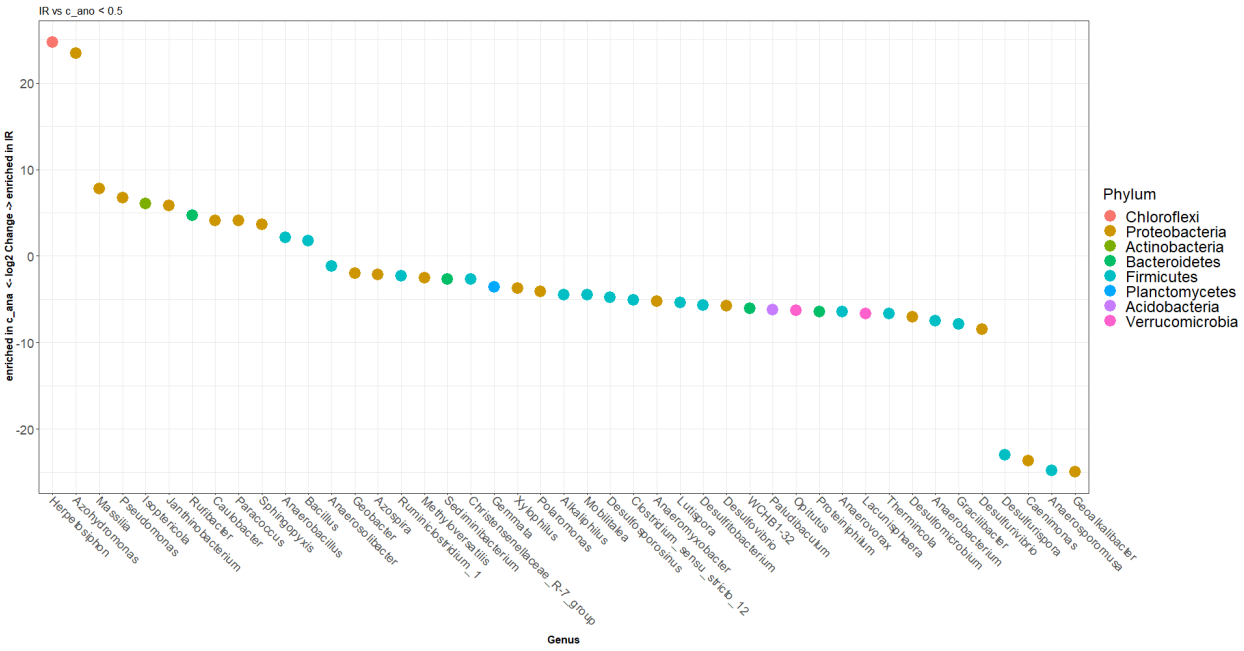

Figure 29: Deseq2 analysis comparing genera in irradiated samples (IR) and anaerobic samples (C-ana) based on the sequencing result.

3.1.3 Microbial metabolic profiles

Based on the known information from the literature, the dominant species detected in the samples in their metabolic requirements and the ability to form the spores were thoroughly characterized. Such analysis helped us to reveal microbial processes ongoing in the studied samples. The metabolic profile analysis of genera detected by NGS agreed well with the conclusion we have driven from the qPCR data analysis. A large difference between the anaerobic samples and irradiated samples was found (*Figure 30*), which we ascribe mostly to the

presence of aerobic conditions in irradiated samples. When the metabolic features of microorganisms detected in VITA water or BaM powder were analyzed, it was found that about a half of the detected genera in VITA water are autotrophic, heterotrophic or mixotrophic genera that use organic compounds, molecular hydrogen or reduced sulfur compounds as electron donors. On the other hand, in BaM powder, we detected mostly heterotrophs using organic compounds. Similarly to BaM, all the suspensions from the beginning of the experiment contained mostly heterotrophic aerobic or facultatively anaerobic species. However, within three weeks, irradiated samples started to be very different from both anaerobic ones. They were dominated by facultative anaerobes that preferentially respire oxygen, but can switch to nitrate reduction or fermentation in the absence of oxygen. In the constant presence of oxygen, which we assume in these samples, we cannot expect any changes in the preferred electron acceptors as oxygen represents the most energetically favorable electron acceptor.

On the other hand, in both types of anaerobic samples, the samples were first dominated by heterotrophic facultatively anaerobic nitrate reducers, but within three weeks obligatory anaerobic chemolithotrophic (using molecular hydrogen as electron donor) or organotrophic iron reducers dominated the community. Biological reduction of nitrate or iron has an adverse impact on the safety of the waste disposal system. Nitrate reduction can possibly influence the mobilization of radionuclide like uranium by reoxidation under the repository condition (Merroun and Selenska-Pobell, 2008). BaM bentonite is rich in iron (Matal et al., 2018) and IRB can thrive on this substrate for several weeks as was also shown in the similar microcosm experiment with bentonite B36, where (Matschiavelli et al., 2017) detected massive ferric ion reduction in the samples during 14 weeks with the highest peak in the sixth week. Microbial reduction of iron can results in illitization of bentonite buffer due to which the bentonite buffers can lose its swelling properties reducing its sealing effect (Meleshyn, 2014; Mulligan et al., 2009).

Towards the end of the experiment, these metabolic groups started to be replaced by the sulfur and sulfate-reducing microorganisms oxidizing organics or molecular hydrogen. There was a slight decrease in the proportion of IRB at the last sampling points in anaerobic samples and a similar decline of *Geobacteraceae* in the second half of the experiment was detected also by qPCR (*Figure 27*). At the same time, faster growth of the SRB was detected since the fifth

week by qPCR. These results might indicate the decrease in available Fe^{3+} in the old suspension causing the gradual shift in microbial composition from IRB to SRB. Bentonite suspension is naturally rich in sulfate (see below), which would become preferred electron acceptor when the ferric ions are unavailable. The existence of such a process was implied both by the qPCR and also by the chemical analyses (see below) and was also experimentally proven by (Matschiavelli et al., 2018) in a one-year bentonite microcosm experiment.

Both VITA water and BaM powder were clearly dominated by Gram-negative non-sporulating microorganisms at the beginning of the experiment. The low proportion of spore-forming genera detected in the bentonite powder is surprising as bentonite is known to contain predominantly spore formers (Fru and Athar, 2008). This result can thus mean that the extraction efficiency of DNA from the spores is actually low and we should focus on these problems further in the future. Nevertheless, the proportion of Gram-negative non-sporulating microorganisms in most aerobic irradiated samples remained high in the whole experiment, very surprising, because spores generally exhibit five times higher radiation tolerance than vegetative cells (van Gerwen et al., 1999) and we have expected to see the increase of spore formers due to irradiation in our samples. On the other hand, both types of anaerobic samples were rather dominated by Gram-positive spore-formers.

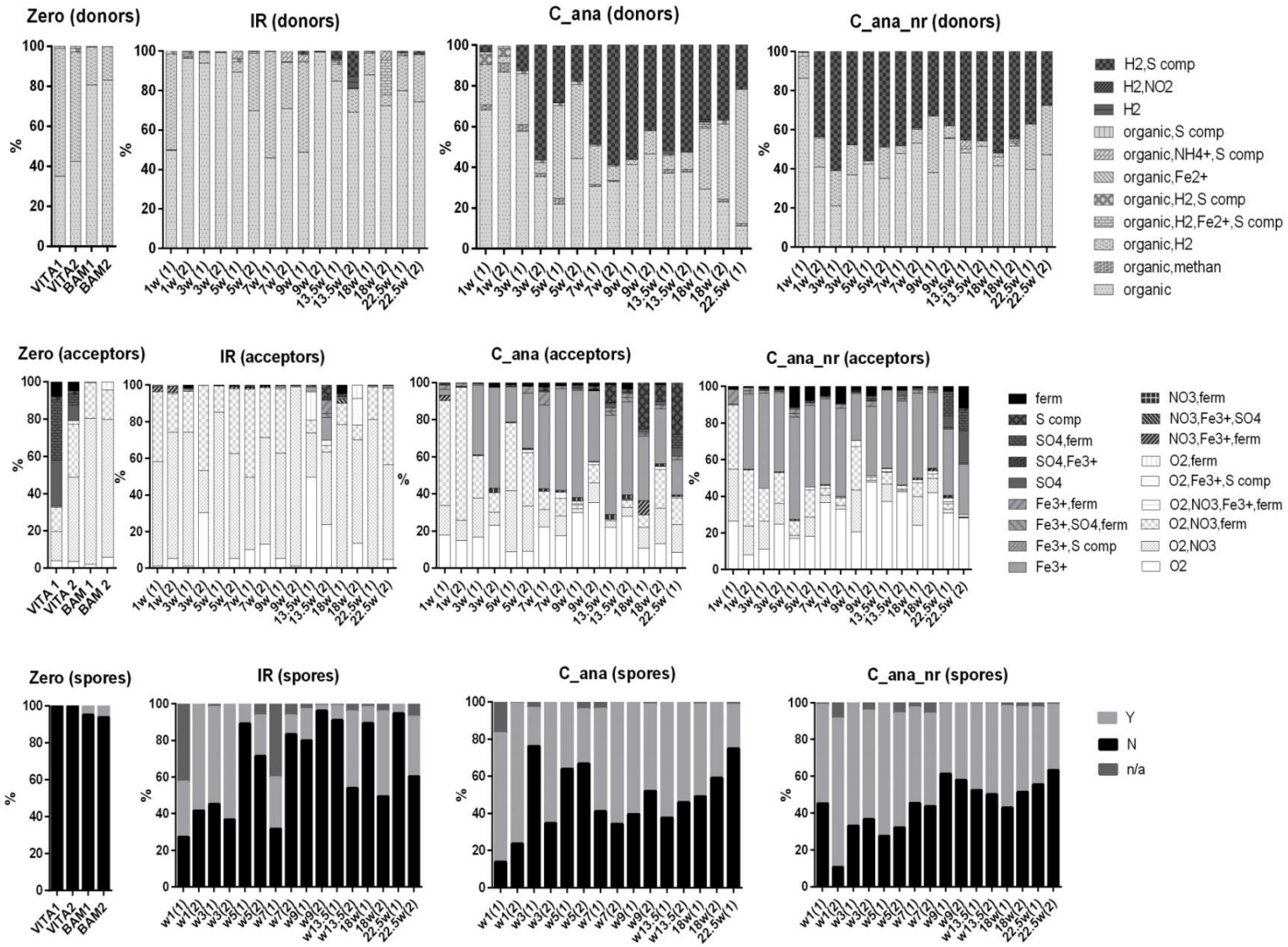

Figure 30: Composition of microbial ecosystems in the original sample (VITA and BaM) at zero time, irradiated and anaerobic samples of bentonite suspension categorized by oxygen requirement, preferred electron donors and acceptors, and spore-forming ability of detected microorganisms. IR- Irradiated samples, C_ana – Anaerobic sample without nutrients, C_ana_nr – Anaerobic sample with nutrients and w – week.

3.2 Chemical analysis

The pH values were generally higher in irradiated samples than in anaerobic samples (*Figure 31***)**. The pH of the irradiated sample remained rather constant throughout the experiment (around 8.5). Anaerobic samples with nutrients have an average pH value of 7.5 while anaerobic samples without nutrients have 7.7. The reason behind the higher pH values in irradiated samples than in anaerobic samples can be attributed to the detected higher population density in anaerobic samples together with different microbial composition and their activity or by different chemical processes ongoing under aerobic and anaerobic conditions.

The concentration of sulfate was highest in anaerobic samples with nutrients which can be attributed to the addition of sulfate at the beginning. The sulfate concentration remained more or less stable (ranging from 285 to 516 mg/l) throughout the whole course of the experiment except for the final sampling point (22.5 w), where its concentration was below the detection limit. In irradiated and anaerobic samples without nutrients, the sulfate concentration ranged from 100 to 130 mg/l. By the end of the experiment, the concentration of sulfate in anaerobic samples without nutrients dropped below the detection similarly to the samples with nutrients. The nitrate concentration was below the detection limit just after the first week in all the samples including the anaerobic samples with added nitrate (figure not shown).

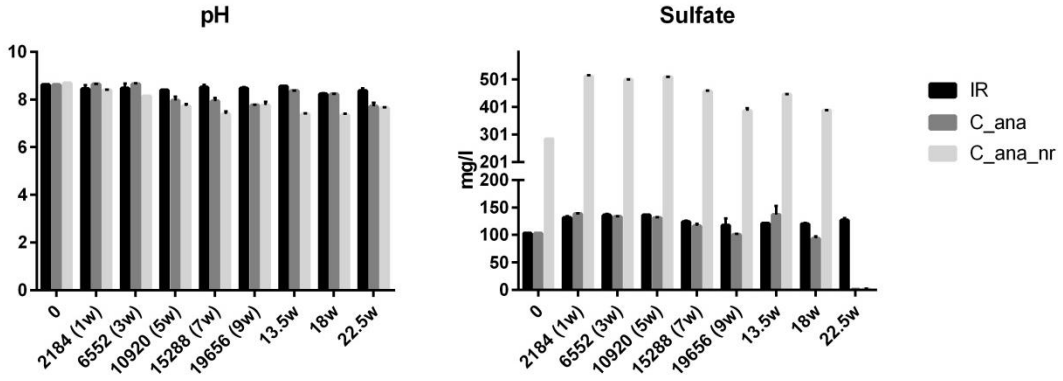

Figure 31: Changes in pH during the experiment and sulfate during the irradiation experiment. For each sampling point were IR- Irradiated samples, C_ana – Anaerobic sample without nutrients, C_ana_nr – Anaerobic sample with nutrients and w – week.

The purpose of adding nitrate and sulfate was to enhance the growth of NRB and SRB, respectively. However, the added concentration of nitrate was too low and was consumed within the first week of the experiment in all samples with no significant effect on the growth of NRB. On the other hand, both natural and added concentrations of sulfate were probably sufficient to promote bacterial growth, but this energetically less favorable electron acceptor was not exploited in the presence of oxygen in aerobic irradiated samples and in the presence of ferric ion in anaerobic samples as discussed above. Massive decrease in sulfate concentration was detected after 22.5 weeks in anaerobic samples which suggests that a longer time frame is required to observe higher microbial sulfate consumption and increase. The rapid increase in the quantity of SRB detected by qPCR in anaerobic samples at the end of the experiment together with the detected decrease of available sulfate indicates the possible transition from the IRB community to the SRB community.

4 Summary

After the closure of the repository, harsh and extreme environment will start to evolve. 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 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 decline in total microbial biomass in time 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. 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 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.

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 conditions

1 Background

The metal container containing waste is the first and the most important barrier that should prevent direct release of radionuclides into the environment for tens of thousands years. 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 microorganisms.

MIC is a synergistic interaction between the metal surface, abiotic corrosion products, and bacterial cells and their metabolites (Beech and Sunner, 2004). MIC can reduce container longevity through two mechanisms, i.e., through direct uptake of electrons from the metal surface by microbial cells or by the production of corrosive metabolites (Černoušek et al., 2020). The former mechanism, involving extracellular electron transfer during microbial metabolism, is termed electrical MIC (E-MIC). Electrogenic microbes, such as SRB, NRB, and acetogenic bacteria, cause E-MIC if there is a local shortage of organic carbon, causing the metal itself to serve as an electron donor. Effective uptake of electrons from the metal can trigger a cathodic reaction, and thus corrosion. The latter mechanism is driven by metabolites secreted by the microbes and hence is termed metabolite MIC (M-MIC). Corrosive metabolites are oxidants, such as protons, organic acids, and sulfides that can attack metal and stimulate cathodic reactions (Pedersen, 2013). Examples of M-MIC reactions include corrosion of steel by acids excreted by acetogenic bacteria in biofilms or copper corrosion by hydrogen sulfide excreted by SRB. In the absence of external oxidants, microbes can perform anaerobic fermentation, which often produces organic acids. Of the two, E-MIC is considered most dangerous for deep geological repositories.

Unlike planktonic community, a biofilm represents fundamentally different conditions for microbial growth, providing better protection against physical, chemical, and biological stresses (Li et al., 2008). Consequently, microbes organized as a biofilm can survive highly irradiated environments. 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. They may be directly involved in electron transfer processes in electrochemical cells

(E-MIC) or be less directly involved through the excretion of metabolites (M-MIC). During each phase, a biofilm may affect the corrosion process in different ways, e.g., corrosion acceleration has been observed in a biofilm isolated from a drinking water system during the first seven-day incubation, but a protecting effect was observed after 30-days incubation (Jin and Guan, 2014). Later-stage biofilms may act as a barrier, therefore, by coating the metal surface and protecting it from further corrosion. It is also possible, however, that such late-stage biofilms may become weak, porous, and fragile, negating their protective effect (Rabus, 2006). This barrier effect may also have less desirable consequences, however, as it may slow down the diffusion of organic carbon sources, resulting in lowered availability of carbon to sessile cells in the bottom layer. Carbon limitation leads to starvation of sessile cells living close to or directly on a metal surface. Owing to the deprivation of an electron donor (carbon source), starved SRB cells switch to consuming elemental iron for generation of energy, leading to severe E-MIC (Xu and Gu, 2014). Many studies (Libert et al., 2014; Paula et al., 2016; Pedersen, 2010; Rajala et al., 2017, 2015) have demonstrated MIC in natural or synthetic water and bentonite associated with high corrosion rates under repository relevant condition though these study typically focused only on the microbial aspect. In-situ MIC experiment was carried in Mot Terri Rock Laboratory (Switzerland) which showed corrosion rates in the range of 2 µm/year higher than in absence of microorganisms after 20 months of exposure in bentonite of different densities (Necib et al., 2018). Similarly, another in-situ MIC study was performed with the MiniCan test series of miniaturized copper-cast iron containers in the Aspo Hard Rock Laboratory (Sweden) with bentonite of two different dry densities (1300 kg/m^3 installed for 5 years and 1600 kg/m^3 installed for 10 years) and copper-cast iron container only with water without bentonite for 10 years. Extensive corrosion of cast iron specimens caused by SRB was observed in all conditions with local attacks corresponding to the loss of hundreds of µm/yr (Johansson et al., 2017). Higher corrosion rates can result in the premature failure of the metal container compromising its structural integrity (Flemming, 1996). Since the bentonite buffer, clay, or host rock are water-conducting, the integrity of the barrier system will be maintained by only undamaged metal containers and the MIC has to be suppressed to lowest rates possible.

The main aim of this study was to determine and understand the contribution of biocorrosion to overall corrosion and to investigate the microbial community composition and identify the bacteria responsible for corrosion and biofilm formation. Two distinct experiments were

conducted to study MIC on carbon steel. The first experiment studied MIC of carbon steel in the presence of anaerobic SRB present naturally in VITA water from Josef URC carried for 240 days (8 months) in both non-sterile (groundwater containing SRB) and sterile anaerobic conditions where the corrosion was determined by electrochemical impedance spectroscopy. Hereafter, this experiment is referred to as corrosion in groundwater. Likewise, the second experiment with the carbon steel comprised synthetic bentonite pore water (SBPOW) inoculated with VITA water from Josef URC in a 9:1 ratio and run for 26 months, the corrosion rate was estimated by weight loss method SBPOW simulates the real environment of a container surrounded by the bentonite. The second experiment is referred to as corrosion in synthetic water hereafter. Microbial community was analyzed using qPCR and next generation sequencing. The experimental work was carried in collaboration with Centrum výzkumu Řež s. r. o. (Research Centre Řež) in Prague. Section 2 *Corrosion of carbon steel in natural groundwater* is fully based on our published research work (Černoušek et al., 2020).

2 Corrosion of carbon steel in natural groundwater

2.1 Materials and methods

2.1.1 Material and groundwater samples

Circular test plates, measuring 15 mm in diameter and 3 mm thick, were constructed of commercial C15E low carbon steel (wt. %, 0.15 C, 0.58 Mn, 0,256 Si, 0.029 S, 0.06 P, and Fe balance, microphotograph shown in *Figure 32*). For the corrosion experiment, the plate surface was mechanically polished with P500 silicon carbide grinding paper in an Argon-purged glove box, following which the plates were cleaned with de-aerated ethanol. Natural groundwater was collected from the VITA source at the Josef URC, Czech Republic. The chemical composition of the groundwater is provided in *Table 10*. Conductivity of the groundwater was 61.1 $mS \cdot cm^{-1}$ and pH 7.2. A sterile abiotic control was obtained through sterile filtration of the same groundwater through a membrane filter with a pore size of 0.22 µm (Madigan et al., 2015).

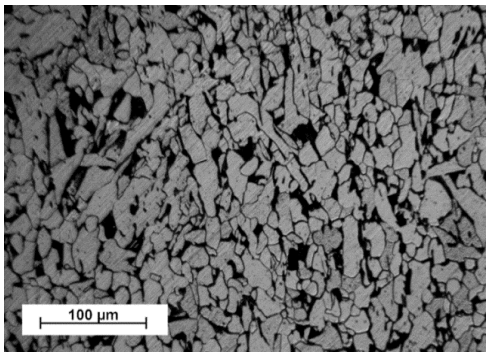

Figure 32: Microphotograph of the carbon steel sample.

Table 10: Chemical composition of the natural groundwater (VITA source, Josef URC).

Analyte	Concentration [mg/l]	Detection limit [mg/l]
Mg^{2+}	12.6	< 0.1
Ca^{2+}	60	< 0.1
Na^{+}	54.7	< 1
K^{+}	1.79	< 0.1
Fe^{2+}	1.01	< 0.02
Mn^{2+}	0.11	< 0.005
Cr^{3+}	< 0.005	< 0.005
TOC	97.0	< 1
NH_4^{+}	< 0.05	< 0.05
Cl^{-}	16.6	< 2
NO_2^{-}	< 0.05	< 0.05
NO_3^{-}	< 2	< 2
SO_4^{2-}	56.4	< 10
PO_4^{3-}	1.0	< 0.05
F^{-}	< 0.05	< 0.05
H_2S	0.08	< 0.01

2.1.2 Electrochemical measurement

The corrosion experiment was carried out in an Argon-purged glove box (gaseous oxygen concentration < 1 ppm volume) at approximately 25°C for 240 days. Open circuit potential (OCP or corrosion potential) measurement and electrochemical impedance spectroscopy (EIS) was undertaken weekly using a Gamry Reference 600 potentiostat/galvanostat/ZRA (GAMRY, USA), to characterize the corrosion process over the 240-day exposure period. Electrochemical measurements were performed with a three-electrode system, using a saturated calomel electrode as a reference and two graphite rods as auxiliary electrodes, *Figure 33*.

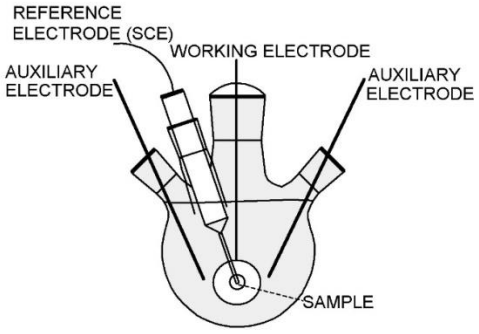

Figure 33: Experimental corrosion cell.

The working electrode had an exposed metal surface area of 1 cm^2. The EIS tests were performed with a sinusoidal signal of 10 mV amplitude over a frequency range of 100 kHz – 5 mHz at the corrosion potential. Later measurements, performed in order to gain information on polarisation resistance under non-sterile conditions, had an increased frequency range from 100 kHz to 10 µHz. Corrosion potential was highly stable and allowed EIS measurement at lower frequencies. Analysis of impedance spectra was performed using Gamry Echem Analyst 6.24 and ZSimpWin 3.50 software.

2.1.3 Surface and cross-section analysis

The steel specimen surface was examined using a LYRA3 scanning electron microscope (SEM) from Tescan, Czech Republic. Changes in surface morphology were observed with secondary electron detectors (SE and In-beam SE mode) and back-scattered electrons (In-beam BSE mode) at 5 kV accelerating voltage. Energy dispersive X-ray spectroscopy (EDS) was used to determine local chemical composition using unprepared samples. Subsequently, the samples were modified by pouring into polyacrylic resin followed by cutting and polishing, after which they were carbon sputtered to a thickness of 10 nm to provide charging reduction. Cross-section analysis was then performed at 20 kV accelerating voltage.

Cross-section analysis of corrosion penetration was carried out using an Olympus PME3 metallographic microscope equipped with AxioVision software. Micro-Raman analysis was then performed using a Thermo Scientific DXR2xi spectrometer with a 532 nm laser line coupled with an optical microscope using a 10x magnification objective lens. Laser power was set at 0.5 mW to minimize the possible phase transition of corrosion products.

2.1.4 Molecular biological analysis

Molecular biological analysis of water samples from the experiment followed the same method that has been described in section 2.4 in chapter II. Isolation of DNA was performed from both water and biofilm samples formed on the surface of the carbon steel. Biofilm samples from the metal surface were collected using sterile swabs sticks, FLOQSwabs (COPAN Diagnostics Inc, USA). Same Power Water DNA Isolation Kit (was used for isolation of DNA from the biofilms as well. qPCR analysis was performed by using a functional marker for bacteria and SRB only.

2.2 Results and Discussion

2.2.1 Electrochemical Impedance Spectroscopy

Time evolution of impedance spectra, measured under both sterile and non-sterile conditions, is provided as Bode representations in *Figure 34* and *Figure 35*, respectively at a frequency range of 100 kHz to 1 kHz appear to be artifacts caused by a parasitic capacitance originating from the electrochemical cell. Consequently, this part of the spectra was disregarded during further analysis. Spectra measured under sterile conditions are characterized by a single capacitive time constant over the whole measurement period, indicating uniform corrosion of the carbon steel. This corrosion stage is modeled in the circuit description code by the equivalent circuit R1 (R2Q1), where R1 is the solution resistance, R2 is the polarisation resistance and Q1 is the dispersive double-layer capacitance characterized by the constant phase element. The *Table 11* shows selected results for EIS measurements performed under sterile conditions. The three equivalent circuits **(***Figure 36***)** were used sequentially for data fitting under non-sterile conditions, representing three corrosion stages. The first corrosion stage is characterized by one capacitive time constant, equivalent to the sterile environment, where R1 is the solution resistance, R2 is the polarisation resistance and Q1 is the dispersive double-layer capacitance.

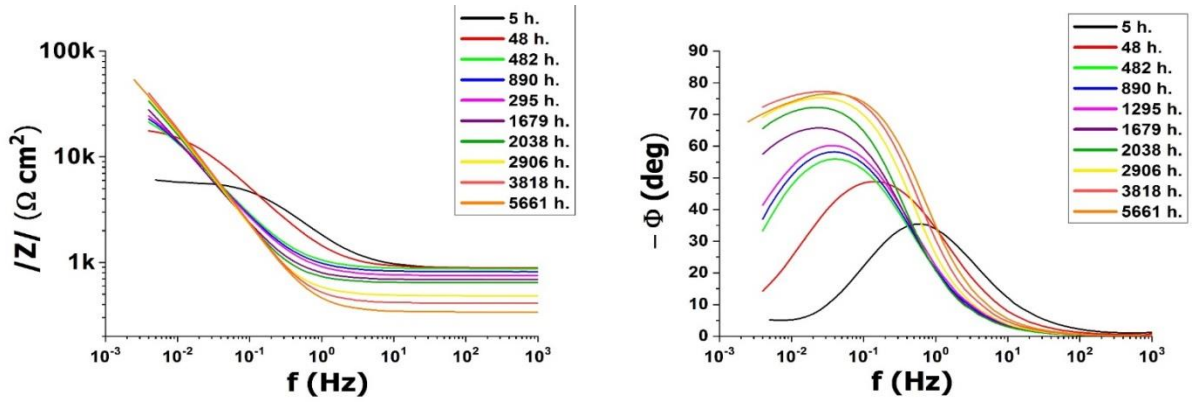

Figure 34: Bode plots of electrochemical impedance spectra time evolution for carbon steel under sterile conditions.

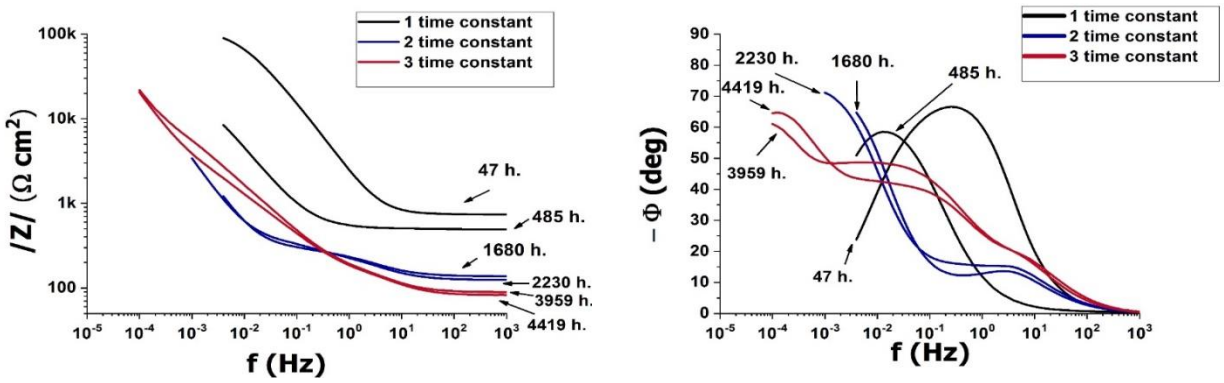

Figure 35: Bode plot of electrochemical impedance spectra time evolution for carbon steel under non-sterile conditions.

Initial EIS measurements under both sterile and non-sterile conditions showed similar corrosion patterns characterized by the presence of a single time constant. In the non-sterile environment, the main differences observed were a more rapid increase in dispersive double-layer capacitance and a rapid decrease in solution resistance over time. A second corrosion stage was observed after 23 days, represented by the occurrence of two-time constants due to biofilm formation. This corrosion stage was modelled in the circuit description code by the equivalent circuit R1(R2Q1)(R3Q2), where R3 represents the resistance of the biofilm and Q2 is the dispersive capacitance of the biofilm. Values n2 of dispersive capacitance Q2 indicating the influence of diffusion. The second biofilm time constant appeared in the high-frequency area. A third-time constant was observed after 112 days which is usually indicative for the change of the corrosion state on the surface. This was confirmed by the results of SEM measurements (see below) revealing the presence of the second biofilm layer. The formation of the second biofilm layer caused a shift in Faradaic charge transfer to very low frequencies of up to tens of µHz linked to an increased contribution of diffusion resistance. This corrosion stage was modelled in the circuit description code by the equivalent circuit R1(R2Q1)(R3Q2)(R4Q3), where R4 represents the resistance of the second biofilm and Q3 is the second biofilm's dispersive capacitance. The impedance of the second biofilm layer differed from that of the first by showing lowered value n3 and increased capacitance. *Table 12* shows selected results for EIS measurements under non-sterile conditions. Approximation accuracy of the experimental data by each equivalent circuit ($\chi 2$ - chi-square goodness of fit test) achieved similar values in sterile and non-sterile environments, ranging in the order of 10^{-4} to 10^{-5}.

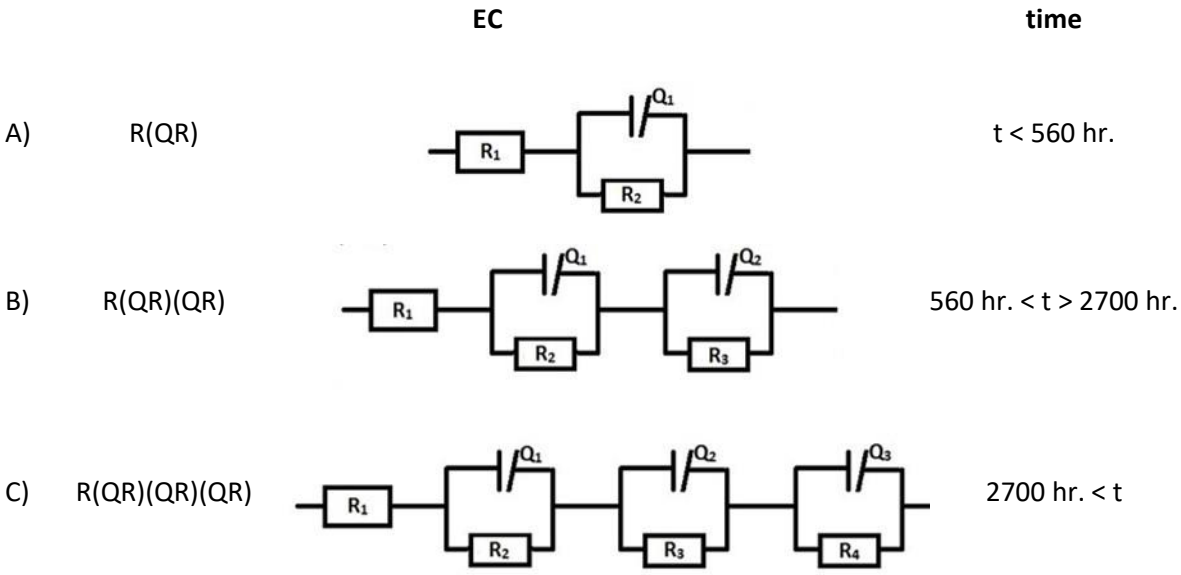

Figure 36: Equivalence circuits used for electrochemical impedance spectroscopy data fitting and time evolution of corrosion stages.

Polarization resistance time dependence under sterile and non-sterile conditions was estimated by fitting nonlinear least squares (*Figure 37*). Increased polarization resistance during the early stages of the experiment under non-sterile conditions can be attributed to sedimentation, which has an inhibitory effect on the corrosion rate compared with the sterile environment without colloidal particles. When SRB was present, carbon steel polarization resistance decreased by a factor of two after 240 days, when compared with sterilized VITA groundwater.

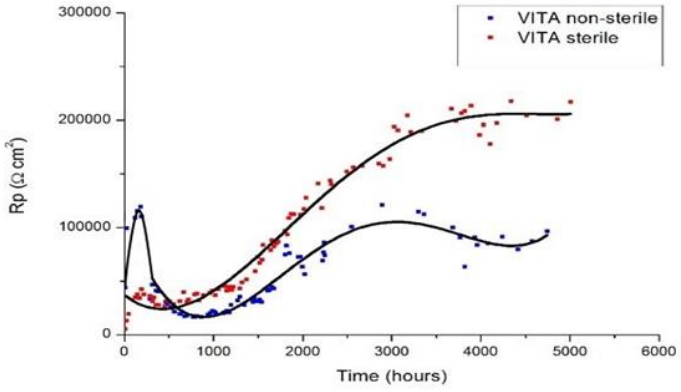

Figure 37: Time evolution of polarization resistance.

Table 11: Results of EIS measurements performed under sterile conditions.

Element →	***R1***	***Q1***	***n1***	***R2***
Time (h) ↓	**[Ohm·cm^2]**	**[Ω^{-1}s^{-n}cm^{-2}]**		**[Ohm·cm^2]**
5	872	1.75E-04	0.7862	5198
48	876.4	2.76E-04	0.7739	19430
482	867.2	5.76E-04	0.8081	32170
890	814.7	5.99E-04	0.8195	36710
1295	747.5	6.10E-04	0.8243	42770
1679	681.5	7.05E-04	0.8473	82160
2038	640.5	7.19E-04	0.8949	127700
2906	484.3	7.13E-04	0.9100	157300
3818	412.4	6.79E-04	0.9191	208500
5661	301.6	7.01E-04	0.9082	209600

Table 12: Results of EIS measurements performed under non-sterile conditions.

Element →	R_1	Q_1	n_1	R_2	Q_2	n_2	R_3	Q_2	n_3	R_4
Time (h) ↓	[Ohm·cm^2]	[Ω^{-1}s^{-} ^{n}cm^{-2}]		[Ohm·cm^2]	[Ω^{-1}s^{-} ^{n}cm^{-2}]		[Ohm·cm^2]	[Ω^{-1}s^{-} ^{n}cm^{-2}]		[Ohm·cm^2]
6	691	9.56E-05	0.7916	43580						
47	724.8	9.71E-05	0.8411	105600						
485	496.1	0.00195	0.7933	27700						
866	308.4	0.0111	0.9356	17300	0.0144	0.4866	297.4			
1295	206.8	0.01756	0.8993	25710	0.00411	0.4387	102.2			
1537	142.6	0.02229	0.8983	30390	0.00175	0.4864	175.8			
2230	100.1	0.02785	0.8932	63370	0.0021	0.4639	298.8			
4239	80.32	0.05035	0.9358	85130	0.00356	0.5608	22.65	0.00113	0.9792	3381
5080	82.21	0.02807	0.8554	125800	0.00374	0.5904	20.06	0.0013	0.9781	6891

2.2.2 Surface and cross-section analysis

The images obtained from SEM indicated that the surface of carbon steel in the presence of bacteria was covered with a relatively thick layer of spherical or rod-shaped microorganisms with many cells visible (*Figure 38*). The biofilm consisted of microorganisms surrounded and held together by an excreted gelatinous matrix of EPS composed of high molecular weight compounds (Bhaskar and Bhosle 2006; Reitner 2011). The bacteria were about 2 μm long and had a typical cylindrical shape. The SE detector mode provided the best surface visualization, showing the bacteria as dark hyaline objects (*Figure 39*). The main disadvantage of this mode was the difficulty in finding bacterial cells. The use of the BSE detector improved the imaging contrast between bacterial cells (comprising elements of lower atomic number) and the steel's surface due to the backscatter of electrons, which made areas, where bacteria were located, appear darker (*Figure 39*). The surface analysis showed a heterogeneous sample surface with some areas covered with biofilm and some with many flat crystals.

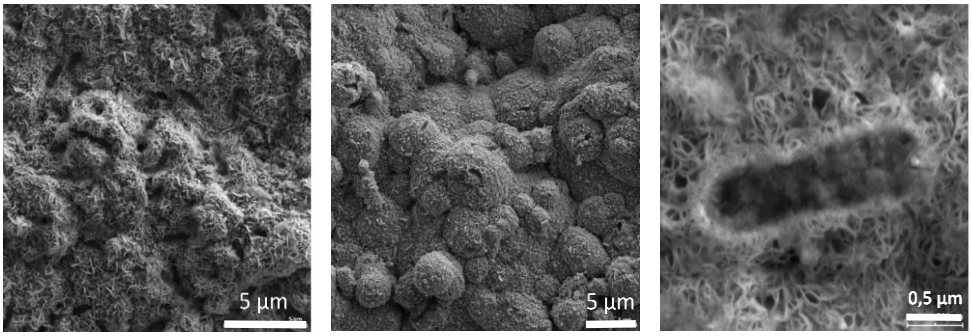

Figure 38: Scanning electron micrographs showing the surface of carbon steel covered by a thick layer of spherical or rod-shaped microorganisms (SE detector).

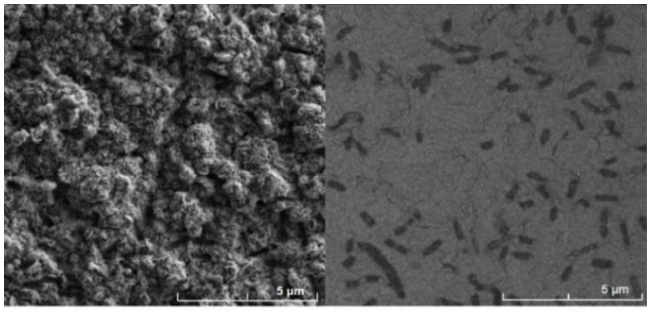

Figure 39: Scanning electron micrograph of the biofilm formed on carbon steel exposed under non-sterile conditions after 240 days incubation – comparison of different detector modes (left SE detector, right In-Beam BSE detector.

Local EDS analysis demonstrated that the corrosion products were mainly composed of iron, oxygen, sulfur, and carbon displayed in. This finding was supported by elemental maps of those areas with bacteria present *Figure 40*. The presence of sulfur and iron suggests iron sulfide minerals formation, thereby indicating bacterial activity. Additionally, black turbidity was observed in the experimental cell (*Figure 41*) and on the surface of the testing coupon under non-sterile conditions. In contrast, no sulfur or signs of microbial activity were detected by SEM/EDS on the sample under sterile conditions.

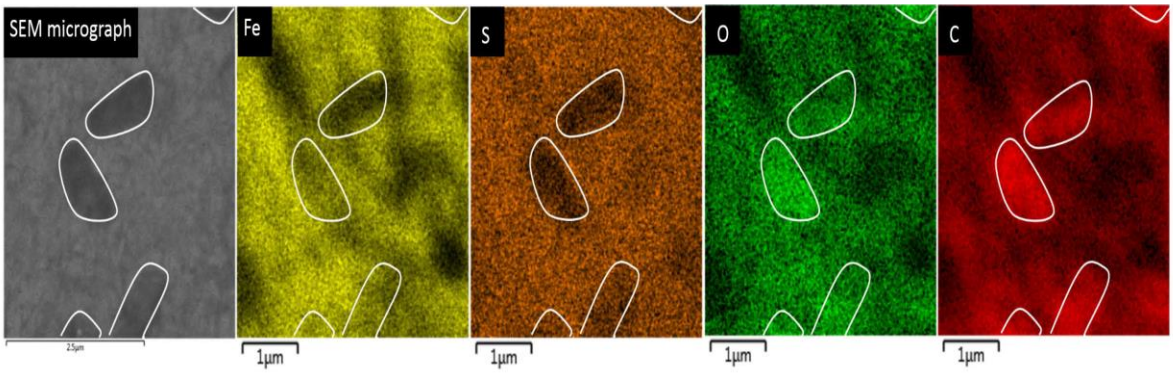

Figure 40: Energy-dispersive X-ray elemental maps of the corroded region with bacteria present (marked with a white line).

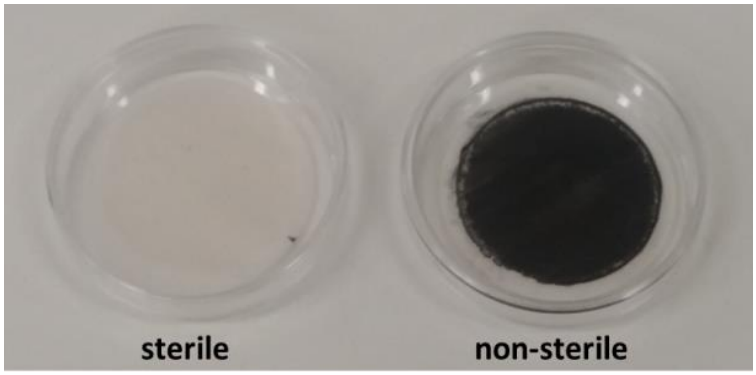

Figure 41: Comparison of filters after the filtration of the water used for the experiment. Left: sterile negative control, right: non-sterile sample.

The SEM analysis indicated that the covering layer was stratified into a thicker (approx. 24.5 µm) inner layer (labeled 2 in *Figure 42*) and a thinner (approx. 3.7 µm) outer layer (labeled 1 in *Figure 42*) representing dry film thickness. The composition of these layers differed, with the inner layer being dominated by iron and oxygen with a small amount of sulfur and the outer

layer comprising iron with oxygen with sulfur dominating for the EDS spectra (the point labeled 3 was dominated by silicon).

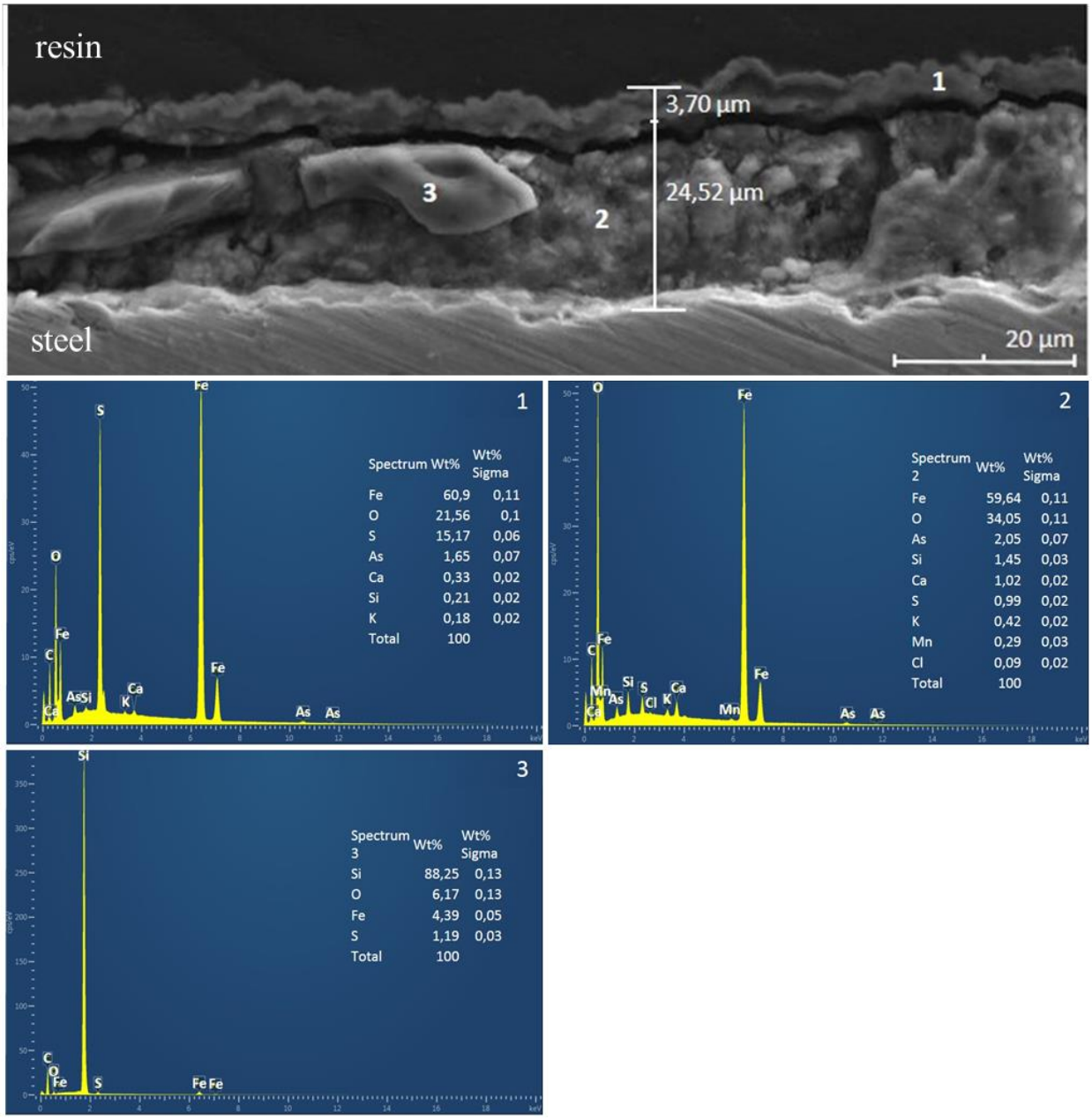

Figure 42: Scanning electron micrograph showing the formation of a corrosion layer. The image shows a sample cross-section with bacteria present (above) and the respective X-ray spectra of positions 1, 2, and 3 (below).

The cross-section images given in *Figure 43* show that corrosion penetration is much lower and thickness more homogeneous in the sterile sample, and much thicker and less homogeneous in the non-sterile sample (evaluation summary in *Table 13*). The average penetration for the sterile sample was 8.36 µm and 27.80 µm for the non-sterile sample, while maximum penetration was 14.01 µm for the sterile sample and 61.31 µm for the non-sterile sample. Extreme penetrations observed at the non-sterile sample surface are probably caused by occluded solution formation under the biofilm, with subsequent localization of the corrosion attack.

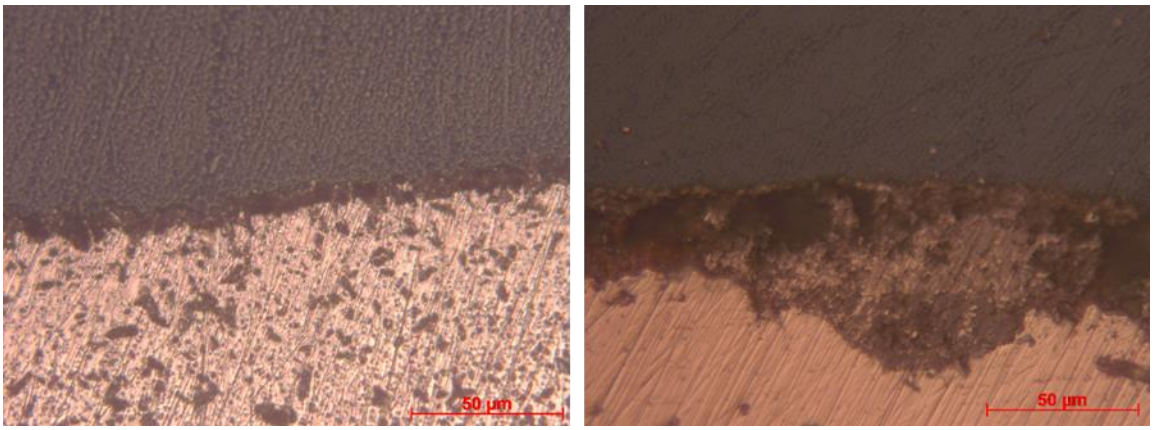

Figure 43: Cross-sections of the sterile (left) and non-sterile (right) steel after exposure.

Table 13: Evaluation of corrosion penetration on cross-cut samples at the end of the experiment.

Environment	Corrosion penetration (µm)			
	average	standard deviation	minimum	maximum
Abiotic (sterile)	8.36	2.69	3.49	14.01
Biotic (non-sterile)	27.80	10.74	10.46	61.31

Sterile and non-sterile samples were also analyzed by micro-Raman spectroscopy, which detected the presence of mackinawite ($(Fe_{1+x}S)$ on the surface of the samples exposed to bacteria (characterized by peaks at 217, 280, 391, 487, 595, 653 and 1286 cm^{-1}; *Figure 44*). Mackinawite and greigite (Fe_3S_4) are corrosion product typically reported from systems exposed to SRB and is therefore considered an indication of microbial activity on the surface of the material (Rémazeilles et al., 2010).

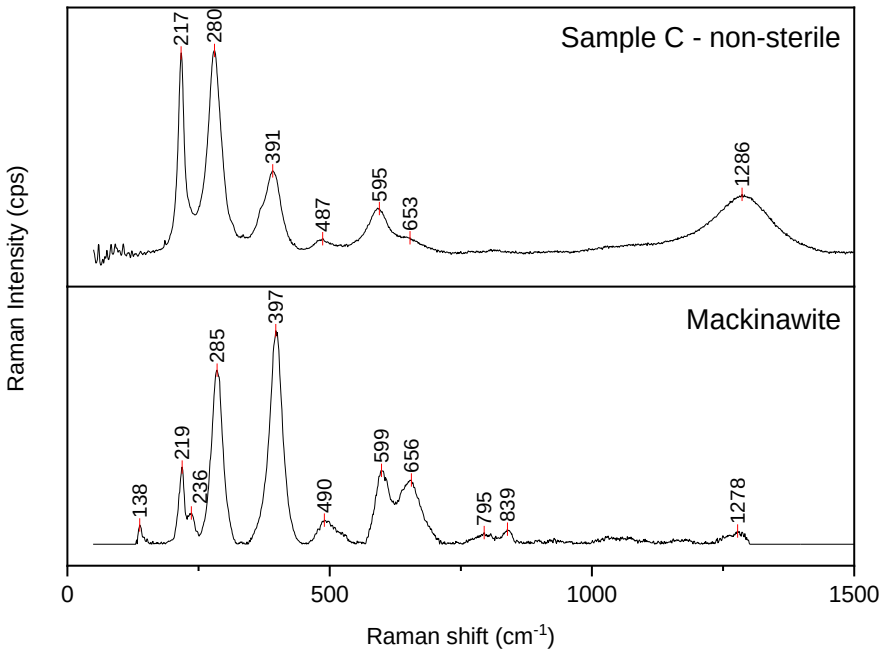

Figure 44: Raman spectra of carbon steel under non-sterile anaerobic conditions in VITA water after 293 days (top) and mackinawite standard (bottom).

2.2.3 Molecular biological analysis

Results of the qPCR analysis indicated that the relative abundance of bacterial biomass (detected by the universal 16S rRNA marker) in the water sample increased slightly compared with the start of the experiment revealed in *Figure 45*. In contrast, the amount of SRB in the water sample remained more or less the same with no remarkable change in the fold. However, when interpreting these results, it should be taken into account that most bacteria contributing to MIC are expected to grow in the biofilm. No bacteria were detected by qPCR under sterile conditions.

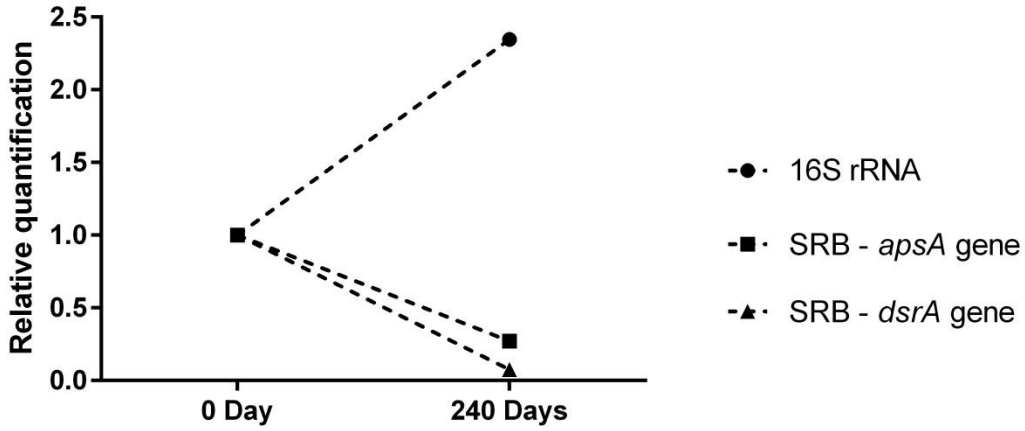

Figure 45: Results of qPCR analysis of the 16S rRNA (total bacterial biomass) and apsA and dsrA genes (SRB), shown as a relative change compared to the start of the experiment.

Microbial community composition of the test water and biofilm after 240 days was determined alongside the initial VITA groundwater source by NGS amplicon sequencing, *Figure 46* . The initial VITA water was dominated by mesophilic and thermophilic SRB such as *Desulfobacula*, *Desulfomicrobium*, *Desulfovibrio,* and *Desulfurivibrio* (all Deltaproteobacteria). The genera *Paludibacter* (Bacteriodetes) and *Thiobacillus* (Betaproteobacteria), along with members of the Rhodocyclaceae and Comamonadaceae families (both Betaproteobacteria), were also common in VITA water. While the taxonomic composition of the microbial community present in both water and biofilm samples collected after 240 days changed, the communities were still dominated by SRB. While both *Desulfobacula* and *Desulfurivibrio* were detected at low numbers in the 240-day water and biofilm samples, *Desulfomicrobium* and *Desulfovibrio* spp. dominated in the biofilm sample. Members of these genera can utilize organic compounds or hydrogen as electron donors and sulfur compounds as electron acceptors (Steger et al. 2002, Dias et al. 2008) and, therefore they participate in MIC (Enning and Garrelfs 2014). Both genera are also able to promote anaerobic corrosion indirectly through the corrosive chemical agent hydrogen sulfide (M-MIC). In addition, the genus *Desulfovibrio* includes many strains that have the ability to corrode metals through a direct withdrawal of electrons from the metal surfaces (E-MIC) (Dinh et al. 2004, Venzlaff et al. 2013, Enning and Garrelfs 2014). Under nutrient limiting conditions, as found in the groundwater examined in this study, the concentration of organic electron donors is relatively low and, as a result, microbes probably attack the steel as it represents a good source of electrons for reduction of sulfate to sulfide (Rajala et al. 2015).

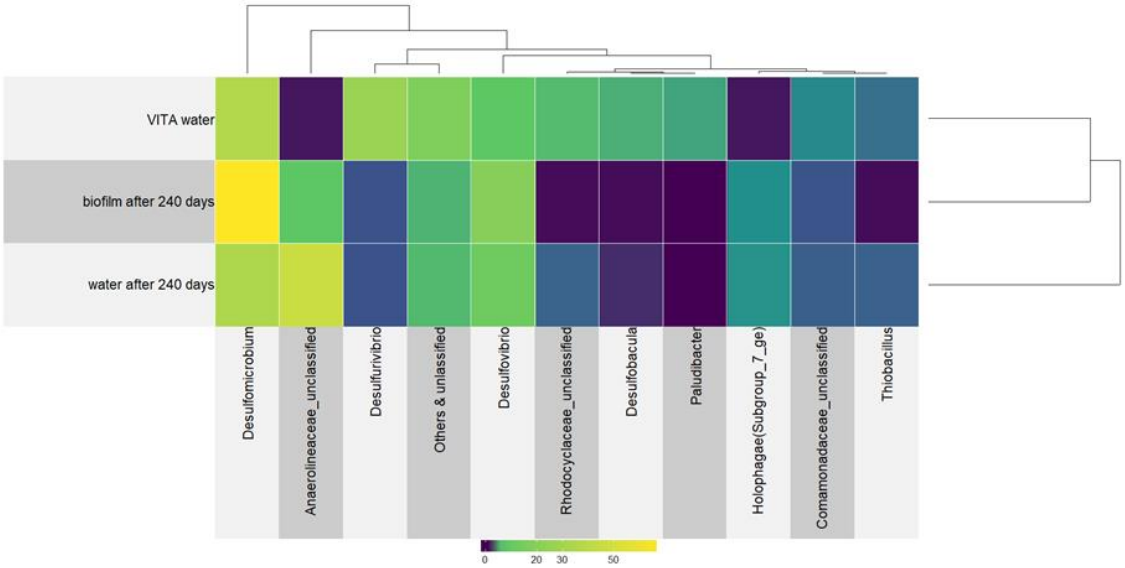

Figure 46: Heat map showing the results of the 16S rRNA gene amplicon sequencing (only taxa with abundance over 1% visualized).

The obligate anaerobes Anaerolinaceae, found in low numbers in the initial VITA water, were detected in relatively high numbers in samples collected after 240 days. Similarly, members of the class Halophagae (subgroup_7_ge; phylum Acidobacteria) were also found in both 240-day water and biofilm samples in higher quantities than at the beginning of the experiment. At the same time, the number of other bacteria present in the initial VITA water sample declined significantly. Natural biofilms are composed of a wide variety of microbes, including bacteria, archaea, and fungi. When multiple corrosion-causing species are present they will act synergistically, causing severe corrosion (Kip and Van Veen 2015, Rajala et al. 2017). A recent study conducted on water from the Yucca Mountains (USA), for example, showed a higher rate of corrosion in the presence of a mixed bacterial population (iron and sulfur-reducing bacteria) than in the presence of single species (Pitonzo et al. 2004).

3 Corrosion of carbon steel in synthetic bentonite pore water inoculated by natural groundwater

3.1 Materials and methods

3.1.1 Materials and experimental set-up

Test coupons for the study were made from commercial C15E low carbon steel (as described in section 2.1.1 of this chapter). The test specimens of the carbon steel were cylindrical with a diameter of 10 mm and a length of 50 mm. The coupon surface was mechanically polished with P500 silicon carbide grinding paper in an argon-purged glove box, following which surface was cleaned with de-aerated ethanol. The design of this experiment is illustrated in *Figure 47*. Synthetic bentonite pore water (SBPOW), which simulates the leachate of the Czech BaM bentonite (Červinka and Gondolli, 2015) was used as a working environment (see *Table 14* for its chemical composition).

Autoclaved distilled water was used for the preparation of the SBPOW. To avoid contamination, the SBPOW was sterilized after preparation by filtration using an autoclaved filter apparatus and 0.22 µm GV Durapore® (Merck, Germany) filter membrane under sterile conditions. The VITA water, groundwater rich in SRB was used as a microbial inoculum. The ratio of SBPOW and VITA water was 9:1. The VITA groundwater was collected from the VITA source at the Josef URC (Czech Republic) a day before the experiment under sterile conditions. The chemical composition of groundwater is stable and thus was similar to the previous experiment (see *Table 10*). The experiments were conducted in 2-L sterilized flasks in separate batches. The abiotic sterile version of the experiment was performed in parallel and consisted of carbon steel and SBPOW only. The experiment was carried out in an Argon-purged glove box (CO_2 < 1 ppm volume) at a laboratory temperature of approx. 25°C. The samples were analyzed at the beginning and after 3, 6, 12, 18, and 26 months of incubation.

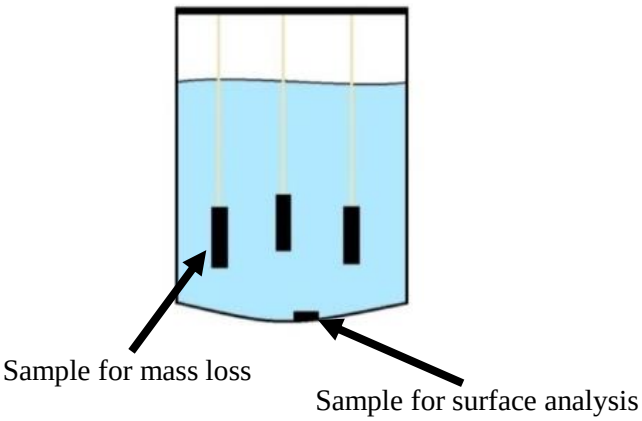

Figure 47: Experimental design for corrosion in SBPOW.

Table 14: Composition of synthetic bentonite pore water in 1 L of distilled water.

Chemicals	Unit, g/l
$MgSO_4$ heptahydrate	2.727
$NaNO_3$	0.816
NaCl	0.419
KNO_3	0.133
Na_2SO_4	0.1462
$KHCO_3$	0.1066
$CaCl_2$	0.0388

3.1.2 Corrosion rate determination

The cylindrical carbon steel samples were used to determine the corrosion rate for long-term immersion by weight-loss methods according to the standard ISO 8407. After exposure, corrosion product of samples was removed by repeated chemical etching in a Clark solution (5 g of tin(II) chloride and 2 g of antimony(III) oxide in 100 ml hydrochloric acid) including subsequently rinsing with distilled water, then in acetone and finally dried in air at room temperature. The weighing was repeated until a constant weight was achieved by repeated treatment.

3.1.3 Surface characterization

The SEM analysis was performed as described in section 2.1.3 of this chapter. The Raman spectra analysis was performed by Raman dispersion spectrometer (Thermo Scientific - model DXR Microscope equipped with an Olympus confocal microscope). The excitation source was a diode Nd: YAG laser was used as the excitation source with a wavelength of 532 nm and an input power of 10 mW. A grid of 900 scratches / mm was used. A multichannel thermoelectrically cooled CCD camera was used as a detector. Samples were measured at 50x magnification with a measuring track of approx. 1 μm^2. Samples were measured through an aperture of 50 µm slit. To exclude the thermal degradation of the sample, measurements were performed at 0.05 mW, 30 sec measurement time, and 40 spectrum accumulations.

3.1.4 Chemical analysis

The concentration of chloride, sulfate, nitrate, and nitrite was measured using ion spectroscopy as described in the section 2.5 of the chapter II. Filtered water obtained after filtration through 0.22 μm (GV Durapore® filter membrane, Germany) was used for the chemical analysis.

3.1.5 Molecular biology analysis

Biofilm from the metal surface was sampled using sterile swabs. DNA was extracted from both water and biofilm that was formed on the surface of the carbon steel same as in previous corrosion experiment (corrosion in natural in groundwater). Molecular biology analysis was done following the methods described in the section 2.4 of the chapter II.

3.2 Results and Discussion

3.2.1. Corrosion rate

All samples exposed to microorganisms had a higher corrosion rate compared to abiotic controls. This effect was observable already after three months (*Figure 48*). The average corrosion rate of carbon steel exposed to microorganisms was 3.81 μm/yr, reaching the highest values after 6 months (i.e., 5.4 μm/yr) and the lowest values (i.e., 0.9 μm/yr) after 18 months. At the end of the experiment, the corrosion rate increased to 3.4 μm/yr. Additionally, in the non-sterile environment; the pits were initiated during the first 6 months and visible local attacks were observed after 12 and 26 months. The non-sterile samples analyzed after 18 months had a significantly lower corrosion rate without visible local attacks. A gradual decrease in corrosion rate was observed under sterile conditions: the initial rate was 3 μm/yr after 3 months and dropped to 0.36 μm/yr after 26 months. Weight loss data of carbon steel in SBPOW solution under sterile conditions confirmed the tendency of the corrosion rate to decrease with increasing exposure time under anaerobic conditions.

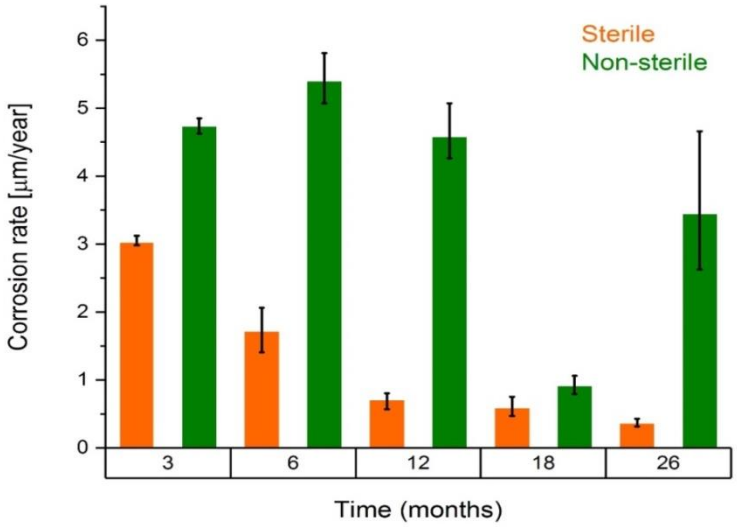

Figure 48: Average corrosion rates based on weight loss measurements for the carbon steel in a sterile environment (orange) and in the environment enriched with microorganisms (green).

Long-term corrosion of copper coupons was studied in compacted bentonite at the Äspö Hard Rock Laboratory (Karnland et al., 2000). The mean corrosion was calculated to be 3 µm /yr after 1-year exposure. In our study, corrosion in the biotic environment was always calculated to be higher than 3 µm /yr except for one sampling point, 18 months. In the Swedish SKB concept, the copper container would be 50 mm thick and expected lifetime of the waste container at least 100,000 years (King et al., 2010). In this context, the carbon steel or copper container would not be safe for at least 100,000 years. However, the experiment with SBPOW and groundwater was held in conditions that cannot be expected in the repository for the first tens of years, where the saturation phase will be strongly influenced by the heat generated by the container. The porewater will thus reach the container after longer time period. Our experiment aimed to show the different impact of sterile SBPOW and SBPOW inoculated with natural microbial community from deep geological environment.

3.2.2 Surface analysis

The visual observations showed that samples exposed to microorganisms had different appearance compared to the sterile controls (*Figure 49* and *Figure 50*). The surfaces of the abiotic specimens were homogenous and relatively smooth, with very little morphological differences between distinct areas. In contrast, the biotic samples were morphologically more heterogeneous.

The specimens from the biotic systems were covered with a layered deposit of corrosion products, which showed local compositional and morphological variations.

SEM

The SEM analysis showed a clear difference in surface characteristics between samples exposed to abiotic and biotic environments (see *Figure 49* for surface and *Figure 50* for cross section). In the abiotic environment, a uniform oxide layer was observed with no local attacks or pits (*Figure 49A and 50A*). On the contrary, the samples exposed to the microorganisms were characterized by the presence of pits and in some cases even by large local attacks in the samples collected after 12 and 26 months (*Figure 49B and 50B)*. The surface of carbon steel in the biotic sample was covered with a relatively thick layer of a non-uniform biofilm with some areas showing many flat crystals.

Similarly, the corrosion penetration data estimated by SEM (*Table: 15*) from cross-section images (*Figure 50*) showed that corrosion penetration was much deeper and more heterogeneous in the biotic samples compared to the sterile samples. Surfaces of the samples exposed to the microorganisms were characterized by clearly higher penetration values (also with significantly higher values of standard deviation, *Table: 15*). Extreme penetrations observed at the non-sterile sample surface were probably caused by the separation of anodic and cathodic sites and occluded solution formation under the biofilm, with subsequent localization of the corrosion attack. The average penetration for the abiotic sample was 38.82 µm and 1,381.65 µm for the biotic sample, while the maximum penetration was 12.57 µm and 985.34 µm for the abiotic and the biotic sample, respectively. Additionally, under non-sterile conditions, black turbidity was observed in the experimental cell and on the surface of the testing coupon (*Figure 51*).

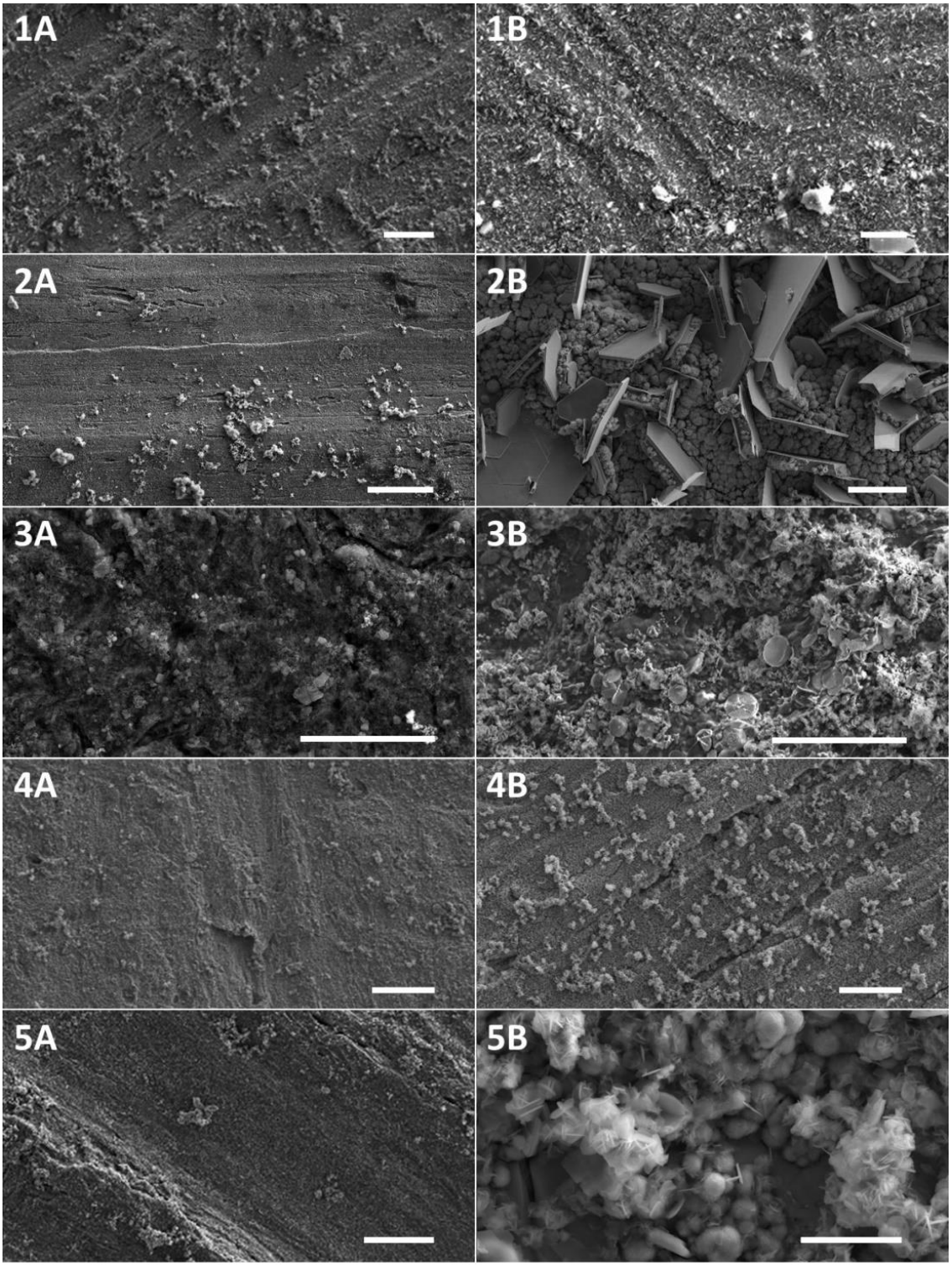

Figure 49: SEM micrograph presenting the surface of carbon steel. A belongs to abiotic (left) and B belongs to biotic samples (right) where the numbers 1, 2, 3, 4, and 5 represents sampling time after 3, 6, 12, 18, and 26 months, respectively. The scale bar used for each image was 20 µm.

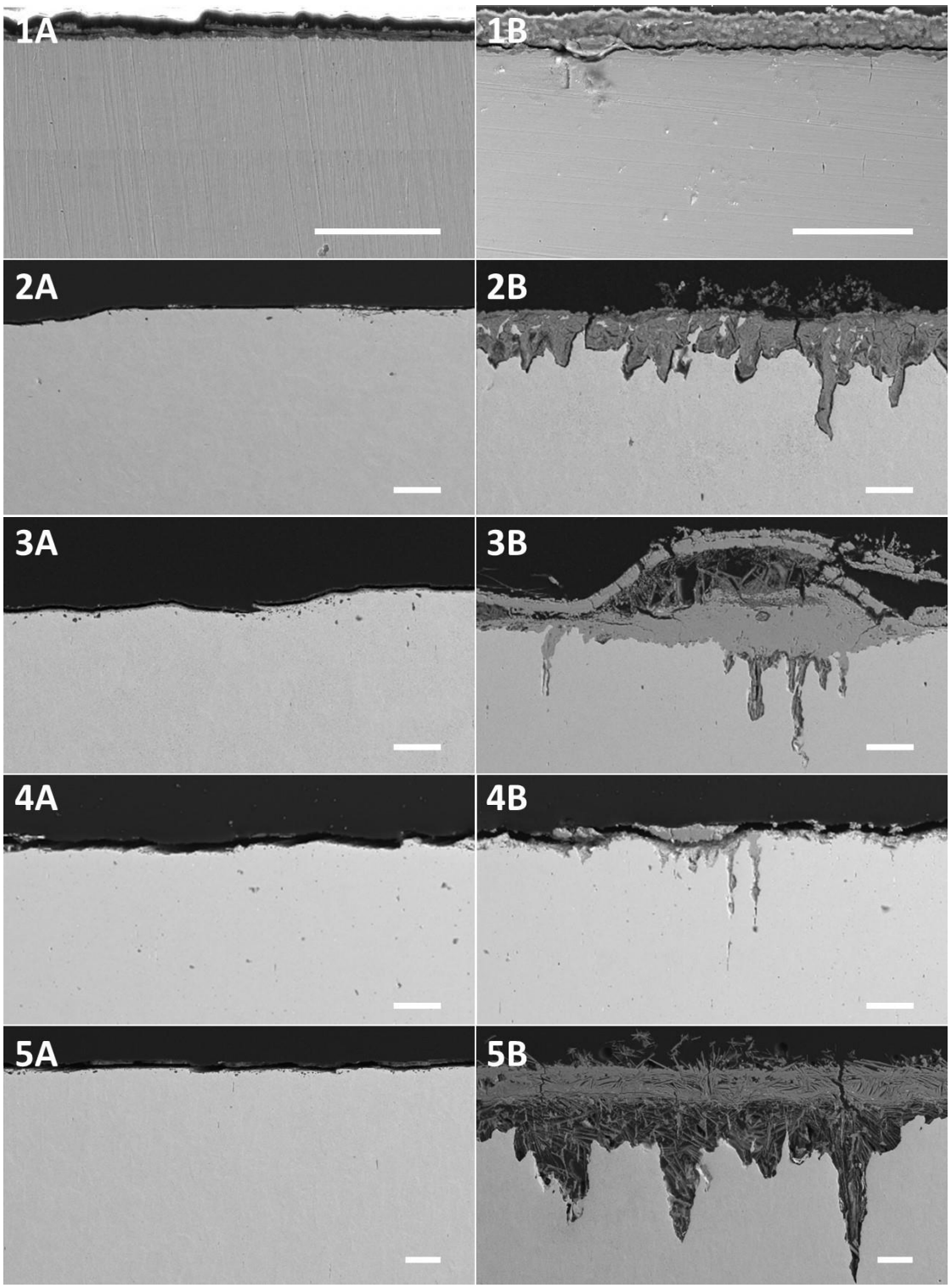

Figure 50: SEM micrograph presenting MIC – a cross-section of the carbon steel A belongs to abiotic (left) and B belongs to biotic samples (right) where the numbers 1, 2, 3, 4, and 5 represents sampling time after 3, 6, 12, 18 and 26 months, respectively. The scale bar used for each image was 50 µm.

Table: 15. Corrosion penetration under abiotic and biotic conditions.

Month	Sample	Corrosion penetration (µm)			
		average	standard deviation	minimum	maximum
3	Sterile	6.99	0.73	5.83	7.7
	Non-sterile	15.95	1.24	14.47	17.72
6	Sterile	5.52	0.52	4.75	6.20
	Non-sterile	82.44	33.81	52.13	138.72
12	Sterile	7.535	1.52	5.61	9.81
	Non-sterile	465.37	383.00	69.55	985.34
18	Sterile	10.80	1.02	9.76	12.16
	Non-sterile	77.65	47.49	11.13	138.28
26	Sterile	10.63	1.59	8.53	12.57
	Non-sterile	365.79	94.35	247.37	507.98

Figure 51: Test specimens of the carbon steel under non-sterile and sterile conditions collected after 26 months. Black turbidity was observed on the surface of the testing coupon. A clear sign of corrosion is seen on the surface of carbon steel under the biotic environment.

Raman spectroscopy

Raman spectroscopy was performed on all the samples from each sampling point. The results are summarized in *Table 16*. The measured spectra were compared with standard spectra from the RRUFF library (Lafuente et al., 2016).

Table 16: Summary of corrosion products.

Sampling time	Corrosion products	
	Abiotic (Sterile)	**Biotic (Non-sterile)**
3 months	Magnetite (Fe_3O_4)	Magnetite, Mackinawite ($FeS_{(1-x)}$)
6 months	Magnetite	Magnetite, Mackinawite
12 months	Magnetite	Magnetite, Mackinawite
18 months	Magnetite, Calcite (Calcium carbonate)	Magnetite, Mackinawite
26 months	Magnetite (partially substituted)	Akaganeite ($Fe^{3+}O(OH,Cl)$), Magnetite, Rozenite $Fe^{2+}SO_4$

The surface of the samples under abiotic conditions was covered with homogeneous magnetite (Fe_3O_4) layer in all sampling times with the peaks at 315, 547, and 670 cm^{-1}. The band shifts 662 -> 670 cm^{-1} and its asymmetry can be caused either by partial substitution of iron with other elements or by a minor presence of other corrosion products (e.g., maghemite). The presence of four intense bands around 153, 280, 711, and 1086 cm^{-1} in the Raman spectra of carbon steel was also identified after 18 months. A narrow intensive band around 1086 is typical for the group carbonate (CO^{2-}_3). According to the RRUFF database, these bands are assigned to calcite (measured chemistry of calcite is $(Ca_{0.99}Mg_{0.01})\ CO_3$). Calcite could occur elsewhere in the sampling point but was not found. The corrosion layer was practically homogeneous on the sample after 26 months, the typical 5ST_1 spectrum (see *Figure 52* on left), was identified as magnetite. The band displacement occurred at 667 -> 671 cm^{-1} and its asymmetry could be due to either partial substitution of iron for other elements (chromium) or minor presence of other corrosion products (e.g. maghemite). Bands around 1350 and 1580 cm^{-1} again indicate the presence of amorphous carbon. A deviation was found in only one case where silicon carbide (see 5ST_2 spectra on *Figure 52* at left) was identified in the spectrum in addition to the common component which could possibly be impurity or an abrasive used.

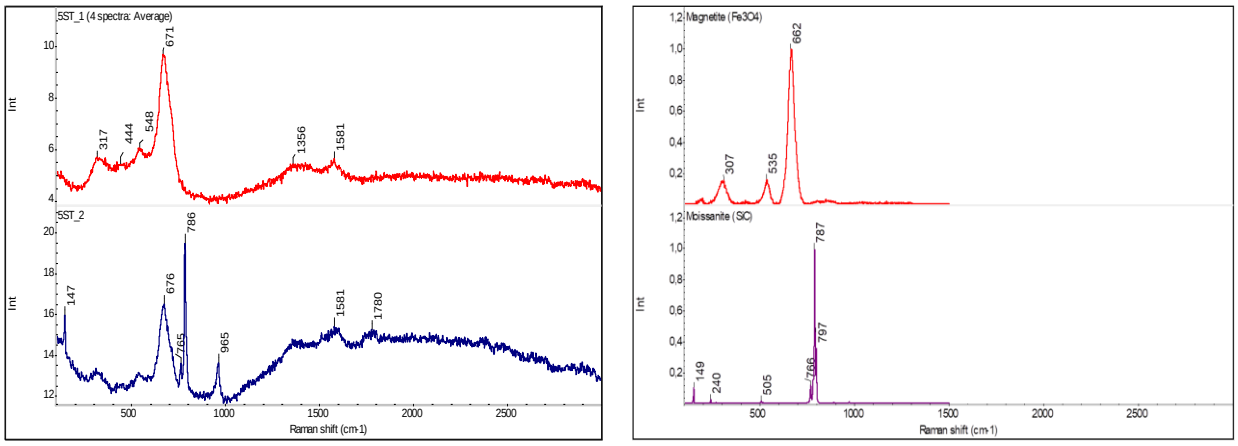

Figure 52: Raman spectra of carbon steel in SBPOW under sterile anaerobic conditions after 26 months denoting magnetite and silicon carbide (SiC) (left) and Standard spectra from the RRUFF library (right) (Lafuente et al., 2016).

Raman spectra of samples exposed to microorganisms were very different compared to sterile controls. Raman signature peaks at 213, 275, 384, and 583 cm^{-1} were observed in all sampling times and they are characteristic for mackinawite ($FeS_{(1-x)}$) which suggested the presence of SRB. Along with mackinawite, magnetite was observed as a corrosion product. Interestingly, after 26 months the Raman spectrum showed two dominant phases in the biotic sample. The first one was the red area formed by acicular crystals, this spectrum labeled as 10NE_1 (see *Figure 53* on left) was not identified using standard libraries, however, according to Colomban and Chiaberge (2011), it corresponds to akaganeite ($Fe^{3+}O(OH, Cl)$). The second one was the black area observed especially on the edges of the sample. This spectrum referred to as 10NE_2 in *Figure 53* (left) corresponds to magnetite. Compared to other samples it is a very exact agreement with the standard spectrum. Furthermore, in one place (10NE_3 in *Figure 53* on left), ferrous sulfate (especially the 990 cm^{-1} bands) was additionally identified in the area of akaganeite indicating the presence of rozenite.

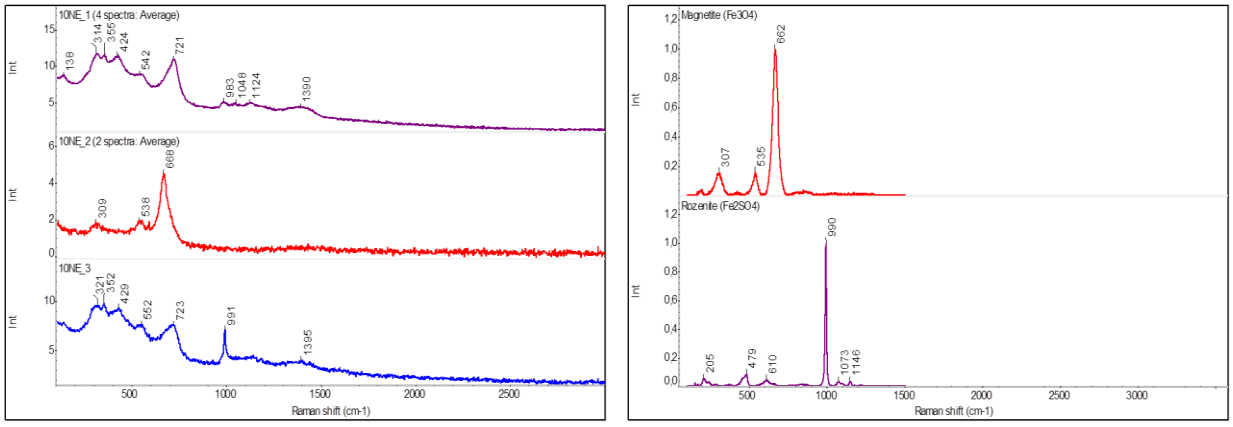

Figure 53: Raman spectra of carbon steel in SBPOW under non-sterile anaerobic conditions after 26 months of indicating the presence of akaganeite, magnetite, and rozenite (left) and standard spectra from the RRUFF library (right) (Lafuente et al., 2016).

Raman spectra under non-sterile conditions detected the presence of mackinawite (FeS_{1-x}) and rozenite ($Fe^{2+}SO_4$) corrosion products indicating the activity of SRB as these corrosion products are composed of iron and sulfur formed by the metabolism of SRB (El Mendili et al., 2013; Smith et al., 2019). Normally, sulfate reducers are considered to be active microorganisms responsible for anoxic corrosion (Rajala et al., 2015). Many reports have shown the presence of mackinawite as a consequence of steel corrosion by SRB (de Romero, 2005; Liu et al., 2000; Sherar et al., 2011). Similarly, studies on the deterioration of iron have confirmed the presence of rozenite as a corrosion product (Smith et al., 2019; Wang, 2007).

Mackinawite layer has also protective nature against corrosion of carbon steel however, the metabolites of SRB like sulfide and organic acids can damage the protective layer and promote corrosion (Liu et al., 2000). Akaganeite (detected only at the end of the experiment under non-sterile condition) is a non-magnetic ferric hydroxide and is understood as an intermediate phase which further transforms into the final product magnetite (Ruhl et al., 2014). This ferric hydroxide with chlorine is considered the main corrosion product in a typical marine environment (Rodrıguez et al., 2002). Likewise, magnetite (Fe_3O_4) was observed under sterile and non-sterile conditions indicating oxidation of iron. Anaerobic corrosion of carbon steel and cast iron in artificial groundwater caused the evolution of hydrogen gas and the formation of magnetite (Smart et al., 2001). Likewise, a study on the MIC of carbon steel demonstrated the

formation of magnetite as a corrosion product under anaerobic conditions in the presence of bacteria (El Hajj et al., 2013).

3.2.3 Chemical analysis

The concentration of chlorides, nitrates, nitrites, and sulfates were determined from every sampling point in both abiotic and biotic samples (see *Table 17*) yet, samples taken after 6 months were not analyzed due to technical problems. In the abiotic samples, the concentration of all measured compounds remained more or less stable throughout the whole experiment, whereas in the biotic samples, only the concentration of chlorides remained similar. Furthermore, in biotic samples, the concentration of nitrates (that can be used as a terminal electron acceptor by NRB) has decreased (from 579 mg/l to 28.9 mg/l) over time with only the exception after 18 months. It is obvious that nitrates have been reduced to nitrites that were below detection limit (5 mg/l) at the beginning in biotic conditions and then increased up to 232 mg/l. Nitrites did not occur in sterile conditions. In comparison to nitrates, only a small amount of sulfate has been consumed by bacteria.

Table 17: Chlorides, nitrates, nitrites, and sulfates concentration.

Sampling time	Sample type	chlorides (mg/l)	nitrates (mg/l)	nitrites (mg/l)	sulfates (mg/l)
start	abiotic	233.0	658.0	<5	1158.0
	biotic	228.8	579.0	<5	1011.1
3 months	abiotic	244.5	600.1	<5	1061.2
	biotic	221.2	455.3	26.0	918.4
12 months	abiotic	236.7	649.6	<5	1143.4
	biotic	217.0	176.6	191.0	980.6
18 months	abiotic	237.5	652.5	<5	1070.6
	biotic	213.3	517.0	15.8	963.1
26 months	abiotic	247.1	672.9	<5	1109.3
	biotic	222.9	28.9	232.1	996.7

The high concentration of nitrates (517 mg/l) and low concentration of nitrites (15.8 mg/l) detected after 18 months could be because the nitrates were not consumed in the same level as it was consumed in other sampling points. The main reason could be that different

microbial community established after 18 months replacing dominant *Methyloversatilis* (see below microbial analysis results). This phenomenon can also be ascribed to the formation of electrostatic isolation by the reduction of the proton from the metal surface and metabolic activity of *Methyloversatilis.*

Interestingly, nitrite (oxidizing agent) has a dual nature in terms of corrosion. Generally, the reduction of nitrite enhances corrosion of steel but a higher concentration of nitrite behaves as a corrosion inhibitor. Above the critical concentration (800 mg/l), it protects the steel against corrosion forming a passivating film, while below the critical concentration; it can stimulate the pitting corrosion (Jones, 1997). Nitrite is often called an anodic inhibitor as it interferes with the anodic reaction (the oxidation of elemental iron to ferrous iron) (Jones, 1997). Additionally, the corrosion inhibitory effect of nitrite at higher concentrations could be partially because of the chemical formation of nitrogen oxide, which has a toxic effect on microorganisms present on the surface of the steel (Kielemoes et al., 2000). In our study, the reduction of nitrite may have enhanced the corrosion of steel as the concentration of nitrate remained lower than the critical concentration.

3.2.4 Molecular biological analysis

qPCR analysis

All sterile control samples remained sterile for the entire period of 26 months. The sterility of the abiotic controls was checked by DNA isolation and analysis in parallel (see *Table 18*). Based on the relative quantification of 16S rRNA, the total bacterial biomass increased in course of time reaching its highest level after 12 months (increased by 750-fold compared to the initial point) (*Figure 54*). Nevertheless, at the end of the experiment, the relative abundance of bacterial biomass dropped down 12-fold. Although VITA groundwater, used as a microbial inoculum, was originally dominated with SRB, this bacterial group did not proliferate and almost disappeared. In contrast, the rapid proliferation of nitrate reducers was observed with all three markers used for the detection of NRB.

Table 18: DNA yield from biotic and abiotic samples.

	biotic			abiotic		
	volume (L)	DNA concentration (ng/µL)	DNA yield (µg DNA/L water)	volume (L)	DNA concentration (ng/µL)	DNA yield (µg DNA/L water)
start	1,00	0.04	0.00400	1.00	0	0
3 months	1.97	12.7	0.64467	1.97	0	0
6 months	1.93	1.09	0.05648	1.86	0	0
12 months	1.88	2.19	0.11680	1.90	0	0
18 months	1.85	9.34	0.49158	1.90	0	0
26 months	1.85	0.38	0.02054	1.06	0	0

The *nirS* gene reached its maximum abundance right after 3 months (increased by 127-fold compared to the initial point) and then it gradually decreased to the level below the limit of detection from 18 months. The relative abundance of the *nirK* gene increased rapidly after 3 months and then declined to the numbers that were similar to the starting point after 6 months. Then again, it increased and reached a maximum value of 686 after 18 months but then decreased by the end. Likewise, the *nosZ* gene, responsible for the expression of nitrous oxide reductase, showed the highest relative abundance after 3 and 12 months when increased 2000 times but decreased unexpectedly after 18 months. It was the only functional marker that was detected even after 26 months. *Geobacteraceae* were not detected in any sample. NRB are involved in the process of denitrification - a complete reduction of nitrates to nitrogen by the consumption of intermediate products like nitrites, nitric oxides, and nitrous oxides ($NO^-_3 \rightarrow NO^-_2 \rightarrow NO \rightarrow N_2O \rightarrow N_2$). Hence, nitrates from synthetic water have been consumed by NRB to carry their metabolic activity.

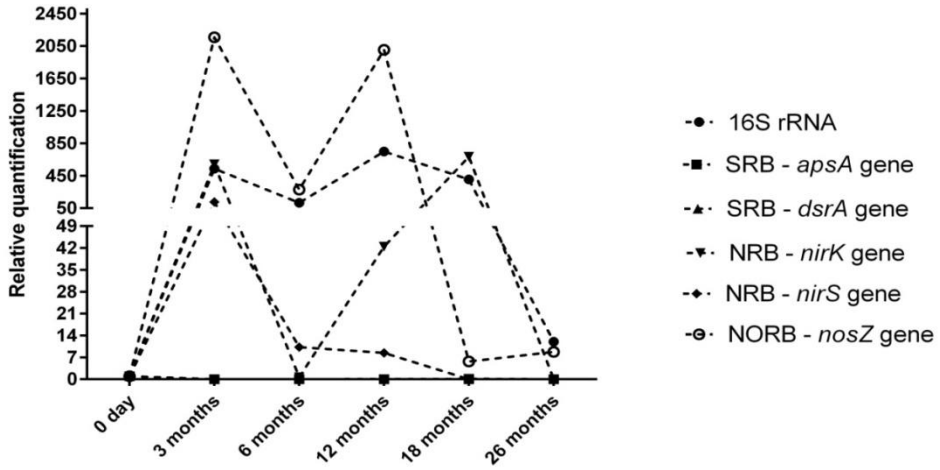

Figure 54: Relative changes of total bacterial biomass (detected by 16S rRNA), SRB (detected by apsA and dsrA genes), and denitrifying bacteria (detected by nirK, nirS, and nos-Z genes) through the experimental period.

The dominance of NRB was clearly caused by the chemical composition of SBPOW, which mimics the Czech BaM bentonite leachate, rich in nitrates, and thus offering thermodynamically favorable terminal electron acceptor for NRB. Like sulfate, nitrate is also naturally present in deep geosphere besides being a constituent of bentonite. However, sulfate is less thermodynamically favorable terminal electron acceptor than nitrates. Therefore, the reduction of sulfate and proliferation of SRB generally starts when the nitrates are consumed following the thermodynamic ladder. When the availability of organic donors in the environment is lower than required, carbon steel is used as means of the electron to produce energy by bacteria (Rajala et al., 2015) and hence, induce the process of corrosion. Some members of NRB are also sulfide oxidizers that are capable to reduce nitrate to nitrite or nitrogen oxides which can result in the formation of highly corrosive elemental sulfur or polysulfides (Ock Joo et al., 2015).

16S rRNA sequencing results

A gradual shift in the microbial community structure was observed over time. Sequencing data correspond to results of qPCR analysis showing similar patterns of microbial community development (*Figure 55*). VITA water, which was used as a natural microbial inoculum for the experiment, was dominated by members of genera *Desulfomicrobium*, *Desulfovibrio* (both SRB), and *Lacunisphaera* (NRB). Later, the structure of microbial community changed dramatically, SRB disappeared and were replaced by different genera of NRB, such as *Methyloversatilis,*

Brevundimonas, *Pseudomonas*, *Phenylobacterium*, *Achromobacter*, *Devosia*, *Acidovorax*, *Lacunisphaera* and *Sphingobium*. In comparison to SRB corrosion, NRB corrosion has been reported only occasionally in the literature. According to bioenergetics, iron oxidation coupled with nitrate reduction provides energy for the respiration for NRB which can lead to MIC (Xu et al., 2013). Regarding iron oxidation and nitrate reduction, two major phenomena have been proposed. The first one is the chemical reduction of nitrate with ferrous/nitrate redox couple (abiotic phenomena) and the second once is induced by denitrifying bacteria owning to oxidation of metallic or ferrous iron (biotic phenomena) (Kielemoes et al., 2000).

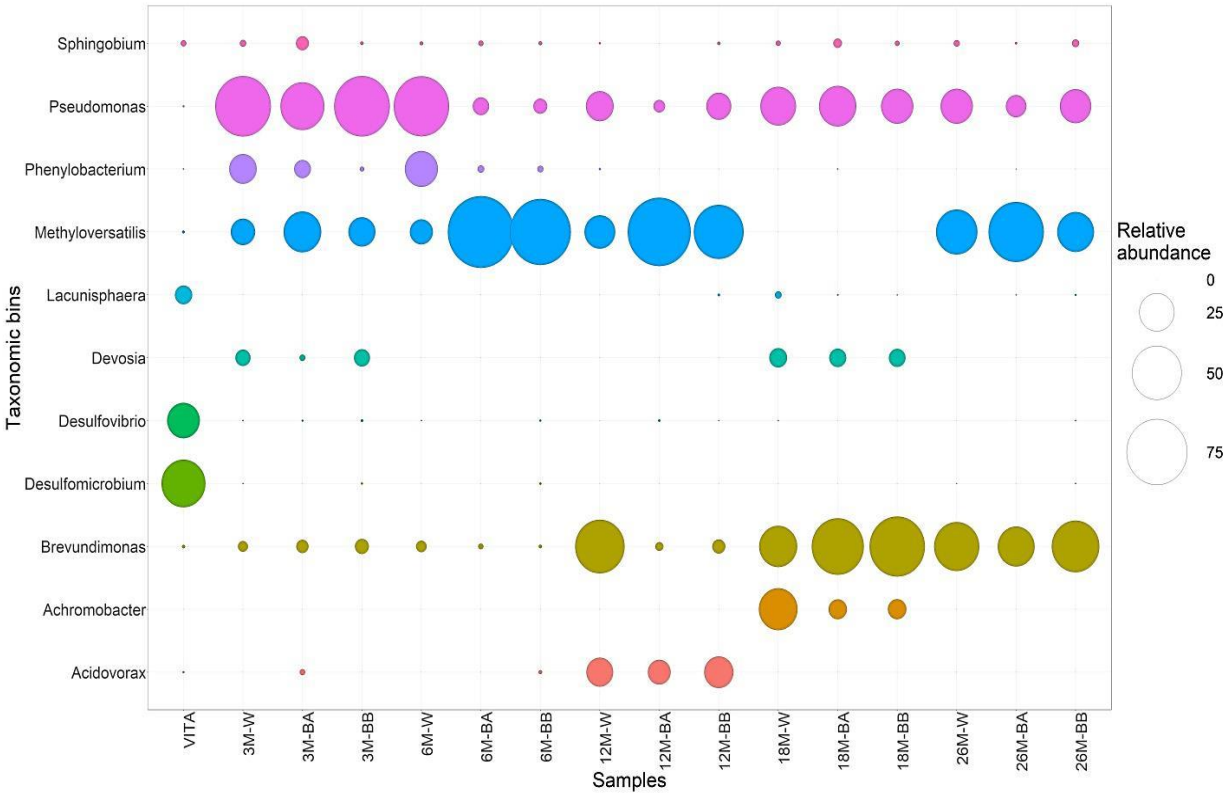

Figure 55: Result of 16S rRNA sequencing of the samples taken after 3, 6 12, 18, and 26 months showing genera with the mean of relative abundance in %. M - months, VITA - composition of initial groundwater inoculum, W- water sample (SBPOW inoculated with VITA), B - is biofilm where A and B are replicates.

Although the activity of sulfate reducers was suggested by Raman spectra under non-sterile conditions, it was not detected by microbial community analysis. After three months, both water and biofilm samples were dominated with bacteria belonging to genus *Pseudomona*s followed by *Methyloversatilis* and *Phenylobacterium*. After six months, the proportion of

detected species differed clearly between biofilm and water samples. Similar to the previous sampling, *Pseudomonas* dominated, but *Methyloversatilis* was the commonest bacterium in the biofilm samples. The same genus, *Methyloversatilis* dominated in the biofilm samples after 12 months followed by *Acidovorax* and *Pseudomonas*, whereas *Brevundimonas* was the most frequent in the water sample. It remained frequent in both water and biofilm samples after 18 and 26 months. In the samples (both water and biofilm) collected after 18 months, unexpectedly any sign of *Methyloversatilis* was detected. In contrast, bacteria belonging to genus *Achromobacter* were detected in this sample and were not observed in any other sampling point. Hence, the community structure of these samples differed significantly compared to all other sampling times and was composed mainly of *Brevundimonas*, *Pseudomonas*, and *Achromobacter*. Interestingly, genera like *Methyloversatilis (*Rhodocyclacea*), Brevundimonas* (Alphaproteobacteria), and *Pseudomonas (*Gammaproteobacteria) were the most frequently detected genera. These denitrifying bacteria are chemoheterotrophic or chemoautotrophic and are mostly mesophilic in nature. Among these genera, *Pseudomonas* was previously reported as denitrifying bacteria responsible for MIC. (Zhou et al., 2018; Jia et al., 2017b). Apart from SRB and NRB many other bacteria like iron-oxidizing bacteria, manganese-oxidizing bacteria, methanogens, and fungal species are also linked to the acceleration of the corrosion process for deterioration of metal (Li et al., 2018).

In the original natural groundwater VITA, most of the NRB genera were not detected at the initial point, which could have fallen below the limit of detection as we have shown the genus of relative mean abundance over 1% only. Nevertheless, by time with the availability of nutrients in water/ SBPOW and the presence of electron donors (carbon steel), they proliferated. The similarity in microbial structure between water and biofilms for the first 3 months and their difference comparing to later sampling suggests that there has been the formation of a biofilm layer with specific bacteria that may be different compared to the surrounding environment. Synergistic interaction of various microorganisms occurs in biofilm which consequences in the sharing of nutrients and energy among themselves causing a severe MIC (Li et al., 2018). In this study, biofilm formation and severe local corrosion contributed by anaerobes and facultative anaerobes of different species of nitrate reducers were confirmed. Under nitrate-reducing conditions, presence of a different type of microbial community in the biofilm can lead not only to uniform corrosion but also to local attacks (Miller et al., 2018) as was seen in this study since

the number of bacteria differed significantly between water and biofilms samples, it is more relevant to take biofilm sample for better understanding of corrosion behavior. Nonetheless, formation biofilm on the steel surface can provide protection to steel against corrosion by the establishment of passivation effect forming a protective layer. However; later the porous biofilm will not continue to form a protective layer and localized corrosion can be initiated when the biofilm become weak, fragile and fractured (Paula et al., 2016). Therefore, the biofilm can modify the chemistry of a protective layer ranging from the acceleration of corrosion to corrosion inhibition (Beech and Sunner, 2004).

Interestingly, the abundance of *Methyloversatilis* positively correlates with the corrosion rates (see *Figure 56*). The *Methyloversatilis* population dominated the samples after 3, 6, 12, and 26 months when the corrosion rates were higher, while no *Methyloversatilis* was detected after 18 months when the corrosion rate was the lowest. *Methyloversatilis* belongs to methylotrophic bacteria capable of using a single carbon compound. In absence of oxygen, *Methyloversatilis* has a unique ability to utilize nitrate as the electron acceptor for the energy generation (Lu et al., 2012) and proliferates well when hydrogen is present as the electron donor (Ontiveros-Valencia et al., 2013) Consequently, it suggests that the *Methyloversatilis* biofilm may work as the cathode and local bare metal within biofilm pores works as anode with much higher corrosion rate accelerated by bacterial metabolism resulting in E-MIC. *Methyloversatilis*, not detected after 18 months, might have faced a lack of hydrogen that is produced by reduction of the proton by the electron from the metal surface (as explained above). This process of hydrogen generation can form a film that could prevent further reduction of protons leading to passivation (Valencia-Cantero and Peña-Cabriales, 2014). Hence, it inhibited corrosion and at the same time, created a scarcity of electron donors in the environment, which most probably had a direct effect on the metabolism of *Methyloversatilis.*

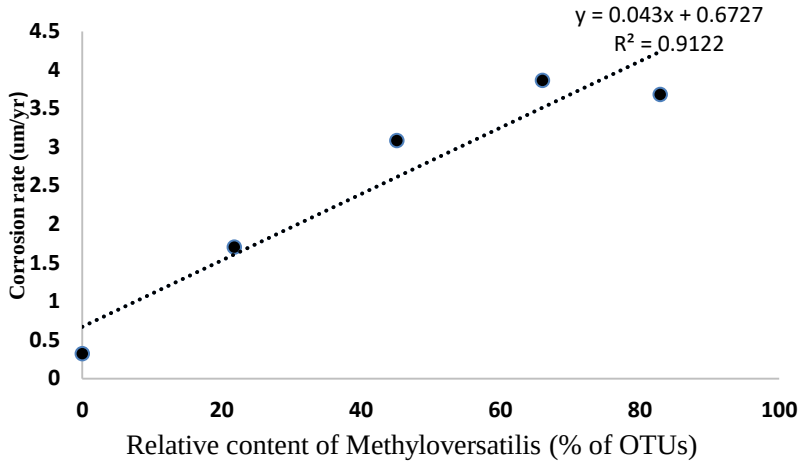

Figure 56: Corrosion rate and the relative abundance of Methyloversatilis.

On the other hand, the presence of *Achromobacter* only in absence of *Methyloversatilis* could be due to their similar methane utilization capacity as a sole source of carbon when present and the ability to consume hydrogen (Davies, 1973; Ontiveros-Valencia et al., 2013). Hence, *Methyloversatilis* most probably over competed the *Achromobacter* and can be considered as the best candidate for MIC under nitrate-rich environmental conditions in our case.

4 Summary

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 biology analysis of both water and biofilm indicated

the dominance of *Desulfomicrobium* and *Desulfovibrio* spp. (both SRB) in the experiment with only VITA water, 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*. 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. 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

1 Background

Concrete, a cementitious material, will be used as an important part of DGR of radioactive waste (Honty et al., 2010). The concrete will be used to construct the sealing plug after the operational part of the repository finishes (Hanusová et al., 2016) and also as filling matrix for LLW and ILW. The most common material used is ordinary Portland cement, in which calcium component comprises the major part (Glasser and Atkins, 1994). A wide variety of indigenous microbial communities with specific metabolic pathways exists both in bentonite buffer and groundwater and therefore may be active in the bentonite layer itself and also at the interfaces between the cementitious material, bentonite buffer and host rock.

Chemical compounds present in concrete generally increase the environmental pH and might also cause an increase in the temperature due to heat produced by the hydration of cement. Resulting shrinkage in the pore size and related decrease in nutrient availability may cause bacterial inactivation (Williams et al., 2017). Thus, the presence of concrete can severely affect the microbial communities that come into contact with these conditions. Recent research has demonstrated that many species of bacteria are capable of surviving in high alkali conditions. Anaerobic alkaliphilic bacteria like *Thialkalivibrio denitrificans* (Sorokin et al., 2001), *Bacillus pseudofirmus* (Janto et al., 2011), *Alkaliphilus transvaalensis* (Takai et al., 2001) and *Alkalitalea saponilacus* (Zhao and Chen, 2012) were isolated from the natural alkaline habitats. Nevertheless, over the period of DGR, the pH of the concrete is expected to decrease gradually by the carbonation and by neutralization with the microbially produced minerals or organic acids consequently resulting in biodegradation of concrete and also metal rebars used to reinforce the concrete (Wei et al., 2013). It is necessary to well understand the long-term structural integrity of concrete to predict its ability to contain waste over a long period because there is a constant possibility of microbially induced degradation of concrete structures (Turick and Berry, 2016). Understanding of interactions between concrete and microorganisms is thus a very important step toward the development of more sustainable, better quality, safer structures in DGR (Bertron, 2014).

Some of the microorganisms are capable of inducing concrete deterioration by generating various acids such as sulfuric acid, nitric acid or organic acids as a result of their metabolism which has a strong capacity to degrade the components of concrete and thus, compromises the

reliability of concrete structure (Turick and Berry, 2016; Wei et al., 2013). Aggressive sulfur-oxidizing bacteria like *Thiobacillus thiooxidans* and other *Thiobacilli* (Nica et al., 2000; Rogers et al., 2003), *Acidithiobacillus* (Ling et al., 2014) or *Thiobacillus ferrooxidans*, acidophilic iron-oxidizing bacteria capable of sulfur oxidation (Yamanaka et al., 2002) are commonly responsible for microbially induced deterioration (MID) of concrete. In contrast, microorganisms can also possess the ability of self-healing and sealing of cracked concrete materials (Wiktor and Jonkers, 2011). This phenomenon is attributed to microbial precipitation of calcium carbonate. Incorporation of calcinogenic bacteria helps in the remediation of cracks in the concrete surface and also improves its durability (Xu and Wang, 2018). Precipitation of calcium carbonate is influenced by ureolytic bacteria; such bacteria are capable of precipitating calcium carbonate by the production of urease enzyme. This enzyme catalyzes the hydrolysis of urea to carbondioxide and ammonia and subsequently, increases the pH and carbonate concentration in the environment (Siddique and Chahal, 2011; Stocks-Fischer et al., 1999). Urea is not a common compound in concrete but is mixed with concrete to promote this microbial effect (Chidara et al., 2014) and also to enhance durability and flowability of concrete (Mwaluwinga et al., 1997). Ureolytic bacteria like *Sporosarcina pasteurii* or some species of *Bacillus* can enhance the compressive strength and reduce the porosity and permeability of the concrete. Bacteria can deposit a layer of calcite on the concrete surfaces and within the pores, which reduce the capillary water uptake and gas permeability (Achal et al., 2011; Chahal et al., 2012; Luo et al., 2018). Microorganisms can severely affect the concrete properties and the knowledge about them is rather extensive. However, the effect of concrete presence on the naturally present microorganisms in the surrounding environment is much less understood through the microorganisms in the environment can represent an important source of possible concrete influencing microflora. This might be especially true in the case of ILW or HLW repository where the bentonite filling and sealing layer rich in indigenous microorganisms could eventually come in contact with the outer concrete layer protecting the waste package or the concrete plague (Koťátková et al., 2017).

The major goal of this study is to investigate the changes in indigenous microbial community composition and their activity caused by the presence of concrete under the LLW/ILW repository relevant conditions. Indigenous Czech BaM bentonite and VITA groundwater's microflora were set to react in the presence of concrete. Such information about

the microbial ecology in the presence of concrete can be highly relevant for the LLW/ILW DGR concept and its safety.

2 Materials and methods

2.1 BaM bentonite and VITA groundwater

BaM bentonite and VITA water from Josef URC as described in section 2.1 of the chapter II were used for the study.

2.2 Concrete

Aged low alkaline concrete from Josef Underground Research Centre (URC), Czechia used in a European DOPAS project (Demonstration of Plugs and Seals, grant agreement No. 323273) (Hanusová et al., 2016) was chosen as a material for this experiment. One of the required limits of this concrete was to reach the pH of leachate < 11.7, in optimal case pH ≤ 11.5. The obtained concrete was crushed in a jaw crusher, milled in a planetary ball mill, and sieved on a vibratory sieve shaker with the porosity ≈ 125 μm so that particles were of similar size to mix with bentonite. The concrete powder was kept in the anaerobic glove box until the start of the experiment to deoxygenize.

2.3 Experimental set-up

The experiment was performed under strictly anaerobic conditions (CO_2 < 1ppm) in the glow box in Research Centre Řež, Prague at laboratory temperature. To avoid contamination during sampling, each sample was prepared in a separate reactor bottle. For samples containing bentonite and concrete eight reactor bottles were prepared, each one consisted of 15 g BaM bentonite (described in chapter I), 15 g of crushed concrete, and 100 ml of VITA water. Further, we prepared four control samples containing 15 g BaM and 100 ml VITA water (further called bentonite control), two control samples without VITA water consisting of 15 g bentonite, 15 g concrete, and 100 ml sterile water (BCW control) and two control samples without the concrete and VITA water (15 g bentonite + 100 ml sterile water), further called BW control. All these controls were included to distinguish between the effects of each of the reactants used. The

experiment run for 2 months and samples were taken in duplicates at the beginning (start), after one week (1 w), two weeks (2 w), one month (1 m) and after two months (2 m).

2.4 Sample processing and performed analysis

At each sampling time, 100 ml of suspension samples were centrifuged at 11,500 × g for 10 min to separate the supernatant. The supernatants were used for chemical analysis, the pellets were used for chemical analyses, surface and porosity analysis, and DNA extraction.

2.4.1 pH and Eh measurement

We measured the pH and Eh of each sample. pH 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.

2.4.2 Chemical analysis

Supernatants of each sample 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 K_2CO_3 a 1 mM $KHCO_3$ 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.

2.4.3 Molecular biological analysis

Molecular biological analyses were performed on the sample pellets obtained from centrifugation and followed the same method as described in section 2.4 in chapter II.

2.4.4 Data analysis

Data processing was performed as described in section 2.4.4 in chapter II. Deseq2 was used to determine taxa being mostly influenced by concrete and no concrete environment. Only taxa with relative abundance over 5% were selected for Deseq2 analysis and for indicator species analysis as well. Indicator species analysis was done using indispecies R library and P values threshold was set to 0.05. Furthermore, principal coordinates analysis (PCoA), analysis of similarities (ANOSIM) was conducted in Phyloseq package.

2.4.5 Surface and porosity analysis

Changes in the concrete surface morphology were observed by using a LYRA3 scanning electron microscope (Tescan, Czech Republic) with secondary electron detectors (SE and In-beam SE mode) and back-scattered electrons (In-beam BSE mode) at 5 kV accelerating voltage. Energy-dispersive X-ray spectroscopy (EDS) was used to determine local chemical composition using unprepared samples. Subsequently, the samples were modified by gold-sputtered with a thickness of 30 nm to provide charging reduction. Cross-section analysis was then performed at 20 kV accelerating voltage.

The specific surface area was measured with the Quadrasorb EVO/SI and calculated by the QuadraWin software according to the DFT/BET isotherm. Porosity was determined by pure liquid N2 adsorption at 77 K. Before the analysis, all samples were degassed under vacuum at 60°C for 24h.

3 Results and Discussion

3.1 pH and Eh measurement

Initial pH was about 9.3 in all experimental samples with concrete (bentonite concrete sample and BCW control). However, by the first week, the pH in these samples increased to 10 and remained at this high pH value until the end of the experiment. On the other hand, in bentonite control samples and BW control without concrete, the pH was lower (approximately 8.9 at the beginning) than in concrete containing samples and the detected pH values further slightly decreased throughout the experiment to 8.5 by the end of the experiment (*Figure 57).*

In the beginning, redox potential (Eh) was around -115 mV in all the experimental samples and the Eh value gradually decreased in time due to the reduction of oxidized compounds by microorganisms. Samples containing concrete (bentonite concrete samples and BCW control) had slightly lower Eh values than samples without concrete (bentonite controls and BW control), *Figure 57*. The lowest detected Eh value was -271 mV in bentonite concrete samples at the end of the experiment and -232 mV in bentonite controls. The value of Eh for BCW and BW changed from -112mV at the beginning to -178 mV and -65 mV, respectively at the end of the experiment.

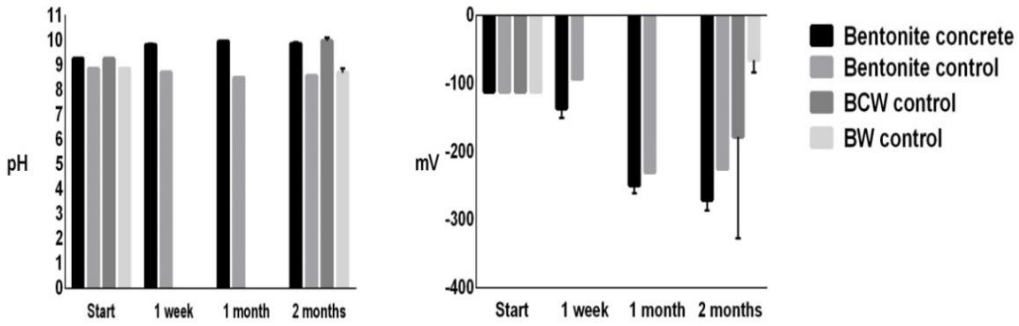

Figure 57: pH and Eh values measured in bentonite concrete (bentonite, concrete, and VITA water), bentonite control (bentonite and VITA water without concrete), BCW control (bentonite, concrete, and sterile water) and BW control (bentonite and sterile water).

Bentonite environment is generally alkaline (Ye et al., 2014) and the pH of cementitious materials is even higher. The concrete we used for this experiment is low alkaline concrete with the pH of leachate below 11.5 in the optimal case due to gypsum and soluble alkali content (Hanusová et al., 2016). An increase in pH was detected in samples containing concrete compared to only bentonite samples as expected and the level remained stable. Constantly high pH levels in the concrete samples regardless of detected microbial activity, which generally reduces pH (see below) imply a high buffering capacity of the concrete environment. The portlandite ($Ca(OH)_2$) and calcite ($CaCO_3$) present in cementitious material establish a chemical restraint on the water phase composition. This chemical restraint is caused by the mineral transformation from one to another at the phase boundary and represents a chemical buffer in cementitious materials (Reardon and Fagan, 2000). Additionally, hyper alkaline matter and

predominance of calcium silicate hydrate (CSH) gel in cement can be attributed to higher pH than control samples (Savage and Benbow, 2007).

In bentonite controls, on the other hand, a slight decrease in pH was detected in two months. The decrease of pH in the samples is often caused by microbes as a result of their respiration, because of the metabolic production of organic (acetic, lactic and succinic) and mineral (sulfuric, nitric) acids that decrease the pH in the environment (Bertron, 2014). Likewise, fermentation under anaerobic conditions produces an acid that lowers the pH. Although the reduction in pH can inhibit the growth of alkaline bacteria, some of these bacteria are resistant to fermentation acid (Russell and Diez-Gonzalez, 1997).

The redox potential reflects the balance between oxidizing and reducing conditions in the environment and is influenced by the chemical species present. Resulting redox conditions in the environment determines the physiological type of microbes present because microbes possess specific metabolic functions based on redox reactions and very sensitively react to the environmental conditions (Turick and Berry, 2016). The detected low value of redox potential in our samples agrees with the anaerobic condition in which the samples were kept during the experiment. The detected Eh was generally lower in concrete containing samples, but this lower value can be attributed to the fact, that in pure aqueous solution the Eh value decreases with a slope of 59 mV per pH unit (Sparks, 2003). The difference thus does not have to be caused by the microbial activity but can be just a function of pH.

3.2 Chemical analysis

LLW and ILW may contain organic compounds, nitrate, iron, metal oxides, or hydrogen (evolved by corrosion of metal waste/ container). Such compounds are crucial for establishing a suitable environmental condition for the growth of microorganisms (Rizoulis et al., 2016). The estimation of the particular concentration of these compounds in different materials and wastes can thus help us to predict potential microbial activity within the repository.

Nitrate concentration was about 4.1 to 4.6 g/l in most of the samples at the beginning of the experiment. However, the nitrates were rapidly consumed within the first week by nitrate reducers and the detected values remained below 0.5 mg/l (detection limit) in all subsequent samples. The small amount of detected nitrate in the samples originated probably from secondary

environmental contamination of bentonite and/or concrete. The concentration of sulfate in the samples containing concrete (bentonite concrete samples and BCW control) was about 4.5-times higher (mean 962.13 mg/l), than in the control samples without concrete (mean 209.66 mg/l), (*Figure 58*). The concrete thus represents a significant source of sulfate in the experimental system. The detected concentration of sulfate was highest after the first week in the bentonite concrete samples when most of the sulfate probably dissociated to the solution. Afterward, it gradually decreased to the nearly initial levels. Generally, microorganisms use sulfate as an electron acceptor when energetically more favorable compounds such as ferric ions (common in BCV bentonite) are very reduced by microbial activity (Bethke et al., 2011). Unfortunately, we were not able to measure the concentration of ferric ions in the samples as it is an analytically very challenging task. However, based on the genetic data, we assume the ongoing iron reduction in our samples (see below). The pattern of sulfate concentration, although variable, between the samples, indicated that sulfate was probably not used as the major electron donor in the microbial metabolism during the experiment yet. Because we have not detected an increase of SRB in concrete containing samples (see below), the detected sulfate decrease in the last sampling point is probably not linked with the microbial activity. In bentonite and BW controls, the sulfate concentration remained low and rather stable during the experiment except for the detected increase in the sulfate concentration in BW control at the end of the experiment. The reason for such an increase remains unclear and could represent measurement error.

Microorganisms need electron donors to reduce the terminal electron acceptors like nitrate, iron, and sulfate. For heterotrophic microorganisms whose metabolism is based on organic carbon sources, DOC naturally present in bentonite, concrete, or waste itself can be the most probable source of carbon and energy in the system (Kirchman et al., 1991). Furthermore, groundwater plays a vital role in driving DOC from the terrestrial environment to the anaerobic underground ecosystem (Fisher and Likens, 1973). Similarly to the sulfate, the detected concentration of dissolved organic carbon (DOC) tends to be higher in samples containing concrete (bentonite concrete samples and BCW control, mean 31.71 g/kg) than in bentonite controls and BW control without concrete (mean 23.7 g/kg), shown in *Figure 58*. This result implies that the concrete we used might be a significant source of dissoluble organic material. Interestingly, the final concentration of DOC in bentonite controls was much higher than the DOC concentration detected in all other samples at the end of the experiment (approximately 44

g/kg at the end compared to 18 g/kg in the beginning). Such an increase can indicate the ongoing microbial acetate production, but we have not measured its concentration in our samples. The final concentrations of DOC in other samples except for bentonite concrete samples were also markedly higher than detected in these samples before. Further investigation would be needed to better understand the DOC concentration evolution in our experimental system.

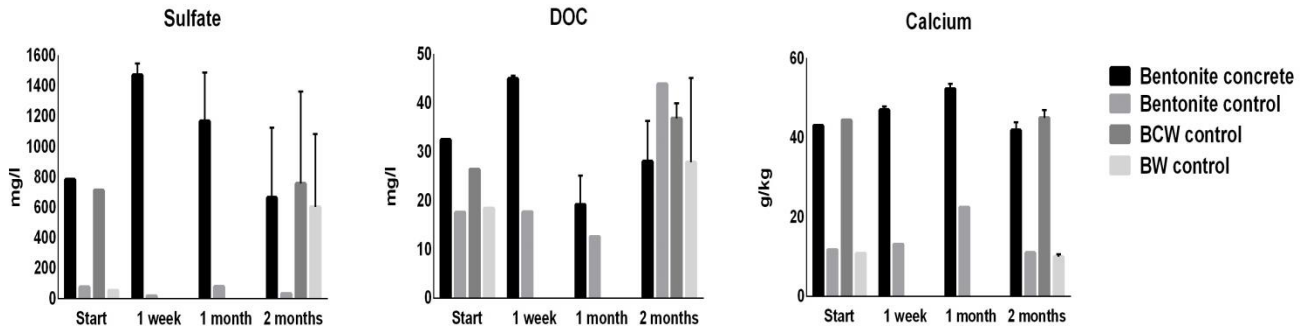

Figure 58: Concentration of sulfate, dissolved organic carbon (DOC) and non-soluble calcium in sample pellet measured in bentonite concrete sample (bentonite, concrete, and VITA water), bentonite control (bentonite and VITA water without concrete), BCW control (bentonite, concrete and sterile water) and BW control (bentonite and sterile water).

The concentration of insoluble calcium detected from the dry mass was, similarly to the sulfate and DOC values, much higher in samples with the concrete (mean 45.98 g/kg in bentonite concrete samples and BCW control) than in the controls without concrete (mean 12.72 g/kg) samples (*Figure 58*), because the type of cement used in this aged concrete was CEM (calcium-enriched mixture) II/B-M (Svoboda et al., 2017). Calcium is one of the major components that maintain the mechanical properties in concrete. However, its concentration was rather constant during the whole course of the experiment in all the treatments suggesting no leaching of calcium or calcification (i.e. the change in the Ca solubility) within the samples in two months. Similarly, SEM analysis did not reveal any structural or mineralogical changes in the samples, see below.

3.3 Molecular biological analysis

3.3.1 Microbial communities characterized by qPCR

The growth of particular bacteria during the experiment indicates the suitability of the environmental conditions to that specific microorganism. An increase in total microbial biomass (detected by 16S rRNA) was revealed by the qPCR analysis in all samples regardless of their composition (*Figure 59*). However, microbial biomass detected at the last sampling point was several times higher in control samples without concrete, than in samples with concrete (total microbial biomass increased 286 times in bentonite control samples and 867 times in BW control compared to the initial values but only 59 times in bentonite concrete samples and 37 times in BCW control). Such a result implies a strong negative effect of concrete on total microbial biomass compared to the bentonite samples.

When we focus on the functional groups of microorganisms, noticeable proliferation (at least 5 times increase in the gene copies) was observed in sulfate, nitrate, and iron-reducing bacteria in bentonite control samples without concrete (*Figure 59*). However, in the presence of concrete, the growth of these bacteria was much lower. We detected only 5.8 times increase in IRB and 4.7 times increase of NRB in bentonite concrete samples. Interestingly, sulfate reducers did not increase their biomass either in the concrete samples (bentonite concrete samples and BCW control) or in BW control, where their level remained under detection limit during the whole experiment. However, a noticeable proliferation of SRB was detected in bentonite controls. These results imply firstly, that the growth of SRB is strongly inhibited by the presence of concrete, although concrete represents a large source of sulfate for microbial metabolism. Secondly, VITA water might be a primary source of SRB in the experimental system.

Our qPCR results, demonstrating the microbial inhibition by concrete, well agree with another study that was carried on microbial fouling and corrosion of carbon steel in deep anoxic alkaline groundwater (Rajala et al., 2017). It showed that the number of bacteria and archaea in the presence of concrete was 1000-fold lower, with 620-times lower corrosion rate in comparison to natural groundwater without concrete. Relatively small microbial growth in the concrete samples might be accredited to the harsh conditions in the concrete compared to the bentonite environment, which itself is rather extreme. Concrete can contain additional chemical

compounds such as calcium formate which has an inhibitory effect especially on sulfide oxidizing bacteria (Turick and Berry, 2016). Similarly, calcium hydroxide may have an inhibition effect on microorganisms as has been reported on anaerobic bacteria (Morrier et al., 2003). On the other hand, loss in bacterial growth or reduction in their metabolic activity can be minimized by the adsorption of bacterial cells into concrete pores by encapsulation which provides a suitable microenvironment to them and protects them in aged concrete (Xu and Wang, 2018).

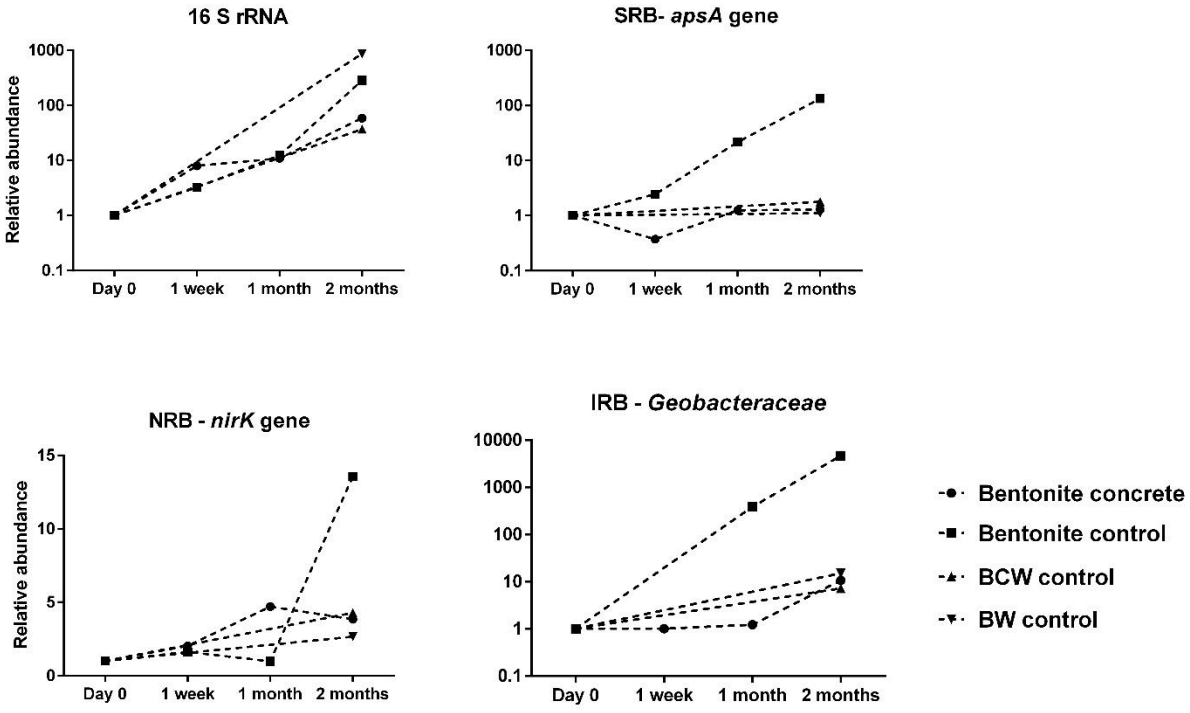

Figure 59: Relative quantification of changes in microbial abundance. Bentonite concrete – bentonite, concrete and VITA water, bentonite control – bentonite and VITA water without concrete. BCW control - bentonite, concrete and sterile water, and BW control - bentonite and sterile water. Only the Cq values above the detection limit were used for the calculations. Only those genes which had a remarkable change in relative value are shown in the figure. Relative values are presented in the log scale except for the nirK gene.

3.3.2 Microbial populations detected by next-generation sequencing

A diverse community of bacteria was detected in all the samples and the microbial composition evolved in time. The exact microbial composition depended on the sample composition and differed a lot between concrete containing samples (bentonite concrete samples and BCW

control) and bentonite samples without concrete (bentonite control and BW control) as revealed in *Figure 60*. For this reason, we will further describe the changes in microbial composition in concrete and samples without concrete separately.

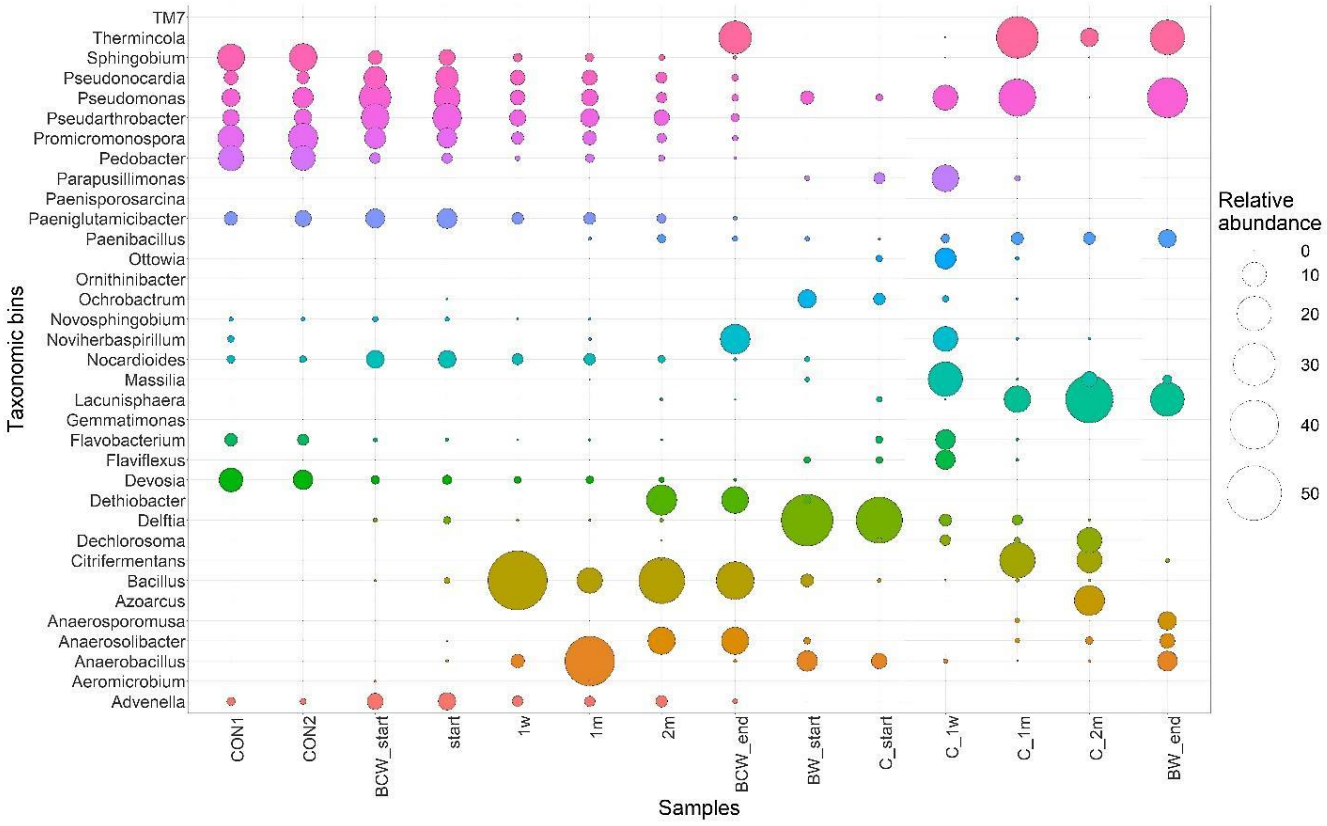

Figure 60: Genera detected by 16S rRNA amplicon sequencing in different samples. 1w – sampling after the first week, 1m and 2m – sampling after the first (second) month. Start, 1w, 1m and 2m – bentonite concrete samples, C – bentonite control samples (bentonite + VITA water), BW – control samples (bentonite + sterile water), BCW – control samples (bentonite, concrete, sterile water), CON – concrete powder. Relative abundance is in %.

Both initial samples containing concrete (BCW_start and start) had a very similar microbial composition and the genera composition in these samples was also very similar to the genera detected in concrete powder (CON1 and 2) The most frequent genera were nitrate-reducing facultative anaerobes *Pseudomonas*, *Pseudarthrobacter*, *Paeniglutamicibacter*, *Pseudonocardia*, *Promicromonospora*, *Shingobium* or *Nocardioides*. Furthermore, aerobic genus *Devosia* and NRB genus *Flavobacterium* were common in concrete powder. Subsequently, we

observed a gradual change in microbial composition in our samples during the experiment. Facultatively anaerobic genus *Bacillus* became the most abundant within the first week of the experiment and remained prominent until the end of the experiment in all concrete containing samples. Within the second month of the experiment, obligately anaerobic chemolithotrophic thiosulfate reducing genus *Dethiobacter* became further significant in concrete containing samples and followed by genus *Anaerosolibacter*. *Dethiobacter* is capable of performing its metabolic activity by oxidation of hydrogen under high alkaline conditions (Sorokin et al., 2008). *Dethiobacter* and *Anaerosolibacter* can cause MIC of a metal container or MID of reinforced concrete containers by the production of sulfide as part of metabolism. Iron reducing genus *Thermincola* and nitrate-reducing *Noviherbaspirillum* was another dominating genus especially in concrete containing BCW control after the second month of the experiment. *Thermincola* is an obligately anaerobic spore former responsible for the reduction of Fe^{3+} by oxidation of hydrogen or thiosulfate (Kunapuli et al., 2007). Iron reducing bacteria are suspicious of altering the mechanical properties of bentonite by the reduction of structural ferric ions to ferrous one, which can lead to illitization of bentonite and decrease of its swelling ability (Kim et al., 2004)

In control samples without concrete, the microbial composition of zero-point samples in both controls (C_start and BW_start) was very similar. Generally, these samples showed high diversity – i.e. high number of genera with very low abundance. Both controls were dominated by facultatively anaerobic heterotrophic NRB genera *Delftia*. This species is a common laboratory contaminant detected in various commercially available kits (Salter et al., 2014). Because the DNA yield in zero-point samples is generally very low, the possible contaminations and PCR bias can play a more significant role in the detected microbial profile of such samples. The other detected genera in zero bentonite samples were *Anaerobacillus, Bacillus, Ochrobactrum,* or *Pseudomonas,* all facultatively anaerobic genera capable of nitrate reduction in the absence of oxygen. During the first week of the experiment the microbial composition changed, and several different nitrate-reducing facultatively anaerobic genera like *Massilia, Parapusillimonas*, and *Pseudomonas* were detected. Within one month, the microbial composition further changed and the samples became dominated by obligate anaerobes like *Thermincola* and *Citrifermentans* respiring ferric iron followed by facultative anaerobes such as *Pseudomonas* and *Lacunisphaera*. In two-months-old control samples without concrete *Thermincola, Lacunisphaera* and *Paenibacillus* were the most dominant genera in both controls.

In the bentonite concrete control, further genera such a genus *Dechlorosoma,* capable of nitrate and chlorate reduction (Achenbach et al., 2001), not-yet described genus *Citrifermentans* closely related to anaerobic iron-reducing genus *Geobacter,* or *Azoarcus* were also very frequent. Genus *Lacusnisphaera* is known as an aerobic alkaliphilic thermophile (Rast et al., 2017). However, because our experiment was run under strictly anaerobic conditions, the detected *Lacunisphaera* representative could belong to some unidentified species of this genus that may have the ability of facultative or fermentative respiration. *Citrifermentans* (*Geobacte*r), iron-reducing bacteria is responsible for the reduction of Fe^{3+} by oxidation of hydrogen or organic compound (Caccavo et al., 1994) similar to *Thermincola*. The nitrogen-fixing genus *Azoarcus* is another common laboratory contaminant (Salter et al., 2014) and as it was not detected in any of the other samples during the experiment, we consider is as sample contamination. In BW control after two months, the detected microbial composition was quite similar to the one and two-month-old bentonite controls but was relatively more enriched in NRB genera *Anaerosolibacter* and *Anaerobacillus* or acetogenic genus *Anaerosporomusa*.

3.3.3 Difference between concrete and without concrete samples

As was mentioned above, the concrete containing samples differed from the bentonite ones in many features such as the availability of nutrients or pH in general. The increased pH due to dissolving of $Ca(OH)_2$ from concrete can specifically support the growth of alkaliphilic bacteria (Luo et al., 2018) Unsurprisingly, the detected difference in microbial composition between concrete containing samples and bentonite controls was relatively high. In all the suspensions, we could generally detect gradual evolution in the microbial community composition from the facultatively anaerobic nitrate reducers toward obligatory anaerobic genera. However, the particular composition was strongly influenced by the presence of concrete as described above. When we focus on the facultative anaerobes, the concrete containing suspensions were dominated by the genus *Bacillus*, while the bentonite controls were dominated by the genera *Lacunisphaera* and *Pseudomonas* that are probably more tolerant to the alkaliphilic environment, *Figure 60*. As for obligatory anaerobic genera belonging to iron reducers, we detected both genera *Thermincola* and *Citrifermentans* in bentonite controls. Furthermore, at least some species of the genus *Pseudomonas,* common in bentonite controls, are also capable of Fe^{3+} reduction (Arnold et al., 1988). On the other hand, in concrete containing samples, the genus

Citrifermentans was not detected and genus *Thermincola* was abundant only in BCW control. Such results indicate that the genus *Citrifermentans* (or *Geobacter* in a broader sense) might be more sensitive to the extreme condition of concrete containing samples.

To better understand the difference in microbial composition patterns in different samples, we performed principal coordinates analysis (PCoA) based on detected operational taxonomic units (OTUs). This method revealed spatial distribution corresponding to the experimental set-up (*Figure 61*). The first axis generally clustered the samples according to the presence of concrete. On the other hand, the distribution of the samples based on the second axis correlated rather with the time and detached the samples from the beginning and the end of the experiment. The BCW control samples clustered well with the other concrete containing samples of similar age, while BW controls clustered with bentonite controls without concrete, again with the time factor being important in the spatial distribution. The PCoA further showed that the microbial composition of the concrete powder samples was very similar to the zero points of all other concrete containing samples. This means, that the microbial composition of all the concrete containing samples was primarily determined by the indigenous concrete microflora at the beginning of the experiment, not by the bentonite one. This was also demonstrated by the analyses below. The described PCoA pattern well agrees with the detected differences in the microbial composition described above. The statistically significant difference between the concrete containing samples and the BW and bentonite controls was confirmed also by analysis of similarities (ANOSIM, $R = 0.2471$, $p = 0.0299$). The presence of concrete thus proves to be the most determining factor in detected microbial composition followed by the time factor. The main reason probably is the different chemical composition of concrete containing samples and markedly increased pH.

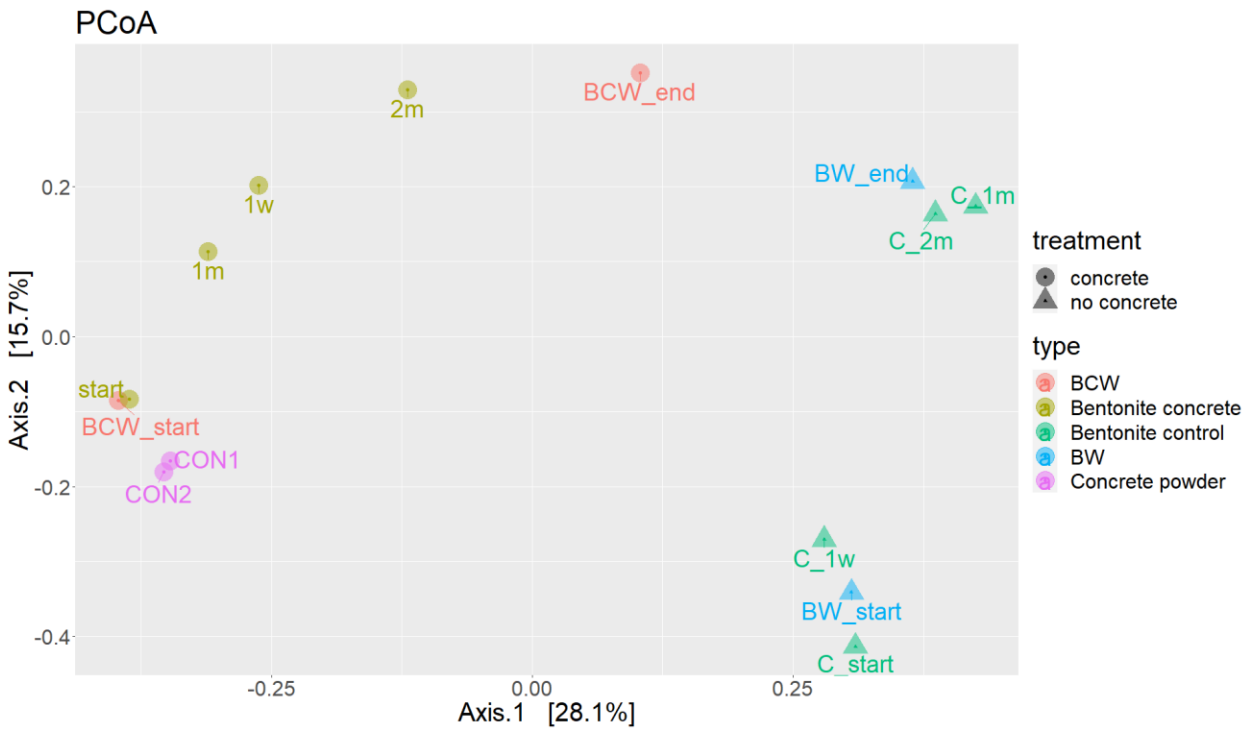

Figure 61: Principal coordinates analysis (PCoA) based on detected operational taxonomic units (OTUs). 1w – sampling after the first week, 1m and 2m – sampling after the first (second) month. Start, 1w, 1m, and 2m – bentonite concrete samples, C – bentonite control samples (bentonite + VITA water), BW – control samples (bentonite + sterile water), BCW – control samples (bentonite, concrete, sterile water).

To further estimate the particular effects of the concrete and bentonite environment on the microbial composition, the Deseq2 analysis and indicator species analyses were performed. For the Deseq2 analysis, we included only the genera with a relative abundance of over 5%. Using this analysis, we detected genera that are significantly ($p \leq 0.05$) more abundant either in concrete containing samples or in bentonite samples. The results are presented in *Figure 62*. Genera such as *Azoarcus*, family *Peptococcaceae*, *Lacunisphaera*, *Citrifermentans*, and *Thermincola*, on the very left top, are bentonite specific while genera such as *Bacillus*, *Nocardioides*, *Pseudarthrobacter or Promicromonospora* are highly specific for the concrete containing samples. Particular representatives of genus *Pseudomonas* are enriched both in bentonite and concrete samples, which implies that this genus includes various species that can be specialized either in concrete or in bentonite environment.

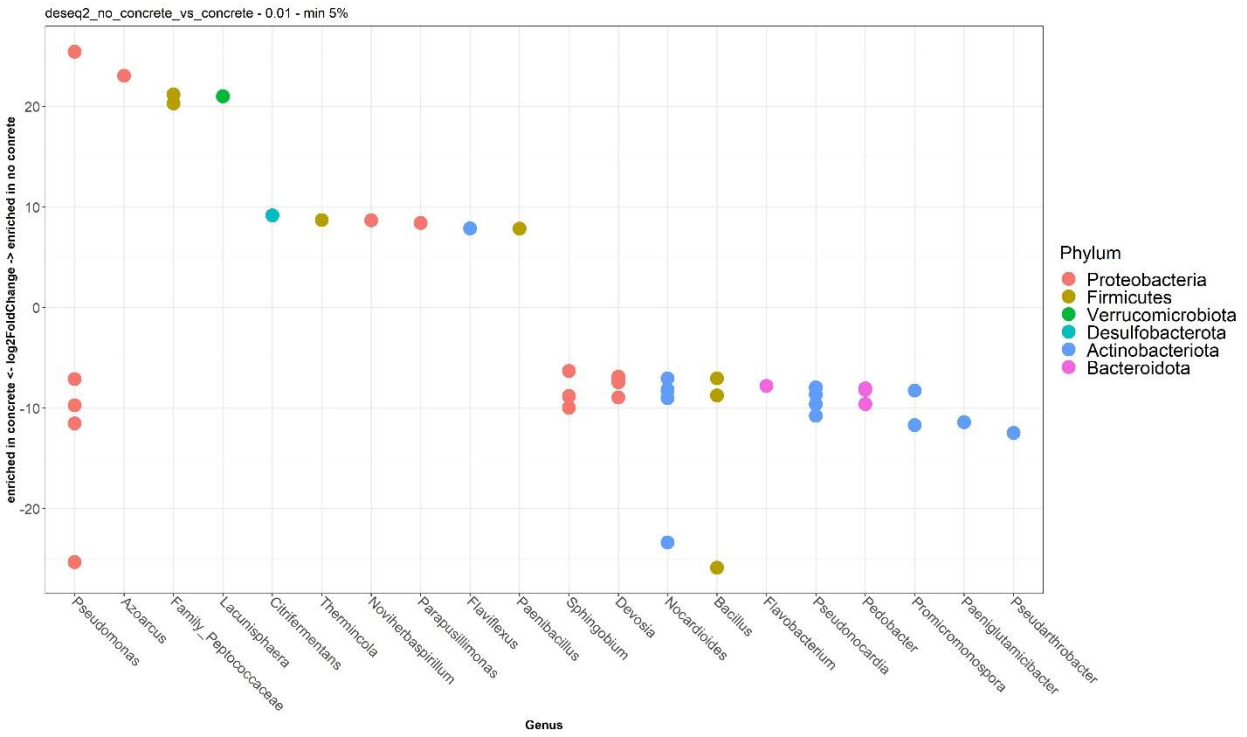

Figure 62: Deseq2 analysis showing the genera specific for the bentonite and concrete samples. Only the genera with the relative abundance over 5% were included. Each dot represents a separate OTU. Bacteria above zero in the y-axis are specific in no-concrete samples (i.e. bentonite) while below are genera specifically enriched in the concrete environment.

Further, indicator species analysis was applied to detect the indicator species in both concrete and non-concrete environment. This analysis revealed 11 indicator genera, 8 for concrete containing environment, and 3 for non-concrete (i.e. bentonite) environment, *Table 19*.

Table 19: Indicator genera for concrete and no-concrete environment.

Group concrete	**stat**	**p-value**	**significance**
Promicromonospora	1.000	0.0003	***
Pseudonocardia	1.000	0.0003	***
Pedobacter	1.000	0.0003	***
Paeniglutamicibacter	1.000	0.0003	***
Devosia	1.000	0.0003	***
Sphingobium	0.999	0.0003	***
Pseudarthrobacter	0.999	0.0003	***
Nocardioides	0.949	0.0017	**
Group no-concrete			
Paenibacillus	0.915	0.0168	*
Massilia	0.866	0.0071	**
Lacunisphaera	0.789	0.0511	.

Except for the genus *Massilia*, all the indicator genera were detected as specific also in Deseq2 analysis, which generally revealed higher diversity of species specifically enriched in both environments.

We also visualized the relative frequency of the detected indicator genera in our samples to see their effect on the overall microbial composition, *Figure 63*. The indicators of the concrete environment dominated the zero-point concrete containing samples, but their frequency markedly decreased with the increasing time in the concrete containing samples. On the other hand, the frequency of non-concrete indicators was negligible at the beginning in bentonite samples, but it gradually increased with the time and the non-concrete indicator genera were most abundant in the end-point bentonite samples. Interestingly, the composition of non-indicator genera in both concrete and non-concrete samples was very different. Because the zero-point concrete samples were obviously dominated by the concrete indicator genera and the detected microbial diversity and abundance in zero-point bentonite samples was generally negligible (probably due to low DNA extraction efficiency in bentonite environment), we can assume, that the majority of the remaining (i.e. non-indicator) genera detected in concrete containing samples might actually originate from bentonite. However, the composition of these non-indicator genera in concrete samples is generally very different from the composition detected in non-concrete samples, which again implies that the evolution of indigenous bentonite microflora is strongly influenced by the presence of concrete.

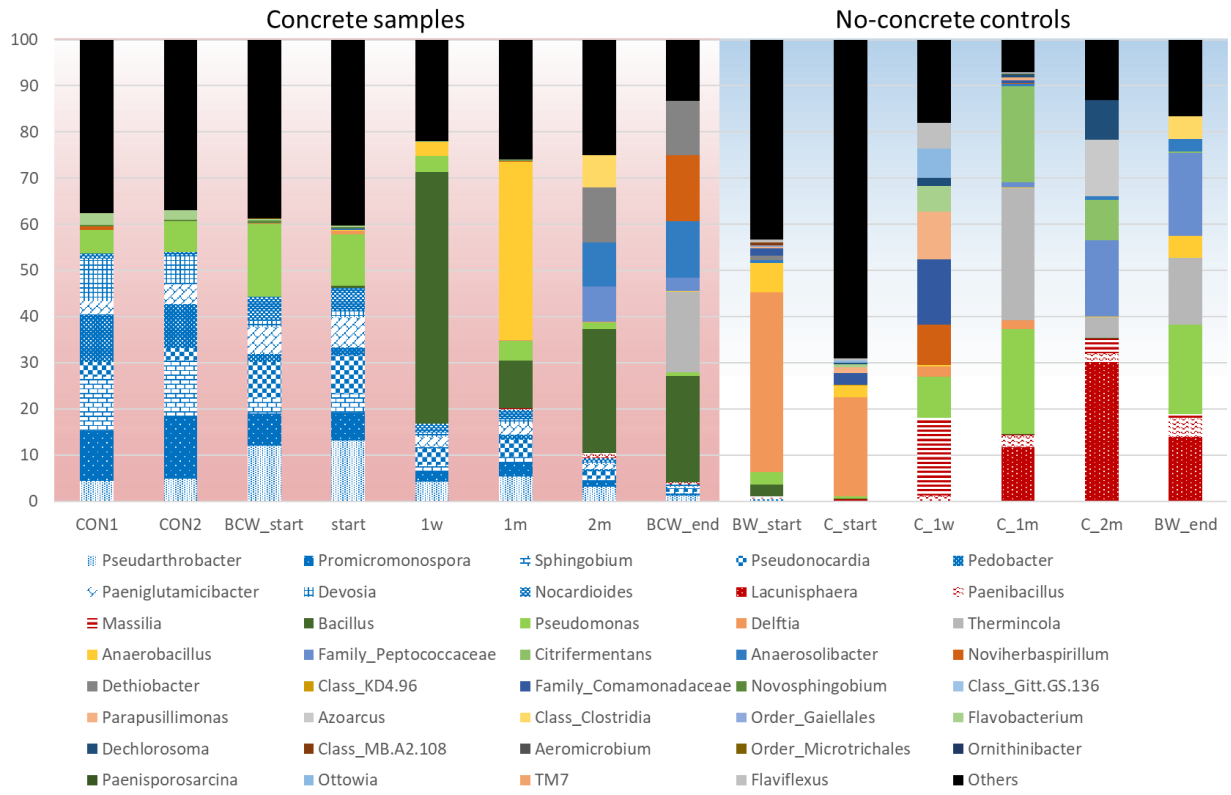

Figure 63: Indicator genera frequency in studied samples. Blue hatching – indicator genera for concrete, red hatching – indicator genera for no-concrete controls. 1w – sampling after the first week, 1m and 2m – sampling after the first (second) month. Start, 1w, 1m and 2m – bentonite concrete samples, C – bentonite control samples (bentonite + VITA water), BW – control samples (bentonite + sterile water), BCW – control samples (bentonite, concrete, sterile water), CON – concrete powder.

3.3.4 Effect of bacteria on concrete

Metabolites (such as organic acids, mineral acids, or sulfur compounds) produced by microorganisms may be chemically aggressive to the cementitious material. However, the impact of microorganisms on the concrete structure is still not well understood in terms of biological deterioration mechanism (Bertron, 2014). Microorganisms can form a biofilm by assembling themselves, which makes them more powerful and resistant to harsh and severe environmental conditions (Bertron, 2014). The formation of such microbial film on the concrete surface may accelerate the biological deterioration. On the other hand, a biofilm on the concrete surface can also form a passive layer and thus also protects the concrete against further deterioration. However, over time the biofilm becomes porous, fragile, and weak to break down having no more protecting effect (Paula et al., 2016).

Although we primarily focused on the effect of the concrete environment on the bentonite microflora, we also attempted to estimate the possible effect of present microorganisms on the concrete. Under the anoxic condition, nitrate reducers can induce $CaCO_3$ precipitation through the reduction of nitrate via dissimilatory nitrate reduction pathway, although the production of $CaCO_3$ by these denitrifying bacteria is much lower than e.g. by ureolytic bacteria (Van Paassen et al., 2010). *Bacillus*, the most abundant NRB genus detected in our bentonite concrete samples, is a spore-forming facultatively anaerobic microorganisms that utilize nitrate in the absence of oxygen as an electron acceptor by oxidation of organic compound (Brenner et al., 2005). Interestingly, some species of *Bacillus* such as *B. sphaericus* and *B. pasteurii* are ureolytic bacteria that are capable of hydrolysis of urea and precipitate $CaCO_3$ which is the most powerful agent to heal the crack concrete biologically (Luo et al., 2018)**.** The reaction rate of enzymatic hydrolysis of urea to precipitate bio-$CaCO_3$ is approximately 10^{14} times faster than the chemical rate (Tziviloglou et al., 2016). Although ureolytic bacteria favor pH values 8 to 9 for the enzymatic activity to precipitate calcite (Stocks-Fischer et al., 1999), a considerable amount of urease activity was still discovered even at pH 10.5 (Qiu et al., 2014).

As genus *Bacillus* was common in our samples, we analyzed the samples for the possible biomineralization effect. Firstly, we performed scanning electron microscopy (SEM), and further, we measured the porosity in the studied samples.

3.4 Surface and porosity analysis

By the SEM analysis, we did not detect any visible morphological differences either in the bentonite concrete samples or in the BCW controls during the experiment (*Figure 64*). No increase in the production of visible calcite minerals was observed, which is in accordance with the stable level of Ca concentration detected during the whole experiment, see above.

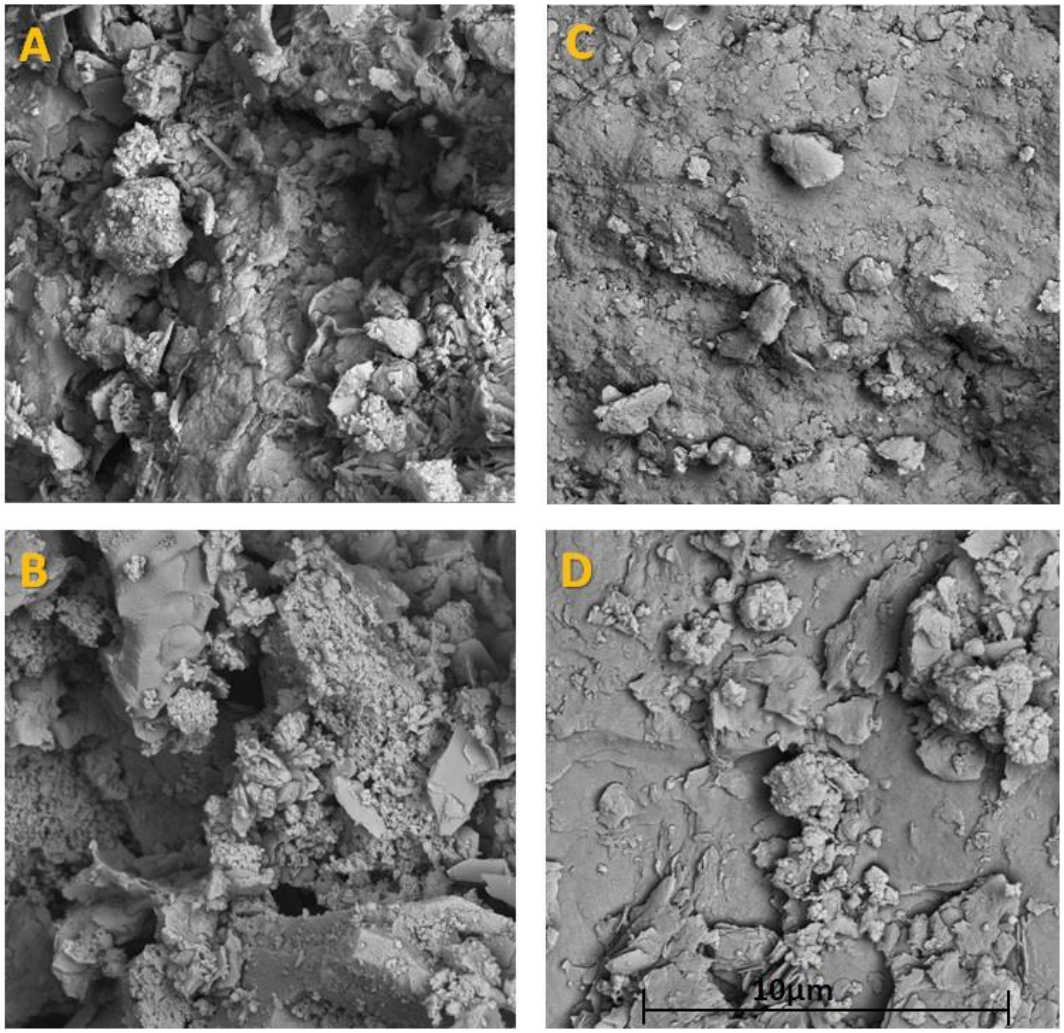

Figure 64: SEM micrograph of the samples: A) bentonite concrete sample (bentonite, concrete and VITA water) start, B) bentonite concrete sample end (2m), C) BCW (bentonite, concrete and sterile water) start, D) BCW end (2m). The scale bar used for each image was 10 µm.

We have not detected any noticeable changes in the pore size distribution in the concrete containing samples during the experiment (*Figure 65*). The analysis has been carried out in a range from 0.96 to 19 nm half pore diameter. The pore size distribution pattern detected in the concrete containing samples copied the pattern typical for bentonite itself with only a negligible effect of the concrete. This result indicates, that no concrete biodegradation or biomineralization resulting in increasing or decreasing pore size probably occurred within the material in our experiment, which agrees with the results of chemical analyses and SEM described above.

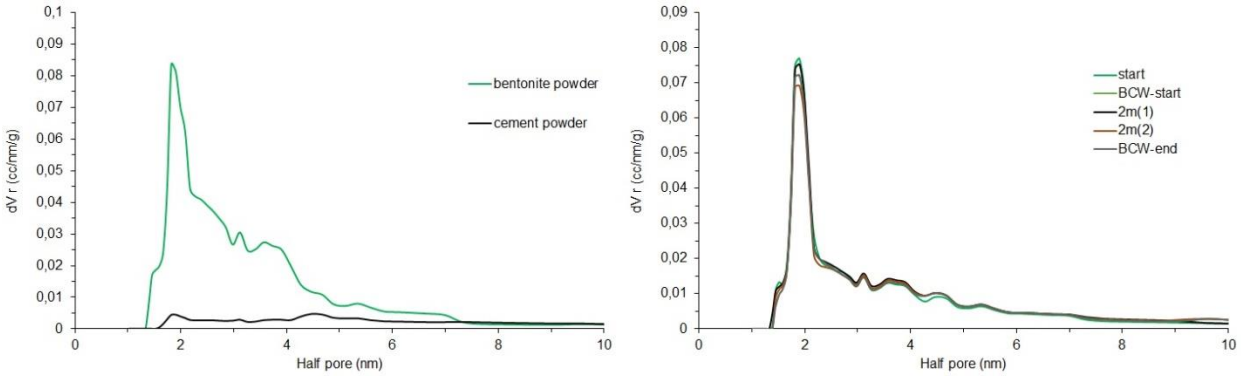

Figure 65: Pore size distribution evaluated by DFT method. On the left - comparison of the pure bentonite and concrete powder. On the right - comparison of the concrete containing samples at the beginning (start and BCW-start) and the end of the experiment (2m, BCW-end). Start and 2m – bentonite concrete samples, BCW – control samples (bentonite, concrete, sterile water).

4 Summary

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 groundwater. 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 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 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 groundwater 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. Hence, the presence of concrete generally has a negative effect on overall microbial activity. 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 radionuclides 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, J. 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., Steinová, J., **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

List of references

AB, S.K., 2011. Long-term safety for the final repository for spent nuclear fuel at Forsmark. SKB Technical Report No TR-11-01. Swedish Nuclear Fuel and Waste Management Co. Stockholm, Sweden.

Achal, V., Mukherjee, A., Reddy, M.S., 2011. Effect of calcifying bacteria on permeation properties of concrete structures. Journal of Industrial Microbiology & Biotechnology 38, 1229–1234.

Achenbach, L.A., Michaelidou, U., Bruce, R.A., Fryman, J., Coates, J.D., 2001. *Dechloromonas agitata* gen. nov., sp. nov. and *Dechlorosoma suillum* gen. nov., sp. nov., two novel environmentally dominant (per)chlorate-reducing bacteria and their phylogenetic position. International Journal of Systematic and Evolutionary Microbiology. 51, 527–533. https://doi.org/10.1099/00207713-51-2-527

Anderson, C., Jakobsson, A.-M., Pedersen, K., 2007. Influence of in situ biofilm coverage on the radionuclide adsorption capacity of subsurface granite. Environmental Science & Technology 41, 830–836.

Anderson, C., Johnsson, A., Moll, H., Pedersen, K., 2011. Radionuclide geomicrobiology of the deep biosphere. Geomicrobiology Journal 28, 540–561.

Aoki, K., Sugita, Y., Chijimatsu, M., Tazaki, K., 2010. Impacts of thermo-hydro-mechanical experiments on the microbial activity in compacted bentonite at the Kamaishi Mine, Northeast Japan. Applied Clay Science 47, 147–154.

Arnold, R.G., DiChristina, T.J., Hoffmann, M.R., 1988. Reductive dissolution of Fe (III) oxides by *Pseudomonas* sp. 200. Biotechnology and Bioengineering 32, 1081–1096.

Aullo, T., Ranchou-Peyruse, A., Ollivier, B., Magot, M., 2013. *Desulfotomaculum* spp. and related gram-positive sulfate-reducing bacteria in deep subsurface environments. Frontiers in microbiology 4, 362.

Azzam, E.I., Jay-Gerin, J.-P., Pain, D., 2012. Ionizing radiation-induced metabolic oxidative stress and prolonged cell injury. Cancer letters 327, 48–60.

Bagnoud, A., Chourey, K., Hettich, R.L., De Bruijn, I., Andersson, A.F., Leupin, O.X., Schwyn, B., Bernier-Latmani, R., 2016. Reconstructing a hydrogen-driven microbial metabolic network in Opalinus Clay rock. Nature communications 7, 12770.

Barker, W.W., Welch, S.A., Chu, S., Banfield, J.F., 1998. Experimental observations of the effects of bacteria on aluminosilicate weathering. American Mineralogist 83, 1551–1563.

Barton, L.L., Fauque, G.D., 2009. Biochemistry, physiology and biotechnology of sulfate-reducing bacteria. Advances in Applied Microbiology 68, 41–98.

Beech, I.B., Sunner, J., 2004. Biocorrosion: Towards understanding interactions between biofilms and metals. Current Opinion in Biotechnology. https://doi.org/10.1016/j.copbio.2004.05.001

Ben-Dov, E., Brenner, A., Kushmaro, A., 2007. Quantification of sulfate-reducing bacteria in industrial wastewater, by real-time polymerase chain reaction (PCR) using *dsrA* and *apsA* genes. Microbial Ecology 54, 439–451.

Benetti, F., 2015. Development of Gold-Magnetite Hybrid Nanoparticles for Advanced Radiotherapy. P.hD. Thesis. University of Trento, Trento, Italy.

Bengtsson, A., Pedersen, K., 2017. Microbial sulphide-producing activity in water saturated Wyoming MX-80, Asha and Calcigel bentonites at wet densities from 1500 to 2000kgm−3. Applied Clay Science 137, 203–212. https://doi.org/10.1016/j.clay.2016.12.024

Bengtsson, A., Pedersen, K., 2016. Microbial sulphate-reducing activity over load pressure and density in water saturated Boom Clay. Applied Clay Science 132, 542–551.

Bennett, D.G., Gens, R., 2008. Overview of European concepts for high-level waste and spent fuel disposal with special reference waste container corrosion. Journal of Nuclear Materials 379, 1–8.

Bergey, D.H., Harrison, F.C., Breed, R.S., Hammer, B.W., Huntoon, F.M. (Eds.), 2015. Bergey's Manual of Systematics of Archaea and Bacteria. Wiley Online Library.

Bertron, A., 2014. Understanding interactions between cementitious materials and microorganisms: a key to sustainable and safe concrete structures in various contexts. Materials and Structures 47, 1787–1806.

Bertron, A., Ranaivomanana, H., Jacquemet, N., Erable, B., Sablayrolles, C., Escadeillas, G., Albrecht, A., 2013. Physico-chemical interactions at the concrete-bitumen interface of nuclear waste repositories, in: EPJ Web of Conferences. EDP Sciences, p. 1002.

Bethke, C.M., Sanford, R.A., Kirk, M.F., Jin, Q., Flynn, T.M., 2011. The thermodynamic ladder in geomicrobiology. American Journal of Science 311, 183–210.

Bracke, G., Kudla, W., Rosenzweig, T., 2019. Status of Deep Borehole Disposal of High-Level Radioactive Waste in Germany. Energies 12, 2580.

Brenner, D.J., Krieg, N.R., Staley, J.T., Garrity, G.M., 2005. Bergey's Manual of Systematic Bacteriology. Volume 2, part B. Springer.

Brown, A.R., 2014. The impact of ionizing radiation on microbial cells pertinent to the storage, disposal and remediation of radioactive waste. P.hD. Thesis. University of Manchester, Manchester, United Kingdom.

Brown, A.R., Boothman, C., Pimblott, S.M., Lloyd, J.R., 2015. The Impact of Gamma Radiation on Sediment Microbial Processes. Applied and Environmental Microbiology 81, 4014–4025. https://doi.org/10.1128/AEM.00590-15

Brown, A.R., Small, J., Pimblott, S.M., Goodacre, R., Lloyd, J.R., 2017. The impact of ionizing radiation on microbial cells pertinent to the storage, disposal and remediation of radioactive waste (No. Report no. RWM/UManchester/01). Nuclear Decommissioning Authority.

Caccavo, F., Lonergan, D.J., Lovley, D.R., Davis, M., Stolz, J.F., McInerney, M.J., 1994. Geobacter *sulfurreducens* sp. nov., a hydrogen-and acetate-oxidizing dissimilatory metal-reducing microorganism. Appl. Environ. Microbiol. 60, 3752–3759.

Callahan, B.J., McMurdie, P.J., Rosen, M.J., Han, A.W., Johnson, A.J.A., Holmes, S.P., 2016. DADA2: High resolution sample inference from Illumina amplicon data. Nat Methods 13, 581–583. https://doi.org/10.1038/nmeth.3869

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.

Černoušek, T., Shrestha, R., Kovářová, H., Špánek, R., Ševců, A., Sihelská, K., Kokinda, J., Stoulil, J., Steinová, J., 2020. Microbially influenced corrosion of carbon steel in the presence of anaerobic sulphate-reducing bacteria. Corrosion Engineering, Science and Technology 55, 127–137.

Červinka, R., Gondolli, J., 2015. Modeling of Pore Water of Compacted Bentonite Bam. ÚJV/SÚRAO report 14407. Prague, Czech Republic.

Chahal, N., Siddique, R., Rajor, A., 2012. Influence of bacteria on the compressive strength, water absorption and rapid chloride permeability of concrete incorporating silica fume. Construction and Building Materials 37, 645–651.

Chan, K.-Y., Xu, L.-C., Fang, H.H., 2002. Anaerobic electrochemical corrosion of mild steel in the presence of extracellular polymeric substances produced by a culture enriched in sulfate-reducing bacteria. Environmental Science & Technology 36, 1720–1727.

Chang, Y.-J., Chang, Y.-T., Hung, C.-H., Lee, J.-W., Liao, H.-M., Chou, H.-L., 2014. Microbial community analysis of anaerobic bio-corrosion in different ORP profiles. International Biodeterioration & Biodegradation 95, 93–101.

Chapman, N.A., McKinley, I.G., Hill, M.D., 1987. The geological disposal of nuclear waste. United Kingdom.

Chidara, R., Nagulagama, R., Yadav, S., 2014. Achievement of early compressive strength in concrete using Sporosarcina pasteurii bacteria as an admixture. Advances in Civil Engineering 2014.

Claesson, M.J., O'Sullivan, O., Wang, Q., Nikkilä, J., Marchesi, J.R., Smidt, H., de Vos, W.M., Ross, R.P., O'Toole, P.W., 2009. Comparative Analysis of Pyrosequencing and a Phylogenetic Microarray for Exploring Microbial Community Structures in the Human Distal Intestine. PLoS ONE 4, e6669. https://doi.org/10.1371/journal.pone.0006669

Clifford, R.J., Milillo, M., Prestwood, J., Quintero, R., Zurawski, D.V., Kwak, Y.I., Waterman, P.E., Lesho, E.P., Mc Gann, P., 2012. Detection of Bacterial 16S rRNA and Identification of Four Clinically Important Bacteria by Real-Time PCR. PLoS One 7. https://doi.org/10.1371/journal.pone.0048558

Collin, F., Li, X.-L., Radu, J.-P., Charlier, R., 2002. Thermo-hydro-mechanical coupling in clay barriers. Engineering Geology 64, 179–193.

Colomban, P., Chiaberge, M., 2011. Potential and drawbacks of Raman (micro) spectrometry for the understanding of iron and steel corrosion. INTECH Open Access Publisher.

Costa, C., Dijkema, C., Friedrich, M., Garcia-Encina, P., Fernandez-Polanco, F., Stams, A., 2000. Denitrification with methane as electron donor in oxygen-limited bioreactors. Applied Microbiology and Biotechnology 53, 754–762.

Dai, Q., Zhao, Y., Dong, F., Wang, B., Huang, Y., 2014. Interaction between bentonite and *Bacillus litoralis* strain SWU9. Applied Clay Science 100, 88–94.

Dall'Agnol, L.T., Cordas, C.M., Moura, J.J., 2014. Influence of respiratory substrate in carbon steel corrosion by a Sulphate Reducing Prokaryote model organism. Bioelectrochemistry 97, 43–51.

Daly, M.J., 2006. Modulating radiation resistance: Insights based on defenses against reactive oxygen species in the radioresistant bacterium *Deinococcus radiodurans*. Clinics in laboratory Medicine 26, 491–504.

David, J.R.D., Merson, R.L., 1990. Kinetic parameters for inactivation of *Bacillus stearothermophilus* at high temperatures. Journal of Food Science 55, 488–493.

Davies, T.R., 1973. Isolation of bacteria capable of utilizing methane as a hydrogen donor in the process of denitrification. Water Research 7, 575–579.

de Romero, M., 2005. The mechanism of SRB action in MIC, based on sulfide corrosion and iron sulfide corrosion products. NACE-05481, NACE International.

Delage, P., Cui, Y.-J., Tang, A.M., 2010. Clays in radioactive waste disposal. Journal of Rock Mechanics and Geotechnical Engineering 2, 111–123.

Dinh, H.T., Kuever, J., Mußmann, M., Hassel, A.W., Stratmann, M., Widdel, F., 2004. Iron corrosion by novel anaerobic microorganisms. Nature 427, 829–832.

Dong, Y., Jiang, B., Xu, D., Jiang, C., Li, Q., Gu, T., 2018. Severe microbiologically influenced corrosion of S32654 super austenitic stainless steel by acid producing bacterium *Acidithiobacillus caldus* SM-1. Bioelectrochemistry 123, 34–44.

Donlan, R.M., 2002. Biofilms: microbial life on surfaces. Emerging Infectious Diseases 8, 881.

Dowd, S.E., Callaway, T.R., Wolcott, R.D., Sun, Y., McKeehan, T., Hagevoort, R.G., Edrington, T.S., 2008. Evaluation of the bacterial diversity in the feces of cattle using 16S rDNA bacterial tag-encoded FLX amplicon pyrosequencing (bTEFAP). BMC Microbiol 8, 125. https://doi.org/10.1186/1471-2180-8-125

Edgar, R.C., 2013. UPARSE: highly accurate OTU sequences from microbial amplicon reads. Nature Methods 10, 996.

Ehrlich, H.L., Newman, D.K., Kappler, A., 2015. Ehrlich's geomicrobiology. CRC press.

El Hajj, H., Abdelouas, A., El Mendili, Y., Karakurt, G., Grambow, B., Martin, C., 2013. Corrosion of carbon steel under sequential aerobic–anaerobic environmental conditions. Corrosion Science 76, 432–440.

El Mendili, Y., Abdelouas, A., Bardeau, J.-F., 2013. Insight into the mechanism of carbon steel corrosion under aerobic and anaerobic conditions. Physical Chemistry Chemical Physics 15, 9197–9204.

Engel, K., Coyotzi, S., Vachon, M.A., McKelvie, J.R., Neufeld, J.D., 2019. Validating DNA Extraction Protocols for Bentonite Clay. mSphere 4. https://doi.org/10.1128/mSphere.00334-19

Enning, D., Venzlaff, H., Garrelfs, J., Dinh, H.T., Meyer, V., Mayrhofer, K., Hassel, A.W., Stratmann, M., Widdel, F., 2012. Marine sulfate-reducing bacteria cause serious corrosion of iron under electroconductive biogenic mineral crust. Environmental Microbiology 14, 1772–1787.

Ewing, R.C., Weber, W.J., Clinard Jr, F.W., 1995. Radiation effects in nuclear waste forms for high-level radioactive waste. Progress in Nuclear Energy 29, 63–127.

Eydal, H.S.C., Pedersen, K., 2007. Use of an ATP assay to determine viable microbial biomass in Fennoscandian Shield groundwater from depths of 3–1000 m. Journal of Microbiological Methods 70, 363–373. https://doi.org/10.1016/j.mimet.2007.05.012

Finneran, K.T., Forbush, H.M., VanPraagh, C.V.G., Lovley, D.R., 2002. *Desulfitobacterium metallireducens* sp. nov., an anaerobic bacterium that couples growth to the reduction of metals and humic acids as well as chlorinated compounds. International Journal of Systematic and Evolutionary Microbiology 52, 1929–1935.

Fisher, S.G., Likens, G.E., 1973. Energy flow in Bear Brook, New Hampshire: an integrative approach to stream ecosystem metabolism. Ecological monographs 43, 421–439.

Flemming, H.C., 1996. Economical and Technical Overview in Microbially Influneced Corrosion of Materials. 5–15.

Francis, A.J., 1990. Microbial dissolution and stabilization of toxic metals and radionuclides in mixed wastes. Experientia 46, 840–851.

Francis, A.J., Dodge, C.J., 2009. Microbial Transformations of Actinides and Other Radionuclides. Brookhaven National Laboratory. Symposium on Nuclear and Radiochemistry. Upton, New York, USA

Frape, S.K., Blyth, A., Blomqvist, R., McNutt, R.H., Gascoyne, M., 2003. Deep fluids in the continents: II. Crystalline rocks. Treatise on Geochemistry 5, 605.

Freiesleben, H., 2013. Final disposal of radioactive waste, in: EPJ Web of Conferences. EDP Sciences, p. 1006.

Fru, E.C., Athar, R., 2008. In situ bacterial colonization of compacted bentonite under deep geological high-level radioactive waste repository conditions. Appl Microbiol Biotechnol 79, 499–510. https://doi.org/10.1007/s00253-008-1436-z

Geets, J., de Cooman, M., Wittebolle, L., Heylen, K., Vanparys, B., De Vos, P., Verstraete, W., Boon, N., 2007. Real-time PCR assay for the simultaneous quantification of nitrifying and denitrifying bacteria in activated sludge. Appl Microbiol Biotechnol 75, 211–221. https://doi.org/10.1007/s00253-006-0805-8

Gittel, A., Mußmann, M., Sass, H., Cypionka, H., Könneke, M., 2008. Identity and abundance of active sulfate-reducing bacteria in deep tidal flat sediments determined by directed cultivation and CARD-FISH analysis. Environmental Microbiology 10, 2645–2658.

Glasser, F., Atkins, M., 1994. Cements in radioactive waste disposal. Mrs Bulletin 19, 33–38.

Glossary., 2020. https://glossary.periodni.com/glossary.php?en=nuklearni+reaktor (accessed 3.2.20).

Grimsel., 2020. *Grimsel test site*. www.Grimsel.com (accessed 3.2.20).

Gu, T., Xu, D., 2010. Demystifying MIC mechanisms. CORROSION, NACE-10213. NACE International.

Hafenbradl, D., Keller, M., Dirmeier, R., Rachel, R., Roßnagel, P., Burggraf, S., Huber, H., Stetter, K.O., 1996. *Ferroglobus placidus* gen. nov., sp. nov., a novel hyperthermophilic archaeum that oxidizes Fe^{2+} at neutral pH under anoxic conditions. Archives of Microbiology 166, 308–314.

Hallbeck, L., Edlund, J., Eriksson, L., 2012. Microbial analyses of groundwater and surfaces during the retrieval of experiment 3, A04, in MINICAN. SKB Technical Report No. P-12-01. Swedish Nuclear Fuel and Waste Management Co. Stockholm, Sweden.

Hanusová, I., Svoboda, J., Večerník, P., 2016. Experimental pressure and sealing plug as part of the European DOPAS project – deep geological repository plug demonstration. Clay Minerals 51, 589–601. https://doi.org/10.1180/claymin.2016.051.4.04

Havlová, V., Rukavičková, L., Staš, L., 2015. Experimental programme at the Bukov underground research facility. Technical Report 6/2015/ENG. Radioactive Waste Repository Administration.

Hersman, L.E., 1997. Subsurface microbiology: Effects on the transport of radioactive waste in the vadose zone. The microbiology of the terrestrial deep subsurface. CRC Press, Boca Raton, FL 299–324.

Hieke, A.-S.C., Pillai, S.D., 2018. *Escherichia coli* cells exposed to lethal doses of electron beam irradiation retain their ability to propagate bacteriophages and are metabolically active. Frontiers in Microbiology 9, 2138.

Hilsen, R.E., Kournikakis, B., Ford, B., 2005. Inactivation of Microorganisms by Gamma Irradiation (No. DRDC-SUFFIELD-TM-2005-236). Defence Research and Development Suffield (ALBERTA).

Honty, M., De Craen, M., Wang, L., Madejová, J., Czímerová, A., Pentrák, M., Stríček, I., Van Geet, M., 2010. The effect of high pH alkaline solutions on the mineral stability of the Boom Clay – Batch experiments at 60°C. Applied Geochemistry 25, 825–840. https://doi.org/10.1016/j.apgeochem.2010.03.002

Hudson, J.A., Stephansson, O., Andersson, J., Tsang, C.-F., Jing, L., 2001. Coupled T–H–M issues relating to radioactive waste repository design and performance. International Journal of Rock Mechanics and Mining Sciences 38, 143–161.

Jankowski, J., Olszewski, J., Kluska, K., 2003. Distribution of equivalent doses to skin of the hands of nuclear medicine personnel. Radiation Protection Dosimetry 106, 177–180.

Janto, B., Ahmed, A., Ito, M., Liu, J., Hicks, D.B., Pagni, S., Fackelmayer, O.J., Smith, T., Earl, J., Elbourne, L.D.H., 2011. Genome of alkaliphilic *Bacillus pseudofirmus* OF4 reveals adaptations that support the ability to grow in an external pH range from 7.5 to 11.4. Environmental Microbiology 13, 3289–3309.

Jia, R., Yang, D., Xu, D., Gu, T., 2017a. Electron transfer mediators accelerated the microbiologically influence corrosion against carbon steel by nitrate reducing *Pseudomonas aeruginosa* biofilm. Bioelectrochemistry 118, 38–46.

Jia, R., Yang, D., Xu, D., Gu, T., 2017b. Anaerobic corrosion of 304 stainless steel caused by the *Pseudomonas aeruginosa* biofilm. Frontiers in Microbiology 8, 2335–2335.

Jin, J., Guan, Y., 2014. The mutual co-regulation of extracellular polymeric substances and iron ions in biocorrosion of cast iron pipes. Bioresource Technology 169, 387–394.

Jin, M., Xiao, A., Zhu, L., Zhang, Z., Huang, H., Jiang, L., 2019. The diversity and commonalities of the radiation-resistance mechanisms of *Deinococcus* and its up-to-date applications. AMB Express 9, 138.

Johansson, A.J., Lilja, C., Sjögren, L., Gordon, A., Hallbeck, L., Johansson, L., 2017. Insights from post-test examination of three packages from the MiniCan test series of copper-cast iron canisters for geological disposal of spent nuclear fuel: impact of the presence and density of bentonite clay. Corrosion Engineering, Science and Technology 52, 54–60.

Johnson, L.F., Osborne, T., 1964. Survival of fungi in soil exposed to gamma radiation. Canadian Journal of Botany 42, 105–113.

Jones, D.R.H., 1997. Corrosion of central heating systems. Engineering Failure Analysis 4, 179–194.

Kakooei, S., Ismail, M.C., Ariwahjoedi, B., 2012. Mechanisms of microbiologically influenced corrosion: a review. World Appl. Sci. J 17, 524.

Karnland, O., 1997. Bentonite swelling pressure in strong NaCl solutions. Correlation between model calculations and experimentally determined data. SKB Technical Report No. 97-31. Swedish Nuclear Fuel and Waste Management Co. Stockholm, Sweden.

Karnland, O., Olsson, S., Nilsson, U., 2006. Mineralogy and sealing properties of various bentonites and smectite-rich clay materials. SKB Technical Report No. TR-06-30. Swedish Nuclear Fuel and Waste Management Co. Stockholm, Sweden.

Kato, S., Goya, E., Tanaka, M., Kitagawa, W., Kikuchi, Y., Asano, K., Kamagata, Y., 2016. Enrichment and isolation of Flavobacterium strains with tolerance to high concentrations of cesium ion. Scientific Reports 6. https://doi.org/10.1038/srep20041

Katoh, K., Asimenos, G., Toh, H., 2009. Multiple alignment of DNA sequences with MAFFT, in: Bioinformatics for DNA Sequence Analysis. Springer, pp. 39–64.

Kielemoes, J., De Boever, P., Verstraete, W., 2000. Influence of denitrification on the corrosion of iron and stainless steel powder. Environmental Science & Technology 34, 663–671.

Kim, J., Dong, H., Seabaugh, J., Newell, S.W., Eberl, D.D., 2004. Role of Microbes in the Smectite-to-Illite Reaction. Science 303, 830–832. https://doi.org/10.1126/science.1093245

King, F., Hall, D.S., Keech, P.G., 2017. Nature of the near-field environment in a deep geological repository and the implications for the corrosion behaviour of the container. Corrosion Engineering, Science and Technology 52, 25–30. https://doi.org/10.1080/1478422X.2017.1330736

King, F., Lilja, C., Pedersen, K., Pitkänen, P., Vähänen, M., 2010. An update of the state-of-the-art report on the corrosion of copper under expected conditions in a deep geologic repository. SKB Technical Report, No. TR-10-67. Swedish Nuclear Fuel and Waste Management Co. Stockholm, Sweden.

Kip, N., van Veen, J.A., 2015. The dual role of microbes in corrosion. The ISME Journal. Nature 9, 542–551.

Kirchman, D.L., Suzuki, Y., Garside, C., Ducklow, H.W., 1991. High turnover rates of dissolved organic carbon during a spring phytoplankton bloom. Nature 352, 612.

Kiyar, D., Wittneben, B.F., 2012. Nuclear energy in the European union after fukushima: Political and economic considerations. CESifo DICE Report 10, 9–15.

Kjeldsen, K.U., Loy, A., Jakobsen, T.F., Thomsen, T.R., Wagner, M., Ingvorsen, K., 2007. Diversity of sulfate-reducing bacteria from an extreme hypersaline sediment, Great Salt Lake (Utah). FEMS Microbiology Ecology 60, 287–298.

Koťátková, J., Zatloukal, J., Reiterman, P., Kolář, K., 2017. Concrete and cement composites used for radioactive waste deposition. Journal of Environmental Radioactivity 178, 147–155.

Kunapuli, U., Lueders, T., Meckenstock, R.U., 2007. The use of stable isotope probing to identify key iron-reducing microorganisms involved in anaerobic benzene degradation. The ISME journal 1, 643.

Kusube, M., Kyaw, T.S., Tanikawa, K., Chastain, R.A., Hardy, K.M., Cameron, J., Bartlett, D.H., 2017. *Colwellia marinimaniae* sp. nov., a hyperpiezophilic species isolated from an amphipod within the Challenger Deep, Mariana Trench. International Journal of Systematic and Evolutionary Microbiology 67, 824–831.

Labalette, T., Harman, A., Dupuis, M., Ouzounian, G., 2013. CIGEO, the French geological repository project. Atw. Internationale Zeitschrift fuer Kernenergie 59, 499–501.

Lafuente, B., Downs, R.T., Yang, H., Stone, N., 2016. The power of databases: the RRUFF project, in: Highlights in Mineralogical Crystallography. Walter de Gruyter GmbH, pp. 1–29.

Laroche, C., Fine, F., Gervais, P., 2005. Water activity affects heat resistance of microorganisms in food powders. International Journal of Food Microbiology 97, 307–315.

Lee, J.S., McBeth, J.M., Ray, R.I., Little, B.J., Emerson, D., 2013. Iron cycling at corroding carbon steel surfaces. Biofouling 29, 1243–1252.

Lee, W., Characklis, W.G., 1993. Corrosion of mild steel under anaerobic biofilm. Corrosion 49, 186–199.

Li, S., Zhang, Y., Liu, J., Yu, M., 2008. Corrosion behavior of steel A3 influenced by *Thiobacillus ferrooxidans*. Acta Physico-Chimica Sinica 24, 1553–1557.

Li, Y., Xu, D., Chen, C., Li, X., Jia, R., Zhang, D., Sand, W., Wang, F., Gu, T., 2018. Anaerobic microbiologically influenced corrosion mechanisms interpreted using bioenergetics and bioelectrochemistry: a review. Journal of Materials Science & Technology 34, 1713–1718.

Libert, M., Schütz, M.K., Esnault, L., Féron, D., Bildstein, O., 2014. Impact of microbial activity on the radioactive waste disposal: long term prediction of biocorrosion processes. Bioelectrochemistry 97, 162–168.

Ling, A.L., Robertson, C.E., Harris, J.K., Frank, D.N., Kotter, C. V, Stevens, M.J., Pace, N.R., Hernandez, M.T., 2014. Carbon dioxide and hydrogen sulfide associations with regional bacterial diversity patterns in microbially induced concrete corrosion. Environmental Science & Technology 48, 7357–7364.

Linhardt, P., 2010. Twenty years of experience with corrosion failures caused by manganese oxidizing microorganisms. Materials and Corrosion 61, 1034–1039.

Litmanen, T., Kari, M., Kojo, M., Solomon, B.D., 2017. Is there a Nordic model of final disposal of spent nuclear fuel? Governance insights from Finland and Sweden. Energy Research & Social science 25, 19–30.

Little, B.J., Lee, J.S., 2007. Microbiologically influenced corrosion. John Wiley & Sons, New Jersey, USA.

Liu, H., Xu, L., Zeng, J., 2000. Role of corrosion products in biofilms in microbiologically induced corrosion of carbon steel. British Corrosion Journal 35, 131–135.

Liu, Hongwei, Fu, C., Gu, T., Zhang, G., Lv, Y., Wang, H., Liu, Hongfang, 2015. Corrosion behavior of carbon steel in the presence of sulfate reducing bacteria and iron oxidizing bacteria cultured in oilfield produced water. Corrosion Science 100, 484–495.

Lloyd, J.R., Macaskie, L.E., 2002. Biochemical basis of microbe-radionuclide interactions, in: Radioactivity in the Environment. Elsevier, pp. 313–342.

Lopez-Fernandez, M., Cherkouk, A., Vílchez-Vargas, R., Jauregui, R., Pieper, D., Boon, N., Sánchez-Castro, I., Merroun, M.L., 2015. Bacterial diversity in bentonites, engineered barrier for deep geological disposal of radioactive wastes. Microbial Ecology 70, 922–935.

Love, M.I., Huber, W., Anders, S., 2014. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. Genome Biology 15, 550.

Lovley, D.R., 2000. Fe (III) and Mn (IV) reduction, in: Environmental Microbe-Metal Interactions. American Society of Microbiology, pp. 3–30.

Lovley, D.R., Chapelle, F.H., 1995. Deep subsurface microbial processes. Reviews of Geophysics 33, 365–381. https://doi.org/10.1029/95RG01305

Lovley, D.R., Phillips, E.J., 1992. Reduction of uranium by *Desulfovibrio desulfuricans*. Applied and Environmental Microbiology 58, 850–856.

Lu, H., Kalyuzhnaya, M., Chandran, K., 2012. Comparative proteomic analysis reveals insights into anoxic growth of *Methyloversatilis universalis* FAM5 on methanol and ethanol. Environmental Microbiology 14, 2935–2945. https://doi.org/10.1111/j.1462-2920.2012.02857.x

Luo, J., Chen, X., Crump, J., Zhou, H., Davies, D.G., Zhou, G., Zhang, N., Jin, C., 2018. Interactions of fungi with concrete: Significant importance for bio-based self-healing concrete. Construction and Building Materials 164, 275–285.

Madigan, M.T., Bender, K.S., Buckley, D.H., Sattley, W.M., Stahl, D.A., 2018. Brock Biology of Microorganisms, Global Edition. New York, NY.

Madigan, M.T., Martinko, J.M., Bender, K.S., Buckley, D.H., Stahl, D.A., 2015. Brock biology of Microorganisms . USA: PearsonEducation.

Männik, J., Driessen, R., Galajda, P., Keymer, J.E., Dekker, C., 2009. Bacterial growth and motility in sub-micron constrictions. Proceedings of the National Academy of Sciences 106, 14861–14866.

Masurat, P., Eriksson, S., Pedersen, K., 2010a. Evidence of indigenous sulphate-reducing bacteria in commercial Wyoming bentonite MX-80. Applied Clay Science 47, 51–57. https://doi.org/10.1016/j.clay.2008.07.002

Masurat, P., Eriksson, S., Pedersen, K., 2010b. Microbial sulphide production in compacted Wyoming bentonite MX-80 under in situ conditions relevant to a repository for high-level radioactive waste. Applied Clay Science 47, 58–64.

Matal, O., Kachlík, M., Maca, K., Vávra, M., Kotnour, P., Pospíšková, I., 2018. Bentonit BaM a jeho některé vlastnosti. Bezpečnost jaderné energie 26(64).

Matschiavelli, N., Kluge, S., Podlech, C., Standhaft, D., Grathoff, G., Ikeda-Ohno, A., Warr, L.N., Chukharkina, A., Arnold, T., Cherkouk, A., 2019. The Year-Long Development of Microorganisms in Uncompacted Bavarian Bentonite Slurries at 30 and 60 °C. Environ. Sci. Technol. 53, 10514–10524. https://doi.org/10.1021/acs.est.9b02670

Matschiavelli, N., Kluge, S., Prause, V., Meleshyn, A., Cherkouk, A., 2018. Evolution of microbial diversity in bentonite - microcosms (Annual Report 2017). HZDR, Institute of Resource Ecology.

Matschiavelli, N., Steglich, J., Kluge, S., Cherkouk, A., 2017. Bentonite–Geotechnical barrier and source for microbial life (Annual Report 2016). HZDR, Institute of Resource Ecology.

Mattimore, V, Battista, J.R., 1996. Radioresistance of Deinococcus radiodurans: functions necessary to survive ionizing radiation are also necessary to survive prolonged desiccation. J Bacteriol 178, 633–637.

McCabe, A., 1990. The potential significance of microbial activity in radioactive waste disposal. Experientia 46, 779–787.

McMurdie, P.J., Holmes, S., 2013. phyloseq: An R Package for Reproducible Interactive Analysis and Graphics of Microbiome Census Data. PLOS ONE 8, e61217. https://doi.org/10.1371/journal.pone.0061217

McNamara, N.P., Black, H.I.J., Beresford, N.A., Parekh, N.R., 2003. Effects of acute gamma irradiation on chemical, physical and biological properties of soils. Applied Soil Ecology 24, 117–132. https://doi.org/10.1016/S0929-1393(03)00073-8

Meleshyn, A., 2014. Microbial processes relevant for the long-term performance of high-level radioactive waste repositories in clays. Geological Society, London, Special Publications 400, 179–194.

Menyah, K., Wolde-Rufael, Y., 2010. CO2 emissions, nuclear energy, renewable energy and economic growth in the US. Energy Policy, The Role of Trust in Managing Uncertainties in the Transition to a Sustainable Energy Economy, Special Section with Regular Papers 38, 2911–2915. https://doi.org/10.1016/j.enpol.2010.01.024

Merroun, M.L., Selenska-Pobell, S., 2008. Bacterial interactions with uranium: An environmental perspective. Journal of Contaminant Hydrology, Radiogeochemical Aspects of Nuclear Waste Disposal 102, 285–295. https://doi.org/10.1016/j.jconhyd.2008.09.019

Miller, R.B., Lawson, K., Sadek, A., Monty, C.N., Senko, J.M., 2018. Uniform and pitting corrosion of carbon steel by *Shewanella oneidensis* MR-1 under nitrate-reducing conditions. Appl. Environ. Microbiol. 84, e00790-18.

Mori, K., Tsurumaru, H., Harayama, S., 2010. Iron corrosion activity of anaerobic hydrogen-consuming microorganisms isolated from oil facilities. Journal of Bioscience and bioengineering 110, 426–430.

Morrier, J.-J., Benay, G., Hartmann, C., Barsotti, O., 2003. Antimicrobial activity of Ca (OH) 2 dental cements: an in vitro study. Journal of Endodontics 29, 51–54.

Motamedi, M., Karland, O., Pedersen, K., 1996. Survival of sulfate reducing bacteria at different water activities in compacted bentonite. FEMS Microbiology Letters 141, 83–87.

Müller-Santos, M., de Souza, E.M., Pedrosa, F. de O., Mitchell, D.A., Longhi, S., Carrière, F., Canaan, S., Krieger, N., 2009. First evidence for the salt-dependent folding and activity of an esterase from the halophilic archaea *Haloarcula marismortui*. Biochimica et Biophysica Acta (BBA)-Molecular and Cell Biology of Lipids 1791, 719–729.

Mulligan, C.N., Yong, R.N., Fukue, M., 2009. Some effects of microbial activity on the evolution of clay-based buffer properties in underground repositories. Applied Clay Science 42, 331–335. https://doi.org/10.1016/j.clay.2008.03.002

Muyzer, G., Stams, A.J.M., 2008. The ecology and biotechnology of sulphate-reducing bacteria. Nature Reviews Microbiology 6, 441.

Mwaluwinga, S., Ayano, T., Sakata, K., 1997. Influence of urea in concrete. Cement and Concrete Research 27, 733–745.

Necib, S., Diomidis, N., Keech, P., Nakayama, M., 2018. Corrosion of carbon steel in clay environments relevant to radioactive waste geological disposals, Mont Terri rock laboratory (Switzerland), in: Mont Terri Rock Laboratory, 20 Years. Springer, pp. 331–344.

Němeček, J., Dolinová, I., Macháčková, J., Špánek, R., Ševců, A., Lederer, T., Černík, M., 2017. Stratification of chlorinated ethenes natural attenuation in an alluvial aquifer assessed by hydrochemical and biomolecular tools. Chemosphere 184, 1157–1167. https://doi.org/10.1016/j.chemosphere.2017.06.100

Nevin, K.P., Lovley, D.R., 2000. Lack of production of electron-shuttling compounds or solubilization of Fe (III) during reduction of insoluble Fe (III) oxide by Geobacter metallireducens. Appl. Environ. Microbiol. 66, 2248–2251.

Nica, D., Davis, J.L., Kirby, L., Zuo, G., Roberts, D.J., 2000. Isolation and characterization of microorganisms involved in the biodeterioration of concrete in sewers. International Biodeterioration & Biodegradation 46, 61–68.

Norris, P.R., Clark, D.A., Owen, J.P., Waterhouse, S., 1996. Characteristics of Sulfobacillus acidophilus sp. nov. and other moderately thermophilic mineral-sulphide-oxidizing bacteria. Microbiology 142, 775–783.

Noynaert, L., De Carrière, P., Volckaert, G., De Bruyn, D., 1998. The CERBERUS project: study of the effect of heat and radiation on the near field of a HLW or spent fuel repository, in: Waste Manage. Symp. pp. 1–7.

Nuclear energy statistics - Statistics Explained [WWW Document], n.d. URL https://ec.europa.eu/eurostat/statistics-explained/index.php/Nuclear_energy_statistics (accessed 4.13.20).

Nuclear Power Today | Nuclear Energy - World Nuclear Association [WWW Document], n.d. URL https://www.world-nuclear.org/information-library/current-and-future-generation/nuclear-power-in-the-world-today.aspx (accessed 4.13.20).

Nuclear shares of electricity generation - World Nuclear Association [WWW Document], n.d. URL https://www.world-nuclear.org/information-library/facts-and-figures/nuclear-generation-by-country.aspx (accessed 4.13.20).

ock Joo, J., Choi, J.-H., Kim, I.H., Kim, Y.-K., Oh, B.-K., 2015. Effective bioremediation of Cadmium (II), nickel (II), and chromium (VI) in a marine environment by using *Desulfovibrio desulfuricans*. Biotechnology and Bioprocess Engineering 20, 937–941.

Ontiveros-Valencia, A., Ilhan, Z.E., Kang, D.-W., Rittmann, B., Krajmalnik-Brown, R., 2013. Phylogenetic analysis of nitrate-and sulfate-reducing bacteria in a hydrogen-fed biofilm. FEMS Microbiology Ecology 85, 158–167.

Pacovská, D., Hausmannová, L., Levorová, M., 2012. The Josef Regional Underground Research Centre (Josef URC). Rudarsko-geološko-naftni zbornik 24, 87–93.

Paget, E., Monrozier, L.J., Simonet, P., 1992. Adsorption of DNA on clay minerals: protection against DNaseI and influence on gene transfer. FEMS Microbiology Letters 97, 31–39.

Paula, M.S. de, Gonçalves, M.M.M., Rola, M.A. da C., Maciel, D.J., Senna, L.F. de, Lago, D.C.B. do, 2016. Carbon steel corrosion induced by sulphate-reducing bacteria in artificial seawater: electrochemical and morphological characterizations. Matéria (Rio de Janeiro) 21, 987–995.

Pedersen, K., 2013. Metabolic activity of subterranean microbial communities in deep granitic groundwater supplemented with methane and H 2. The ISME journal 7, 839.

Pedersen, K., 2010. Analysis of copper corrosion in compacted bentonite clay as a function of clay density and growth conditions for sulfate-reducing bacteria. Journal of Applied Microbiology 108, 1094–1104.

Pedersen, K., 2005. Microorganisms and their influence on radionuclide migration in igneous rock environments. Journal of Nuclear and Radiochemical Sciences 6, 11–15.

Pedersen, K., 2000. Microbial processes in radioactive waste disposal . SKB Technical Report No. TR-00-04. Swedish Nuclear Fuel and Waste Management Co. Stockholm, Sweden.

Pedersen, K., 1999. Subterranean microorganisms and radioactive waste disposal in Sweden. Engineering Geology 52, 163–176.

Pedersen, K., Albinsson, Y., 1991. Effect of Cell Number, pH and Lanthanide Concentration on the Sorption of Promethium by *Shewanella putrefaciens*. Radiochimica Acta 54, 91–96.

Pedersen, K., Bengtsson, A., Blom, A., Johansson, L., Taborowski, T., 2017. Mobility and reactivity of sulphide in bentonite clays–implications for engineered bentonite barriers in geological repositories for radioactive wastes. Applied Clay Science 146, 495–502.

Pedersen, K., Motamedi, M., Karnland, O., Sandén, T., 2000. Cultivability of microorganisms introduced into a compacted bentonite clay buffer under high-level radioactive waste repository conditions. Engineering Geology 58, 149–161.

Perdrial, Julia N, Warr, L.N., Perdrial, N., Lett, M.-C., Elsass, F., 2009. Interaction between smectite and bacteria: Implications for bentonite as backfill material in the disposal of nuclear waste. Chemical Geology 264, 281–294.

Persson, J., Lydmark, S., Edlund, J., Pääjärvi, A., Pedersen, K., 2011. Microbial incidence on copper and titanium embedded in compacted bentonite clay. SKB Technical Report No. R-11-22. Swedish Nuclear Fuel and Waste Management Co. Stockholm, Sweden.

Pettersson, S., Loennerberg, B., 2008. Final repository for spent nuclear fuel in granite - the KBS-3V concept in Sweden and Finland. International Conference, Underground Disposal Unit Design & Emplacement Processes for a Deep Geological Repository, 16-18 June 2008, Prague.

Pikuta, E., Lysenko, A., Suzina, N., Osipov, G., Kuznetsov, B., Tourova, T., Akimenko, V., Laurinavichius, K., 2000. *Desulfotomaculum alkaliphilum* sp. nov., a new alkaliphilic, moderately thermophilic, sulfate-reducing bacterium. International Journal of Systematic and Evolutionary Microbiology 50, 25–33.

Pitonzo, B.J., Amy, P.S., Rudin, M., 1999a. Effect of Gamma Radiation on Native Endolithic Microorganisms from a Radioactive Waste Deposit Site. Radiation Research 152, 64. https://doi.org/10.2307/3580050

Pitonzo, B.J., Amy, P.S., Rudin, M., 1999b. Resuscitation of Microorganisms after Gamma Irradiation. Radiation Research 152, 71. https://doi.org/10.2307/3580051

Plötze, M., Kahr, G., Dohrmann, R., Weber, H., 2007. Hydro-mechanical, geochemical and mineralogical characteristics of the bentonite buffer in a heater experiment: The HE-B project at the Mont Terri Rock Laboratory. Physics and Chemistry of the Earth, Parts A/B/C 32, 730–740.

Polanecký, K., Haverkamp, J., Heinrich-Böll-Stiftung (Eds.), 2010. Energie budoucnosti jaderná energetika ve střední Evropě. Heinrich-Böll-Stiftung, Praha.

Pospiskova, I., Dobrev, D., Kouril, M., Stoulil, J., Novikova, D., Kotnour, P., Matal, O., 2017. Czech national programme and disposal canister concept. Corrosion Engineering, Science and Technology 52, 6–10.

Qiu, J., Tng, D.Q.S., Yang, E.-H., 2014. Surface treatment of recycled concrete aggregates through microbial carbonate precipitation. Construction and Building Materials 57, 144–150.

Rabus, R., 2006. Dissimilatory sulfate-and sulfur-reducing prokaryotes. The prokaryotes: a handbook on the biology of bacteria 2, 659–768.

Rabus, R., Venceslau, S.S., Woehlbrand, L., Voordouw, G., Wall, J.D., Pereira, I.A., 2015. A post-genomic view of the ecophysiology, catabolism and biotechnological relevance of sulphate-reducing prokaryotes, in: Advances in Microbial Physiology. Elsevier, pp. 55–321.

Radioactive wastes and radioactive waste handling, 2009. Energetika (Prague) 59, 244–246.

Rajala, P., Bomberg, M., Vepsäläinen, M., Carpén, L., 2017. Microbial fouling and corrosion of carbon steel in deep anoxic alkaline groundwater. Biofouling 33, 195–209.

Rajala, P., Carpén, L., Vepsäläinen, M., Raulio, M., Sohlberg, E., Bomberg, M., 2015. Microbially induced corrosion of carbon steel in deep groundwater environment. Frontiers in Microbiology 6, 647.

Rao, T., Sairam, T., Viswanathan, B., Nair, K., 2000. Carbon steel corrosion by iron oxidising and sulphate reducing bacteria in a freshwater cooling system. Corrosion Science 42, 1417–1431.

Rast, P., Glöckner, I., Boedeker, C., Jeske, O., Wiegand, S., Reinhardt, R., Schumann, P., Rohde, M., Spring, S., Glöckner, F.O., 2017. Three novel species with peptidoglycan cell walls form the new genus *Lacunisphaera* gen. nov. in the family Opitutaceae of the verrucomicrobial subdivision 4. Frontiers in Microbiology 8, 202.

Ratto, M., Itavaara, M., 2012. Microbial activity in bentonite buffers. Literature study (No. VTT-TECHNOLOGY--20). VTT Technical Research Centre of Finland. Espoo, Finland.

Reactor Database Global Dashboard - World Nuclear Association [WWW Document], n.d. URL https://www.world-nuclear.org/information-library/facts-and-figures/reactor-database.aspx (accessed 4.13.20).

Reardon, E., Fagan, R., 2000. The calcite/portlandite phase boundary: enhanced calcite solubility at high pH. Applied Geochemistry 15, 327–335.

Rehner, R., McCauley, D., 2016. Security, justice and the energy crossroads: Assessing the implications of the nuclear phase-out in Germany. Energy Policy 88, 289–298. https://doi.org/10.1016/j.enpol.2015.10.038

Reisz, J.A., Bansal, N., Qian, J., Zhao, W., Furdui, C.M., 2014. Effects of ionizing radiation on biological molecules—mechanisms of damage and emerging methods of detection. Antioxidants & Redox Signaling 21, 260–292.

Rémazeilles, C., Saheb, M., Neff, D., Guilminot, E., Tran, K., Bourdoiseau, J., Sabot, R., Jeannin, M., Matthiesen, H., Dillmann, P., 2010. Microbiologically influenced corrosion of archaeological artefacts: characterisation of iron (II) sulfides by Raman spectroscopy. Journal of Raman Spectroscopy 41, 1425–1433.

Riley, M., Staley, J.T., Danchin, A., Wang, T.Z., Brettin, T.S., Hauser, L.J., Land, M.L., Thompson, L.S., 2008. Genomics of an extreme psychrophile, Psychromonas ingrahamii. BMC genomics 9, 210.

Riley, P.A., 1994. Free radicals in biology: oxidative stress and the effects of ionizing radiation. International Journal of Radiation Biology 65, 27–33.

Rizoulis, A., Milodowski, A.E., Morris, K., Lloyd, J.R., 2016. Bacterial diversity in the hyperalkaline Allas Springs (Cyprus), a natural analogue for cementitious radioactive waste repository. Geomicrobiology Journal 33, 73–84.

Rodrıguez, J.S., Hernández, F.S., González, J.G., 2002. XRD and SEM studies of the layer of corrosion products for carbon steel in various different environments in the province of Las Palmas (The Canary Islands, Spain). Corrosion Science 44, 2425–2438.

Rogers, R.D., Knight, J.J., Cheeseman, C.R., Wolfram, J.H., Idachaba, M., Nyavor, K., Egiebor, N.O., 2003. Development of test methods for assessing microbial influenced degradation of cement-solidified radioactive and industrial waste. Cement and Concrete Research 33, 2069–2076.

Ross, C.S., Shannon, E.V., 1926. The minerals of bentonite and related clays and their physical properties 1. Journal of the American Ceramic Society 9, 77–96.

Ruhl, A.S., Franz, G., Gernert, U., Jekel, M., 2014. Corrosion product and precipitate distribution in two-component Fe (0) permeable reactive barriers. Chemical Engineering Journal 239, 26–32.

Ruppert, K., Mata, J.F., Brookeman, J.R., Hagspiel, K.D., Mugler III, J.P., 2004. Exploring lung function with hyperpolarized 129Xe nuclear magnetic resonance. Magnetic Resonance in Medicine: An Official Journal of the International Society for Magnetic Resonance in Medicine 51, 676–687.

Russell, J.B., Diez-Gonzalez, F., 1997. The effects of fermentation acids on bacterial growth, in: Advances in Microbial Physiology. Elsevier, pp. 205–234.

Rutqvist, J., Chijimatsu, M., Jing, L., Millard, A., Nguyen, T., Rejeb, A., Sugita, Y., Tsang, C., 2005. A numerical study of THM effects on the near-field safety of a hypothetical nuclear waste repository—BMT1 of the DECOVALEX III project. Part 3: Effects of THM coupling in sparsely fractured rocks. International Journal of Rock Mechanics and Mining Sciences 42, 745–755.

Salter, S.J., Cox, M.J., Turek, E.M., Calus, S.T., Cookson, W.O., Moffatt, M.F., Turner, P., Parkhill, J., Loman, N.J., Walker, A.W., 2014. Reagent and laboratory contamination can critically impact sequence-based microbiome analyses. BMC Biology 12, 87. https://doi.org/10.1186/s12915-014-0087-z

Savage, D., Benbow, S., 2007. Low pH Cements. SKI Report 2007: 32, in: Swedish Nuclear Power Inspectorate (SKI), Stockholm, Sweden.

Schleper, C., Puehler, G., Holz, I., Gambacorta, A., Janekovic, D., Santarius, U.T.E., Klenk, H.-P., Zillig, W., 1995. *Picrophilus* gen. nov., fam. nov.: a novel aerobic, heterotrophic, thermoacidophilic genus and family comprising archaea capable of growth around pH 0. Journal of Bacteriology 177, 7050–7059.

Schloss, P.D., Westcott, S.L., Ryabin, T., Hall, J.R., Hartmann, M., Hollister, E.B., Lesniewski, R.A., Oakley, B.B., Parks, D.H., Robinson, C.J., Sahl, J.W., Stres, B., Thallinger, G.G., Horn, D.J.V., Weber, C.F., 2009. Introducing mothur: Open-Source, Platform-Independent, Community-Supported Software for Describing and Comparing Microbial Communities. Appl. Environ. Microbiol. 75, 7537–7541. https://doi.org/10.1128/AEM.01541-09

Schütz, M.K., Schlegel, M.L., Libert, M., Bildstein, O., 2015. Impact of iron-reducing bacteria on the corrosion rate of carbon steel under simulated geological disposal conditions. Environmental Science & Technology 49, 7483–7490.

Selenska-Pobell, S., Kampf, G., Flemming, K., Radeva, G., Satchanska, G., 2001. Bacterial diversity in soil samples from two uranium waste piles as determined by rep-APD, RISA and 16S rDNA retrieval. Antonie van leeuwenhoek 79, 149–161.

Setlow, P., 2014. Spore resistance properties. Microbiol Spectr 2 (5): TBS-0003-2012.

Setlow, P., 2006. Spores of *Bacillus subtilis*: their resistance to and killing by radiation, heat and chemicals. Journal of Applied Microbiology 101, 514–525.

Ševců, A., Steinová,J., Burkartová,K., Černá, K., 2018. Microbial diversity in aged bentonites. MIND Deliverable 2.07.

Sherar, B.W.A., Power, I.M., Keech, P.G., Mitlin, S., Southam, G., Shoesmith, D.W., 2011. Characterizing the effect of carbon steel exposure in sulfide containing solutions to microbially induced corrosion. Corrosion Science 53, 955–960.

Shukla, A., Parmar, P., Saraf, M., 2017. Radiation, radionuclides and bacteria: An in-perspective review. Journal of Environmental Radioactivity 180, 27–35.

Siddique, R., Chahal, N.K., 2011. Effect of ureolytic bacteria on concrete properties. Construction and Building Materials 25, 3791–3801.

Sikora, A., Detman, A., Chojnacka, A., Błaszczyk, M.K., 2017. Anaerobic digestion: I. A common process ensuring energy flow and the circulation of matter in ecosystems. II. A tool for the production of gaseous biofuels. Fermentation Processes 271–271.

Simoni, S.F., Bosma, T.N.P., Harms, H., Zehnder, A.J.B., 2000. Bivalent cations increase both the subpopulation of adhering bacteria and their adhesion efficiency in sand columns. Environmental Science & Technology 34, 1011–1017.

Slobodkin, A., 2005. Thermophilic microbial metal reduction. Microbiology 74, 501–514.

Smart, N.R., Blackwood, D.J., Werme, L., 2001. The anaerobic corrosion of carbon steel and cast iron in artificial groundwaters. SKB Technical Report No. TR-01-22. Swedish Nuclear Fuel and Waste Management Co. Stockholm, Sweden.

Smart, N.R., Reddy, B., Rance, A.P., Nixon, D.J., Frutschi, M., Bernier-Latmani, R., Diomidis, N., 2017. The anaerobic corrosion of carbon steel in compacted bentonite exposed to natural Opalinus Clay porewater containing native microbial populations. Corrosion Engineering, Science and Technology 52, 101–112. https://doi.org/10.1080/1478422X.2017.1315233

Smith, M., Bardiau, M., Brennan, R., Burgess, H., Caplin, J., Ray, S., Urios, T., 2019. Accelerated low water corrosion: the microbial sulfur cycle in microcosm. npj Materials Degradation 3, 1–11.

Sorokin, D.Y., Kuenen, J.G., Jetten, M.S.M., 2001. Denitrification at extremely high pH values by the alkaliphilic, obligately chemolithoautotrophic, sulfur-oxidizing bacterium *Thioalkalivibrio denitrificans* strain ALJD. Archives of Microbiology 175, 94–101.

Sorokin, D.Y., Tourova, T.P., Mußmann, M., Muyzer, G., 2008. Dethiobacter alkaliphilus gen. nov. sp. nov., and *Desulfurivibrio alkaliphilus* gen. nov. sp. nov.: two novel representatives of reductive sulfur cycle from soda lakes. Extremophiles 12, 431–439.

Sparks, D.L., 2003. Environmental Soil Chemistry. 2nd edition. Elsevier.USA

Stocks-Fischer, S., Galinat, J.K., Bang, S.S., 1999. Microbiological precipitation of CaCO3. Soil Biology and Biochemistry 31, 1563–1571.

Stoodley, P., Sauer, K., Davies, D.G., Costerton, J.W., 2002. Biofilms as complex differentiated communities. Annual Reviews in Microbiology 56, 187–209.

Stroes-Gascoyne, S., 2010. Microbial occurrence in bentonite-based buffer, backfill and sealing materials from large-scale experiments at AECL's Underground Research Laboratory. Applied Clay Science, Advanced smectitic clay research 47, 36–42. https://doi.org/10.1016/j.clay.2008.07.022

Stroes-Gascoyne, S., Hamon, C.J., Maak, P., 2011. Limits to the use of highly compacted bentonite as a deterrent for microbiologically influenced corrosion in a nuclear fuel waste repository. Physics and Chemistry of the Earth, Parts A/B/C 36, 1630–1638.

Stroes-Gascoyne, S., Hamon, C.J., Maak, P., Russell, S., 2010. The effects of the physical properties of highly compacted smectitic clay (bentonite) on the culturability of indigenous microorganisms. Applied Clay Science, Advanced smectitic clay research 47, 155–162. https://doi.org/10.1016/j.clay.2008.06.010

Stroes-Gascoyne, S., Lucht, L.M., Borsa, J., Delaney, T.L., Haveman, S.A., Hamon, C.J., 1995. Radiation Resistance of the Natural Microbial Population in Buffer Materials. MRS Online Proceedings Library Archive 353. https://doi.org/10.1557/PROC-353-345

Stroes-Gascoyne, S., Lucht, L.M., Borsa, J., Delaney, T.L., Haveman, S.A., Hamon, C.J., 1994. Radiation resistance of the natural microbial population in buffer materials. MRS Online Proceedings Library Archive 353.

Stroes-Gascoyne, S., West, J.M., 1997. Microbial studies in the Canadian nuclear fuel waste management program. FEMS Microbiology Reviews 20, 573–590. https://doi.org/10.1111/j.1574-6976.1997.tb00339.x

SÚRAO., 2016. *DGR design.* https://www.surao.cz/en/experts/dgr-design/ (accessed 3.5.16).

Svensk Kärnbränslehantering AB, 2006. Buffer and backfill process report for the safety assessment SR-Can. SKB Technical Report No. TR-06-18. Swedish Nuclear Fuel and Waste Management Co. Stockholm, Sweden.

Svoboda, J., Pacovský, J., Dvořáková, M., Hanusová, I., Večerník, P., Trpkošová, D., 2017. DOPAS EPSP experiment. Geological Society, London, Special Publications 443, 59–72.

Takai, K., Moser, D.P., Onstott, T.C., Spoelstra, N., Pfiffner, S.M., Dohnalkova, A., Fredrickson, J.K., 2001. *Alkaliphilus transvaalensis* gen. nov., sp. nov., an extremely alkaliphilic bacterium isolated from a deep South African gold mine. International Journal of Systematic and Evolutionary Microbiology 51, 1245–1256.

Tsang, C.-F., Barnichon, J.D., Birkholzer, J., Li, X.L., Liu, H.H., Sillen, X., 2012. Coupled thermo-hydro-mechanical processes in the near field of a high-level radioactive waste repository in clay formations. International Journal of Rock Mechanics and Mining Sciences 49, 31–44.

Tuck, V.A., Edyvean, R.G.J., West, J.M., Bateman, K., Coombs, P., Milodowski, A.E., McKervey, J.A., 2006. Biologically induced clay formation in subsurface granitic environments. Journal of Geochemical Exploration 90, 123–133.

Turick, C.E., Berry, C.J., 2016. Review of concrete biodeterioration in relation to nuclear waste. Journal of Environmental Radioactivity 151, 12–21.

Tzeng, C.-C., Kuo, Y.-Y., Huang, T.-F., Lin, D.-L., Yu, Y.-J., 1998. Treatment of Radioactive Wastes by Plasma Incineration and Vitrification for Final Disposal. Journal of Hazardous Materials 58, 207–220.

Tziviloglou, E., Van Tittelboom, K., Palin, D., Wang, J., Sierra-Beltrán, M.G., Erşan, Y.Ç., Mors, R., Wiktor, V., Jonkers, H.M., Schlangen, E., 2016. Bio-based self-healing concrete: from research to field application, in: Self-Healing Materials. Springer, pp. 345–385.

Uchiyama, T., Ito, K., Mori, K., Tsurumaru, H., Harayama, S., 2010. Iron-corroding methanogen isolated from a crude-oil storage tank. Appl. Environ. Microbiol. 76, 1783–1788.

Valencia-Cantero, E., Peña-Cabriales, J.J., 2014. Effects of iron-reducing bacteria on carbon steel corrosion induced by thermophilic sulfate-reducing consortia. J Microbiol Biotechnol 24, 280.

van GERWEN, S.J.C., Rombouts, F.M., Riet, K. van't, Zwietering, M.H., 1999. A data analysis of the irradiation parameter D 10 for bacteria and spores under various conditions. Journal of food protection 62, 1024–1032.

Van Paassen, L.A., Daza, C.M., Staal, M., Sorokin, D.Y., van der Zon, W., van Loosdrecht, M.C.M., 2010. Potential soil reinforcement by biological denitrification. Ecological Engineering 36, 168.

Venzlaff, H., Enning, D., Srinivasan, J., Mayrhofer, K.J., Hassel, A.W., Widdel, F., Stratmann, M., 2013. Accelerated cathodic reaction in microbial corrosion of iron due to direct electron uptake by sulfate-reducing bacteria. Corrosion Science 66, 88–96.

Větrovský, T., Baldrian, P., 2013. Analysis of soil fungal communities by amplicon pyrosequencing: current approaches to data analysis and the introduction of the pipeline SEED. Biology and Fertility of Soils 49, 1027–1037.

Vigneron, A., Alsop, E.B., Chambers, B., Lomans, B.P., Head, I.M., Tsesmetzis, N., 2016. Complementary microorganisms in highly corrosive biofilms from an offshore oil production facility. Appl. Environ. Microbiol. 82, 2545–2554.

Villar, M.V., Lloret, A., 2004. Influence of temperature on the hydro-mechanical behaviour of a compacted bentonite. Applied Clay Science 26, 337–350.

von Wolzogen Kühr, C., Van der Vlugt, L., 1964. Graphitization of cast iron as an electrobiochemical process in anaerobic soils. Translation No. 1021. Army Biological Labs Frederick, Maryland, USA.

Wang, H., Ju, L.-K., Castaneda, H., Cheng, G., Newby, B.Z., 2014. Corrosion of carbon steel C1010 in the presence of iron oxidizing bacteria *Acidithiobacillus ferrooxidans*. Corrosion science 89, 250–257.

Wang, Q., 2007. An investigation of deterioration of archaeological iron. Studies in conservation 52, 125–134.

Wei, N., Finneran, K.T., 2011. Influence of Ferric Iron on Complete Dechlorination of Trichloroethylene (TCE) to Ethene: Fe(III) Reduction Does Not Always Inhibit Complete Dechlorination. Environ. Sci. Technol. 45, 7422–7430. https://doi.org/10.1021/es201501a

Wei, S., Jiang, Z., Liu, H., Zhou, D., Sanchez-Silva, M., 2013. Microbiologically induced deterioration of concrete: a review. Brazilian Journal of Microbiology 44, 1001–1007.

Welle (www.dw.com), D., n.d. Nuclear power faces uncertain future in Europe | DW | 26.04.2016 [WWW Document]. DW.COM. URL https://www.dw.com/en/nuclear-power-faces-uncertain-future-in-europe/a-19215273 (accessed 3.1.20).

Werme, L., 1998. Design premises for canister for spent nuclear fuel. SKB Technical Report No. TR-98-08. Swedish Nuclear Fuel and Waste Management Co. Stockholm, Sweden.

Wersin, P., Spahiu, K., Bruno, J., 1994. Time evolution of dissolved oxygen and redox conditions in a HLW repository. SKB Technical Report No.94-02. Swedish Nuclear Fuel and Waste Management Co. Stockholm, Sweden.

West, J.M., McKinley, I.G., Stroes-Gascoyne, S., 2002. Microbial effects on waste repository materials, in: Radioactivity in the Environment. Elsevier, pp. 255–277.

What is nuclear waste and what do we do with it? - World Nuclear Association [WWW Document], n.d. URL https://www.world-nuclear.org/nuclear-essentials/what-is-nuclear-waste-and-what-do-we-do-with-it.aspx (accessed 4.13.20).

Wikieł, A.J., Datsenko, I., Vera, M., Sand, W., 2014. Impact of *Desulfovibrio alaskensis* biofilms on corrosion behaviour of carbon steel in marine environment. Bioelectrochemistry 97, 52–60.

Wiktor, V., Jonkers, H.M., 2011. Quantification of crack-healing in novel bacteria-based self-healing concrete. Cement and Concrete Composites 33, 763–770.

Williams, S.L., Kirisits, M.J., Ferron, R.D., 2017. Influence of concrete-related environmental stressors on biomineralizing bacteria used in self-healing concrete. Construction and Building Materials 139, 611–618.

Xu, D., Gu, T., 2014. Carbon source starvation triggered more aggressive corrosion against carbon steel by the *Desulfovibrio vulgaris* biofilm. International Biodeterioration & Biodegradation 91, 74–81.

Xu, D., Li, Y., Gu, T., 2016. Mechanistic modeling of biocorrosion caused by biofilms of sulfate reducing bacteria and acid producing bacteria. Bioelectrochemistry 110, 52–58.

Xu, D., Li, Y., Song, F., Gu, T., 2013. Laboratory investigation of microbiologically influenced corrosion of C1018 carbon steel by nitrate reducing bacterium *Bacillus licheniformis.* Corrosion Science 77, 385–390. https://doi.org/10.1016/j.corsci.2013.07.044

Xu, J., Wang, X., 2018. Self-healing of concrete cracks by use of bacteria-containing low alkali cementitious material. Construction and Building Materials 167, 1–14.

Yamanaka, T., Aso, I., Togashi, S., Tanigawa, M., Shoji, K., Watanabe, T., Watanabe, N., Maki, K., Suzuki, H., 2002. Corrosion by bacteria of concrete in sewerage systems and inhibitory effects of formates on their growth. Water Research 36, 2636–2642.

Ye, W.-M., Zheng, Z.J., Chen, B., Chen, Y.-G., Cui, Y.-J., Wang, J., 2014. Effects of pH and temperature on the swelling pressure and hydraulic conductivity of compacted GMZ01 bentonite. Applied Clay Science 101, 192–198.

Zhang, P., Xu, D., Li, Y., Yang, K., Gu, T., 2015. Electron mediators accelerate the microbiologically influenced corrosion of 304 stainless steel by the *Desulfovibrio vulgaris* biofilm. Bioelectrochemistry 101, 14–21.

Zhao, B., Chen, S., 2012. *Alkalitalea saponilacus* gen. nov., sp. nov., an obligately anaerobic, alkaliphilic, xylanolytic bacterium from a meromictic soda lake. International Journal of Systematic and Evolutionary Microbiology 62, 2618–2623.

Zhou, E., Li, H., Yang, C., Wang, J., Xu, D., Zhang, D., Gu, T., 2018. Accelerated corrosion of 2304 duplex stainless steel by marine *Pseudomonas aeruginosa* biofilm. International Biodeterioration & Biodegradation 127, 1–9.

Zhou, Z., 2012. Modeling of Microbial Induced Corrosion on Metallic Pipelines Resulting from Biomethane and the Integrity Impact of Biomethane on Non-Metallic Pipelines. GTI Technical Report. No. DOT Prj# 293. Gas Technology Institute, Illinois, USA.

Zinkle, S.J., Was, G.S., 2013. Materials challenges in nuclear energy. Acta Materialia, The Diamond Jubilee Issue 61, 735–758. https://doi.org/10.1016/j.actamat.2012.11.004