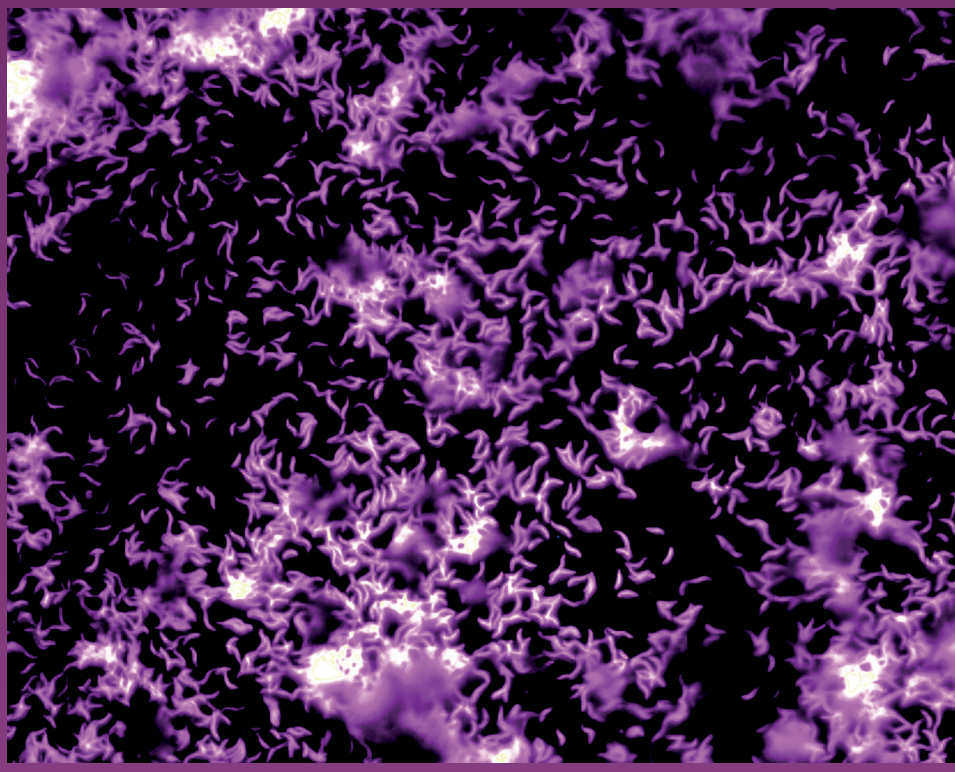
MIND
Microbiology In Nuclear waste Disposal

MIND Project Annual Meeting 1 Extended Abstracts

Granada May 2–4, 2016



PREFACE

The idea of the MIND (Microbiology In Nuclear waste Disposal) project was born at the IGD-TP EF4 in Prague 2013, where the opportunity was provided to gather a working group for microbiology. Representatives from Waste Management Organisations (WMOs), academic institutions, research institutes, consulting companies and one regulator (FANC) attended. The participants came from 8 European countries. Although there are differences between the different repository concepts, there are many unresolved issues in common between the different WMOs. The MIND working group identified a number of specific microbial processes and effects that are of significance to "high urgency" and "high importance" topics highlighted in the most recent IGD-TP Strategic Research Agenda, namely; processes in waste forms, and the technical feasibility and long-term performance of repository components.

It was therefore decided to write a proposal to Horizon2020 focussing on the above mentioned key issues. The project MIND officially started June 1, 2015.

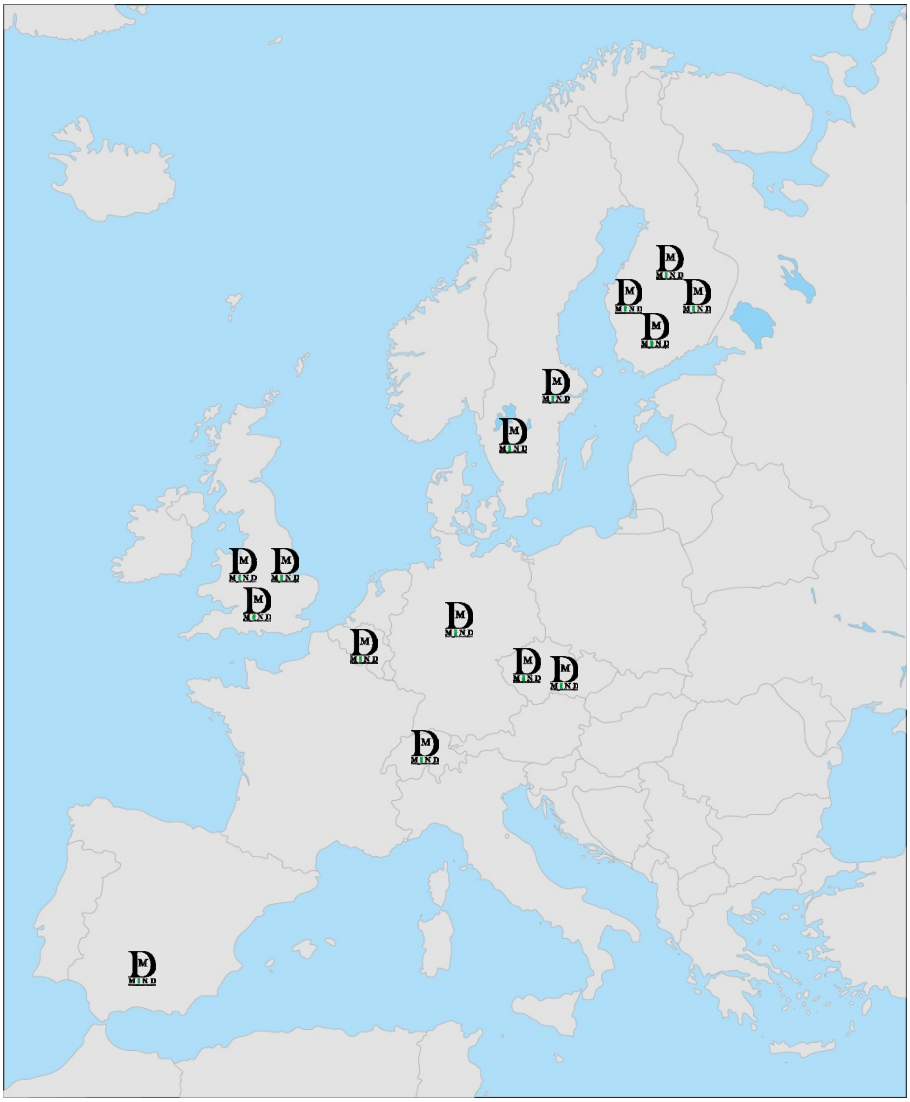


Figure 1: Fifteen organisations from eight countries.

Fifteen European groups are currently working on the impact of microbial processes on the safety cases for geological repositories across Europe, focusing on key questions posed by waste management organisations (WMOs). The emphasis is to quantify specific measureable impacts of microbial activity on safety cases under repository-relevant conditions, thus altering the current view of microbes in repositories and leading to significant refinements of safety case models currently being implemented to evaluate the long-term evolution of radioactive waste repositories. Representatives from academic institutions, research institutes, consulting companies, national laboratories and eight WMOs contributed to formulate the application. So far the following end users have accepted to be part of the review process of the project: SKB, Posiva, TVO, Andra NWMO, Nagra, RWM, ONDRAF/NIRAS and IRSN.

The first Project Annual Meeting (PAM) took place May 2 –4, 2016 and was hosted by the University of Granada.

The meeting started with a pre-workshop organized by Micans on May 2nd. The focus was on these key subjects: Molecular protocols, metagenomics and bio-informatics pipelines for clays and ground water and research design working with buffers and back-fill. The pre-workshop was well attended and very much appreciated by the participants.

The first day of the PAM started with a welcome and a brief presentation of the MIND project by the coordinator and a summary of the pre-workshop discussion by Micans. The morning session started with an introduction by the WP1-leader. WP1 handles questions regarding *Improving the safety case knowledge of organic long-lived intermediate level waste (ILW)*. Nine presentations were given by NNL, UNIMAN, EPFL, SCK•CEN, VTT, HZDR and UGR.

In the afternoon, the WP3-leader gave an introduction of WP3, focusing on *integration, communication and dissemination of results*. There were five presentations given by GTK, UNIMAN-NNL, SCK•CEN-PAS, MICANS and SCK•CEN- PISA. The session ended with a questionnaire for the participants to discuss. The participants were encouraged to motivate the MIND project to themselves as well as to the general public. Valuable input was given in particular by the end users representing Andra and NWMO. In addition, the interest in the MIND project shown by IRSN by sending two representatives that participated both in the pre-workshop and the PAM was greatly appreciated by the MIND consortium.

The day ended with a poster session with fifteen posters from UNIMAN, TUL, CVREZ, EPFL, UGR, MICANS, NNL, SCK•CEN and SKB.

The second day started with an introduction by the WP2-leader. WP2 concerns *Improving the safety case knowledge base about the influence of microbial processes on high level waste (HLW) and spent fuel geological disposal.* Eight presentations were given by GTK, TUL & CVREZ, EPFL, HZDR, VTT, NERC, SCK•CEN and MICANS respectively.

The PAM was closed by the project coordinator, where special thanks were given to Mohamed Merroun and his students at the University of Granada for their excellent organisation of the workshop and meeting.

For more information about the project please visit www.mind15.eu

Follow us on Twitter @MINDH2020

The coordination team would like to thank all participants for their contribution to our first annual meeting! See you all in Prague, May 3-5 2017.

The abstracts of this volume have not been peer viewed and should be regarded as minutes from the meeting.

Birgitta Kalinowski (SKB) project coordinator

Jan Gugala (SKB) administrative coordinator

Joe Small (NNL), WP1-leader

Karsten Pedersen (MICANS), WP2-leader

Katinka Wouters (SCK•CEN), WP3-leader

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MIND Pre-workshop

Monday 2nd May *(please note that all banks are closed on Monday)*

08:30 *Meet up at the lobby at Hotel Granada Centre for joint walk to the University*

The MIND-project will host an open for all pre-meeting workshop at the University of Granada focused on two of our key subjects:

09.00 - 12.30 Molecular protocols, metagenomics and bio-informatic pipelines for clays and groundwater.

12.30 - 14.00 Lunch at Hotel Granada Centre

14.00 - 17.30 Research design working with buffers and back-fill - incubation systems, densities and swelling properties, detection of microbial activity and diversity

The workshop will be organized by MICANS and the MIND-project.

18.30 - 19.00 Registration at Hotel Granada Centre

19.00 - 20.00 Icebreaker (open bar for 1 hour for those who have registered and received a name badge)

MIND Project Annual Meeting, Granada

Tuesday 3rd May

8.30 *Meet up at the lobby at Hotel Granada Centre for joint walk to the University*

9.00 Welcome and Introduction to the MIND-project Birgitta Kalinowski (**SKB**)

9.20 Summary of the pre-meeting workshop Karsten Pedersen (**Micans**)

Session I WP1 9.40 -13.00

9.40 Joe Small (**NNL**) Introduction to WP1

9.45 Liam Abrahamsen (**NNL**) Summary of D1.1; A review of anthropogenic organic wastes and their degradation behaviour

10.00 Naji Bassil (**UNIMAN**) Microbial degradation of cellulose and its alkali degradation products under high pH conditions

10.20 Sophie Nixon (**UNIMAN**) Bioavailability of irradiated PVC for microbial nitrate reduction at high pH

10.40 Rizlan Bernier-Latmani (**EPFL**) Methanogenesis in Opalinus Clay borehole water and products of resin irradiation

11.00 Coffee

Break

11.20 Hugo Moors (**SCK•CEN**) Bitumen degradation products as a source for microbial activity

11.40 Minna Vikman (**VTT**) Microbiological degradation of LLW under repository conditions

12.00 Henry Moll (**HZDR**) Speciation studies of Rn/Ln with selected degradation products of organic LILW – New spectroscopic insights into the uranyl-acetate system

12.20 Miguel Angel Ruiz Fresneda (**UGR**) Molecular scale characterization of the interactions of Se(IV) with bacterial strains isolated from Spanish bentonites

12.40 Joe Small (**NNL**) Coupling microbial processes in geochemical models

13.00 Close of Session

13.00-14.00 LUNCH at Hotel Granada Centre

Session II WP3 14:30 -17.00

14.30 Katinka Wouters (**SCK•CEN – MIC**) Introduction to WP3

14.40 Lasse Ahonen (**GTK**) Microbiological studies in radioactive waste disposal

15.00 Jon Lloyd (**UNIMAN** & NNL) A case study: the BIGRAD project

15.20 Eef Weetjens (**SCK•CEN – PAS**) Performance assessment in Belgium and the potential role of microbes

15.40 Karsten Pedersen (**MICANS**) Education and Training in MIND

16.00 *Coffee break*

16.20 Nicolas Rossignol (**SCK•CEN – PISA**) What's on your Mind? Following microbes, microbiologists, and microbiology into nuclear waste management

17.00 Close of Session

17.00-18:00 Poster session

19.00 Meet up at Hotel Granada Centre for joint walk to the conference dinner at Carmen de la Victoria

20.00 Conference dinner at Carmen de la Victoria

Wednesday 4th May

8.30 *Meet up at the lobby at Hotel Granada Centre for joint walk to the University*

Session III WP2 9.00 -12.40

9.00 Karsten Pedersen, **Micans**, Introduction to WP2

9.20 Riikka Kietäväinen, **GTK**, Deep groundwater in Finland - Towards the inventory of gas.

9.40 Petr Polívka and Jana Steinová, **TUL & CV-REZ**, Overview of the anaerobic biocorrosion experiments (steel canister).

10.00 Rizlan Bernier-Latmani, **EPFL**, In situ steel corrosion and H_2 consumption within bentonite

10.20 Nicole Matschiavelli, **HZDR**, Microbial influence on bentonite transformation

10.40 Break

11.10 Merja Itävaara, **VTT,** Microorganisms in compacted bentonite after 15 years of incubation in aerobic and anaerobic conditions

11.30 Simon Gregory **NERC,** Overview of planned experiments on microbial interactions with steel and bentonite

11.50 Katinka Wouters **SCK•CEN**, Microbes and cement: for better or worse?

12.10 Karsten Pedersen **MICANS**, Microbial sulphide-producing activity in bentonite clays at densities from 750 – 1600 kg m^{-3}

12.30 Close of Session

12.30 – 12.40 Birgitta Kalinowski**, SKB** Summary and Closing of meeting

Posters

WP1

1. Naji Bassil, **UNIMAN** Can microbes help stabilise radioactive waste in the subsurface.
2. Polívka Petr, Jana Steinova, Tomáš Černoušek and Alena Sevcu, **TUL & CV-REZ** Effect of irradiation on polystyrene and microbial community: Experimental design and first results.
3. Carla Perez Mon, Rizlan Bernier-Latmani, **EPFL** Can methanogenesis result from hydrogen accumulation in an Opalinus Clay bioreactor?
4. Ruiz-Fresenda, MA, Gomez-Bolivar, J, Merroun, M, **UGR** Speciation of Se(IV)/U(VI) with bacterial strains isolated from Spanish bentonites: multidisciplinary approach characterization.

WP2

1. Rojina Shrestha, Jana Steinova, Alena Sevcu, **TUL & CV-REZ** Deep groundwater sources in CZ - characterization of microbial diversity.
2. Rojina Shrestha, Jana Steinova, Alena Sevcu, **TUL & CV-REZ** Molecular analysis of microorganisms in Czech bentonite.
3. Černoušek Tomáš, Váňová Hana, Polívka Petr **CV-REZ** Microbially induced corrosion of stainless steel 316L under anaerobic condition.
4. Trevor Taborowski, **MICANS,** Development of methods for separation of microorganisms from bentonite clays.
5. Johanna Edlund-Strömquist, **MICANS,** Bioinformatic analysis of nucleic acid data with focus on safety functions.
6. Haydn Haynes, **UNIMAN**, microbiology within bentonite barrier materials.
7. Nicole Matschiavelli, Andrea Cherkouk **HZDR**, Approach of elucidating the microbial impact on bentonite transformation.

Presentations of the WPs

1. WP1: Joe Small (**NNL**) Improving the safety case knowledge of organic long-lived ILW WP1

2. WP2: Karsten Pedersen (**Micans**) Improving the safety case knowledge base about the influence of microbial processes on HLW and spent fuel geological disposal.

3. WP3: Katinka Wouters (**SCK•CEN**) Integration, communication and dissemination

4. WP4: Birgitta Kalinowski (**SKB**) The MIND project

Appointment of Implementers Review Board

The MIND-project has in the Consortium Agreement (5.2) clearly stated that there should be an End-users' group (Implementers Review Board). The coordinator has contacted a number of key Implementers in Europe and over-seas) and has received a favourable response from the organisations listed below. In addition Posiva and TVO are official members of the MIND project.

The Implementers Review Board/ End-users

- **SKB**
  - **Johan Andersson (HLW)**
  - **Klas Källström**
- **ANDRA**
  - **Achim Albrecht**
- **Niras/ONDRAF**
  - **Benny de Blochouse**
- **NWMO**
  - **Jennifer McKelvie**
- **NAGRA**
  - **Irina Gaus**
- **RWM**
  - **Rebecca Beard**
- **IRSN**
  - **Margot Flatchet and Charles Wittebroodt**

Summary of molecular biology and clay round tables MIND 2016 Project Annual Meeting Granada

Karsten Pedersen, Johanna Edlund-Strömquist, Trevor Taborowski

Microbial Analytics Sweden AB, Mölnlycke, Sweden

Corresponding author e-mail: kap@micans.se

Molecular biology

The molecular round table at the Project Annual Meeting (PAM) meeting in Granada offered the members of the MIND project to come together and present their molecular protocols and discuss the differences amongst the members. Before the panel gathered for the discussion all members voted which topics should be discussed at the panel. The topics that were up to vote were: Collection, Extraction, Quantification, Primer, Sequencing and Bioinformatics. The majority of the members voted for the topics Extraction, Primer and Bioinformatics. At the panel Andrea Cherkouk (HZDR), Nicole Matschiavelli (HZDR), Mohamed Merroun (University of Granada), Johanna Edlund (Micans), Katinka Wouters (SCK CEN), Hugo Moors (SCK CEN), Carla Perez Mon (EPFL), Merja Itävaara (VTT) Jana Steinová (TUL) and Naji M. Bassil (University of Manchester) answered and discussed the question raises by Karsten Pedersen or the audience. The main focus of the first discussion point "Extraction" was that clay and even rocks cause different challenges when extracting bacterial DNA. The main questions where "Which type of protocol should be used amongst the members?" and "The classical phenol-chloroform extraction or commercial DNA extraction kits?" The two approaches have different advantages and disadvantages. The conclusion was that a mock sample with 4-5 different species should be prepared and send out to all partners of MIND. This mock sample will work as a positive control sample and makes it possible to evaluate if we have to change our DNA extraction protocols and if MIND would benefit from all partners having to us the same protocol.

The second discussion point was "Primers". The panel discussed which primer region of the 16S rRNA is the best for our analyses and which gives the highest resolution, is it possible to reach down to species level. The discussion delivered no clear conclusion rather question that have to be take into consideration before you select a primer like: "Do you use combinations of primers or technical replication?"; "What is your research question?" and "Do you focus on a specific group?"

The last point to discuss was the field of "Bioinformatics". The discussion focused mostly on which pipeline is the best to analyse the sequence data and which database should be used. It was stated that running the same sequence results in different pipelines and databases results in different output. Different pipelines are used amongst the partners and the question came up if all partners should use the same pipeline and database.

Overall the discussion led to following points:

- A small mock community with about 4 species should be send to all partners.
- As well as a clay reference. For example, a FEBEX clay core sample from the same position for all partner, but different primers should be used.
- All partners should preferable use the same pipeline and database if possible.

Clay

The Clay round table at the PAM in Granada intended to converge in discussions regarding experiments with various clays relevant for each repository for high level waste. First each research group presented their current and upcoming work where after a panel discussion was arranged on two subjects chosen to be of highest priority. The meeting chose clay properties (density, swelling pressure or loading pressure) and carbon energy from the suggested subjects; clay properties (density, swelling pressure or loading pressure), experimental approach, different clay types, physical variables, carbon energy, sampling, analysis. The front panel consisted of Katinka Wouters (SCK CEN), Hugo Moors (SCK CEN), Trevor Taborowski (MICANS), Andrea Cherkouk (HZDR), Nicole Matchiavelli (HZDR), Mohamed Merroun (University of Granada) and Markus Olin (VTT). They answered and discussed questions from the audience.

The discussion on variables important for microbial activity in compacted clays centered around different properties of clays, experiment setups for densities and swelling pressure or loading pressure. Almost every group had ongoing work on finding thresholds for buffer densities above which microbial activity is inhibited. Since different conditions are applied to experiments a discussion on how to conclude on parameters to control and evaluate studies was initiated.

The second subject that was discussed was: How to deal with additions of carbon sources to microbial activity experiments. The main aspects of the discussion where: when and how it should be done, could a conservative approach be chosen or should only organic matter within the studied clay be considered. Experimental setups differed a lot between the partners of the MIND project. However almost all had ongoing work on finding which organic compound naturally occurring microorganisms may biodegrade. It is also of interest to investigate which organic carbon sources such as acetate, lactate, methanol and formate may be enhance microbial activity in clay.

At the end of the session the concept to model and calculate on processes in high level radioactive repositories were discussed. Modeling aims at adding the timescale aspect for long term calculations. One question that was raised was, is it in the MIND project to do these calculations and if it is possible to do them in such a way that they will be useful. Most groups concluded that modelling has been done and can be done. However, it has mostly only included geochemical properties, reaction speed and kinetics. Microbiology has to be added to these models and therefore modelers have to be informed on microbial processes and the difficulties of adding microbes to the models. First will it be microbial activity at site and will they promote reactions? Further the timing of changes at site is difficult to interpret on timescale bases.

SUMMARY OF D1.1; A REVIEW OF ANTHROPOGENIC ORGANIC WASTES AND THEIR DEGRADATION BEHAVIOUR

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Abstract

A review has been carried out of the range of anthropogenic organic wastes present in low level and intermediate level waste (LLW and ILW) destined for geological disposal in Europe. Based on national inventory data, three key classes of materials have been highlighted for further study based on their expected quantities within the wastes: ion-exchange resins, halogenated plastics and bitumen. In addition cellulose is important in regard to the effect of the strong complexing properties of its degradation products formed under high pH conditions. The review considers the range of waste forms and the storage and disposal conditions expected based on different countries' disposal concepts. Important factors include pH and redox chemistry and radiation dose levels. These will affect the degradation mechanisms of the wastes, as well as influence the growth and behaviour of microbial communities, which themselves may affect the waste degradation mechanisms. The review summarises the current knowledge of the degradation modes of the three groups of organic wastes given above under relevant conditions. Finally, a review is provided of the current knowledge of the radionuclide complexation properties of the expected organic degradation products arising from these wastes. The output from the review is to be used to inform the design of experiments within the MIND project to study the degradation of these wastes and the influence that microbes have on these processes.

Introduction

Work Package 1 of the MIND project addresses remaining key issues for the geological disposal of LLW and ILW concerning the long-term behaviour, fate and consequences of organic materials in the waste, along with hydrogen generated by corrosion and radiolysis. The review of anthropogenic organic wastes and their degradation behaviour [1] begins this work by collating information concerning the inventory and nature of organic materials present in wastes requiring geological disposal.

The review has the following objectives:

- Refine selection of organic materials for irradiation studies.
- Refine conditions of experiments considering encapsulants used, storage and disposal conditions.
- Define current state of knowledge of organic polymer degradation at start of the project.

The review's output provides a list of organic polymers to be studied, and the experimental conditions to be utilised in subsequent irradiation and degradation experiments (Tasks 1.2 and 1.3). Detailed review of cellulose materials was not included in the review, due to the more advanced state of knowledge concerning the generation of isosaccharinic acid and other strong complexants that are formed under high pH cement buffered conditions. Information concerning the distribution of cellulose was however collated.

Review process and key findings

The following tasks were undertaken in the production of the review:

1) The national inventories of LLW and ILW for disposal were collated to establish the distribution of organic materials present. Data was collated for; Belgium, Czech Republic, Finland, France, Spain, Sweden, Switzerland, Netherlands and the UK. The review confirmed that the main materials selected

for study within the MIND project (ion exchange resins, halogenated polymers and bitumen) are widely present in the inventories, although the proportions of the materials do vary between countries (see Figure 1). Cellulose is present in generally lower amounts in most inventories, but it is already established that it is of significance with regard to the strong complexation effect of its alkaline hydrolysis products.

2) Information was collated concerning physical and chemical conditions during treatment, storage and disposal of organic wastes for member states with operating near surface facilities (e.g. SFR, Sweden, VLJ, Finland) and designs for deep geological disposal. This information has been used to constrain the chemical conditions of relevance to study the combined effects of radiolytic, chemical and biodegradation. pH conditions range from pH 12.5 buffered by $Ca(OH)_2$ present in cementitious materials used to treat wastes and in the repository construction and backfill, to neutral conditions representative of groundwater and un buffered regions of heterogeneous waste and unconditioned wastes. A wide range of redox conditions is anticipated from aerobic to methanogenic. Information relevant to considering the total dose and dose rate was provided by available radionuclide inventory information. The review discusses how a compromise must be made to use high dose rates to achieve a total dose over experiment time scales.

3) For ion exchange resins, halogenated polymers and bitumen published information was reviewed concerning the nature and degradation processes and mechanisms of interest. This included information concerning the degradation of cationic and anionic ion exchange resins based on a divinyl benzene-styrene copolymer with sulfonyl or amine functional groups respectively. Halogenated polymers mainly concern polyvinyl chloride (PVC) materials, which contain plasticisers and other additives which are leachable from the PVC structure. Bitumen is used to encapsulate ion exchange resins, evaporator concentrates and sulfate and nitrate salts from reprocessing. Bitumen is a complex mixture of high molecular weight hydrocarbons with functional groups containing N, S and O.

4) A final task reviewed the current knowledge concerning the ability of known degradation products to complex with radionuclides and hence affects their speciation and mobility in groundwater. The focus of this review task was on degradation products of ion exchange resins, halogenated polymers and bitumen as information for cellulose degradation products is better constrained.

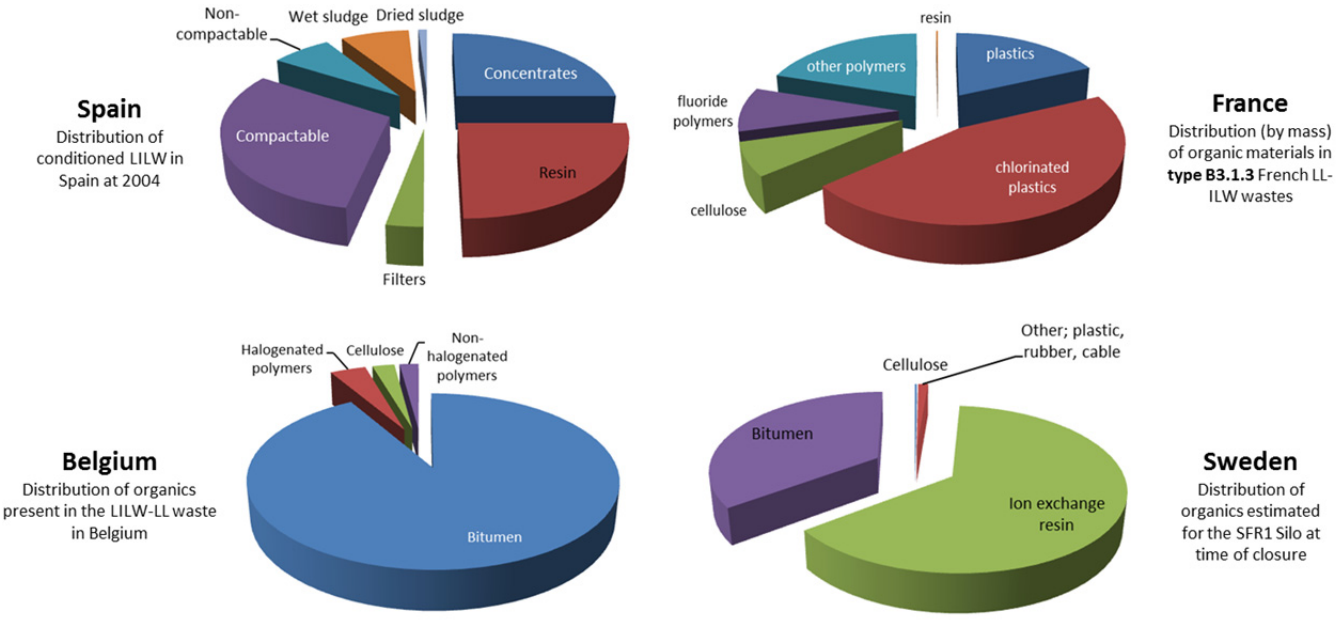

Figure 1 Example inventories of organic materials present in LLW/ILW[1]

Conclusions and following work

This review of organic ILW has collated information relevant to the radiation dose, timescale and chemical conditions that the organic waste and encapsulant materials will be subjected to during storage and

geological disposal. This information, together with information regarding the physical and chemical nature of the organic materials and current understanding of the effects of chemical and radiolytic degradation is used to refine the design of radiolytic and biodegradation experiments that are being undertaken in MIND Work Package 1.

In the next phase of the MIND project ion exchange resin, PVC, bitumen and cellulose materials will be irradiated using ^{60}Co ϒ radiation at a range of dose rates. The first irradiation experiments will be undertaken under a range of relevant pH conditions with air and inert gas headspace. The first experiments will be to ensure that sufficient soluble organic material is produced in order to characterise the compounds present and to use the leachate in subsequent biodegradation experiments. Further experiments will then be undertaken at lower doses. The biodegradation experiments will be inoculated by various cultures, including consortia adapted to high pH conditions and indigenous microbes sampled from underground rock laboratories. Biodegradation studies will utilise a range of electron acceptors, including nitrate and sulfate identified by the review as being of most significance. The process of methane generation under cement buffered ILW conditions will also be explored. The soluble organic species will be characterised before and after biodegradation to identify compounds for complexation studies to draw conclusions regarding the degradation of known complexants such as the cellulose degradation product ISA. The solid residues and biomass materials will also be utilised for microscopy studies including interactions with radionuclides.

References

(1) Abrahamsen, L., Arnold, T., Brinkmann, H., Leys, N., Merroun, M., Mijnendonckx, K., Moll, H., Polvika, P., Ševcu, A., Small, J., Vikman, M., Wouters, K. 2015. A review of anthropogenic organic wastes and their degradation behaviour. MIND Project Deliverable D1.1.

Acknowledgements

This project has received funding from the Euratom research and training programme 2014-2018 under Grant Agreement no. 661880.

MICROBIAL DEGRADATION OF CELLULOSE AND ITS ALKALI DEGRADATION PRODUCTS AT HIGH PH

Naji M. Bassil, Jonathan R. Lloyd

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Abstract

Intermediate-level radioactive waste from the nuclear fuel cycle, will be disposed of via a deep geological disposal facility (GDF). This wasteform is highly heterogeneous and is expected to contain cellulosic material encapsulated in cement. Under the high pH conditions imposed by the cement, cellulose will be chemically hydrolysed to short chain organic acids, the most abundant being isosaccharinic acid (ISA). ISA can bind to various radionuclides and increase their mobility, thereby increasing the probability of their release from the GDF.

As cellulose and ISA are organic molecules, there is the potential for their degradation by microorganisms and the subsequent mitigation of ISA promoted radionuclide mobility. Cellulose (tissue paper) was found to be degraded by bacteria, which stopped ISA production. Enrichment cultures prepared at pH 10 and inoculated using sediments from a high pH lime-kiln site, showed that alkaliphilic bacteria can degrade ISA under aerobic and anaerobic conditions. A novel obligate alkaliphile belonging to the *Bacillus* genus was isolated from these cultures and was able to degrade ISA. Ongoing work, including genome and transcriptome sequencing, TEM and XAS is helping identify the mechanisms of ISA degradation by this novel *Bacillus* species. Similar processes in or around the GDF may potentially stabilise radioactive waste in the subsurface.

Introduction

The deep geological disposal facility (GDF) that is being proposed for the safe disposal of radioactive waste in the UK, is expected to house high-level, intermediate-level and small amounts of low-level radioactive wastes. The largest volume of waste deposited into the GDF will be intermediate-level waste (ILW), which contains cellulosic material in a variety of products including clothes, paper, tissue etc. (*1*). This wasteform will be immobilised in steel containers backfilled with cementitious material to form a physical barrier in the form of a cement matrix. The chemical and biological processes in and around this facility remain poorly understood, and therefore, the impact of this facility on its surrounding environment remains uncertain.

Hyperalkaline conditions will dominate after resaturation of an ILW-GDF with groundwater, due to the extensive use of cement (*2*). Under these hyperalkaline conditions, abiotic hydrolysis of the cellulose present in ILW will take place, leading to the formation of water-soluble low molecular weight compounds, in particular isosaccharinic acid (ISA) (*3*). ISA is a particular concern as it has the potential to complex with a number of metals and radionuclides (*4*, *5*), making them potentially more mobile (*6*, *7*) and more likely to reach the biosphere.

The GDF has previously been considered to be an extreme environment where stresses including hyperalkalinity, radiation and radionuclide toxicity may play a role in limiting microbial colonisation. However, it is becoming clear that microbes may tolerate such extreme conditions (*8*–*10*); and as ILW will contain substantial amounts of organic molecules including cellulose and its alkali degradation

products and other organic chelating agents, microbial colonisation should not be discounted. Alkaliphilic and alkalitolerant cellulolytic bacteria that have been identified previously (*11*, *12*), can play an important role in influencing their surrounding environment through the breakdown of cellulose and the fermentation of its component glucose monomers. In addition, a large number of the alkali cellulose hydrolysis products (i.e. lactate, acetate, formate, succinate, etc.) are known to be degraded by taxonomically diverse bacteria. Additionally, evidence that ISA can be oxidised by microorganisms to drive the reduction of a broad range of electron acceptors, including oxygen, nitrate, Fe(III) and sulfate is growing in recent years (*13*–*16*).

The aim of this work is to study the microbial degradation of cellulose (and ISA) under high pH conditions. Additionally, we intend to identify potential molecular mechanisms of ISA biodegradation and radionuclide immobilisation in ISA-degrading pure cultures.

Results

Microbial degradation of cellulose under hyperalkaline conditions

Incubation of tissue paper in a saturated solution (1 g/L) of $Ca(OH)_2$ (starting pH of 12.2) at 25 ^{o}C for 2.5 years induced hydrolysis of cellulose polymers by alkali and the production of ISA as the main hydrolysis product (Figure 1). The addition of a 2.5% inoculum from a high pH field site in Harpur Hill, Buxton, UK, induced structural degradation of the tissue paper (data not shown) and a significant drop in pH and cessation of ISA production (Figure 1).

Analysis of the 16S rRNA gene sequences showed a shift in the bacterial community in the sample that showed degradation of tissue paper, from a diverse community to one that was dominated by *Clostridia* (Figure 2).

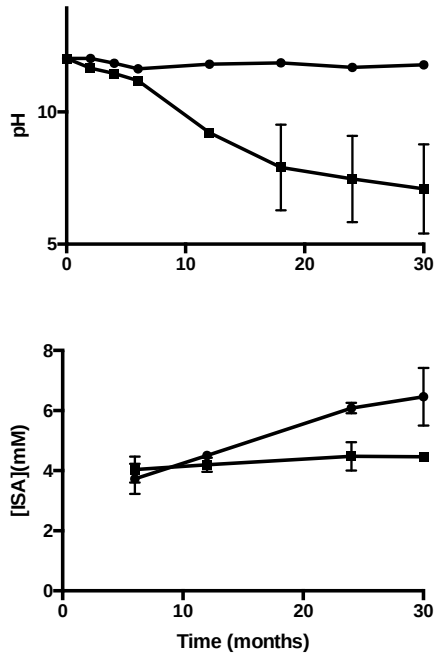

Figure 1: Tissue paper incubated at 25^{o}C in a saturated solution (1 g/L) of $Ca(OH)_2$ showed ISA production in the absence of an inoculum from the high pH field site in Harpur Hill, Buxton, UK ( ). *In the presence of the inoculum* ( )*, ISA production stopped.*

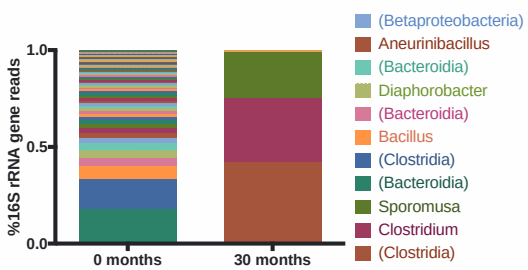

Figure 2: 16S rRNA gene sequence analysis of the sample that showed microbial degradation of tissue paper, showed that the bacterial community became dominated by Clostredia after 30 months of incubation at 25°C.

Microbial ISA degradation at high pH

Enrichment cultures prepared in minimal media at pH 10 and containing Ca(ISA)2 as the only electron donor showed microbial degradation of ISA under aerobic, nitrate-reducing and Fe(III)-reducing conditions (Figure 3).

Analysis of the 16S rRNA gene sequences showed that bacterial diversity dropped under anaerobic conditions (Figure 4).

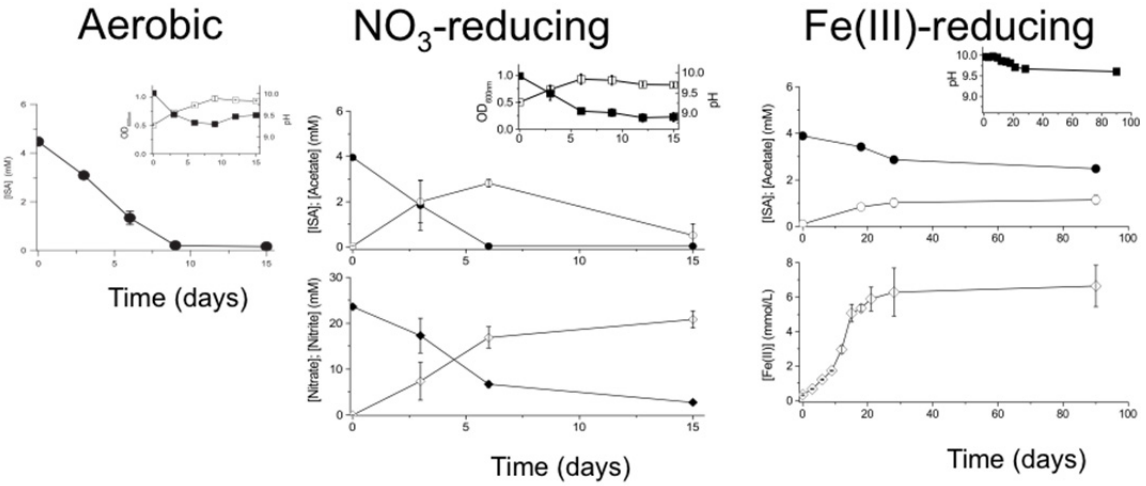

Figure 3: Enrichment cultures containing minimal media at pH 10, supplemented with $Ca(ISA)_2$ as the only electron donor show ISA degradation under aerobic, nitrate-reducing and Fe(III)-reducing conditions.

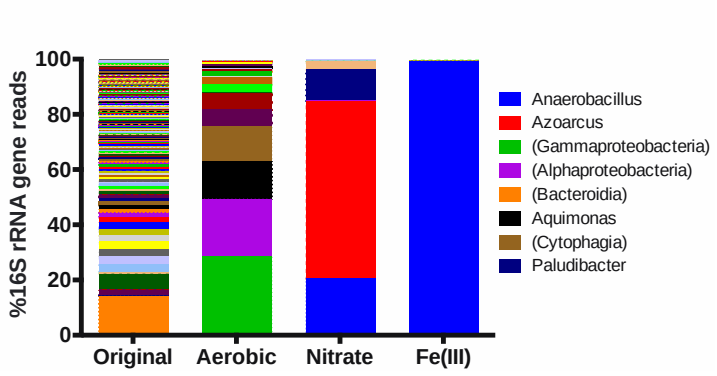

Figure 4: 16S rRNA gene sequence analysis of the samples under aerobic, nitrate-reducing, and Fe(III)-reducing conditions showed a drop in bacterial diversity under anaerobic conditions.

Discussion and Conclusions

Although the extreme conditions in the ILW-GDF (i.e. hyperalkalinity, reducing conditions, radiation and radionuclide toxicity) are expected to hinder microbial colonisation, the ILW-GDF will also

contain materials that can be used as electron donors and acceptors to support microbial growth. Indeed, there is a growing body of evidence supporting the hypothesis that bacteria may be able to survive and even flourish under the conditions expected in and around the ILW-GDF (*9*, *10*, *17*, *18*).

Here we show that bacteria found in sediments sampled from a legacy lime-kiln were able to degrade tissue paper under hyperalkaline conditions and ferment the degradation products.

We also show that bacteria can utilise ISA as a carbon and electron source to reduce a number of relevant electron acceptors. We have also isolated a novel bacterium belonging to the *Bacillus* genus that oxidises ISA and reduces nitrate under high pH conditions. Further studies, including electron microscopy, and whole genome and transcriptome sequencing will help identify the enzymes and mechanisms utilised by this and other ISA-degrading bacteria to metabolise this molecule and immobilise priority radionuclides.

Overall, two biological mechanisms that may help reduce the enhanced radionuclide mobility due to complexation with ISA have been identified in these studies. 1) Bacteria may be able to degrade cellulosic material under hyperalkaline conditions, and ferment the degradation products, thereby preventing cellulose hydrolysis by alkali leading to ISA production; 2) Bacteria can degrade ISA in its free or complexed form and thereby prevent its complexation with radionuclides.

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BIOAVAILABILITY OF PVC FOR MICROBIAL NITRATE REDUCTION AT HIGH PH

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Abstract

Microcosms were established to assess whether a high pH-adapted microbial community can use PVC (powder or film) to fuel nitrate reduction at pH 10. Parallel tests were conducted to assess the effect of gamma irradiation on the bioavailability of organic carbon for this metabolism. PVC was found to fuel nitrate reduction, though significant microbial activity was found only with PVC film, which is chemically distinct from PVC powder, owing to the presence of plasticisers and other additives. Separate microcosms targeting two specific additives detected in organic analysis do not account for the nitrate reduction observed with PVC film. The focus of this on-going study will shift to identifying the organic compounds fuelling this nitrate reduction, accordingly.

Introduction

The safe disposal of nuclear waste relies on a thorough understanding of the role that microbial activity plays in geological disposal. In particular, it is crucial to constrain the microbial metabolism associated with nuclear waste forms, and determine the effect such activity might have on the safety case. Although organic materials represent a significant volume of intermediate level waste (ILW), little is known about the microbial metabolism of such materials, and the associated effects of radiation. Here we focus on one such organic material, polyvinyl chloride (PVC), a widely used plastic in the handling and processing of nuclear waste. Research questions driving this work are twofold: 1) is PVC bioavailable as a source of carbon and electron donors to fuel microbial metabolism by a high pH-adapted microbial consortium? 2) What effect does radiation have on PVC bioavailability? Nitrate reduction was chosen as the target microbial metabolism with which to address these questions.

Materials and Methods

The bioavailability of chemically pure PVC was assessed in the form of PVC powder. For comparison, a sheet of PVC film was used to address the effect of PVC additives (plasticisers, flame retardants) on bioavailability. Samples of both forms of PVC were irradiated using a ^{60}Co gamma source at high pH to a final dose of 1 MGy. All PVC materials were analysed using a range of geochemical and spectroscopic techniques to detect bulk changes in organic composition.

Irradiated and unirradiated PVC powder and film were supplied to anaerobic microcosms buffered to pH 10 containing sodium nitrate as the electron acceptor. Microcosms were inoculated with sediment from a high pH environment known to harbour a diverse microbial consortium. Additional parallel microcosms were initiated to assess the bioavailability of triphenyl phosphate (TPP) and phthalate, two additives in the PVC film detected. All microcosms were setup in triplicate and incubated at 20°C in the dark. Samples from all replicates were taken at regular intervals and analysed using geochemical techniques.

Results

Bulk analyses showed that the organic chemistry of PVC film and powder did not change significantly after irradiation. PVC film was found to fuel nitrate reduction at pH 10, whether irradiated or not. Unirradiated PVC film fuelled greater nitrate reduction than its irradiated counterpart. Minor nitrate reduction was detected with irradiated PVC powder, although unirradiated powder did not fuel nitrate reduction. Initial results from parallel microcosm experiments suggest that additives do not account for the amount of nitrate reduction detected with PVC film.

Conclusions

Results from this preliminary study demonstrate that plasticised PVC film, a significant fraction of organic-containing nuclear waste, can fuel microbial metabolism at high pH. The results further indicate that the major PVC additives identified during organic analyses are not responsible for the nitrate reduction observed with PVC film, and hence the electron donors fuelling this metabolism remain to be identified.

These findings have implications for the safety case of nuclear waste disposal, since the metabolic products of this activity may influence the mobility of radionuclides and contribute towards further microbial activity.

Acknowledgements

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WP1 PROGRESS FROM EPFL IN TWO PARTS:

A- METHANOGENESIS IN OPALINUS CLAY BOREHOLE WATER AND

B- L/ILW RESINS AS A SOURCE OF ORGANIC COMPOUNDS

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Abstract

We report on progress achieved in WP1 of the MIND Project. The first experiment entails the establishment of methanogenic conditions in the subsurface relevant for Swiss Nuclear Waste Disposal. This goal is relevant because, while there is evidence of the presence of methanogens in the rock, there is direct evidence for methanogenesis. We propose that this is because sulfate-reduction is the dominant metabolism and is more thermodynamically favorable than methanogenesis. Thus, we are artificially decreasing the sulfate concentration in a borehole at the Mont Terri Rock Laboratory while amending with H_2. The second experiment entails evaluating the release of organic compounds able to support microbial growth, as a result of irradiation of ion-exchange resins, a major component of low and intermediate level waste. Resins are gamma irradiated and the products of this process characterized by various mass spectrometry tools.

General introduction

In Switzerland, the concept of long-term deep geological repositories for intermediate and low level nuclear waste (I/LLW) involves three barriers in order to prevent the release of radioactivity to the environment[1]. The first barrier consists on 200L steel drums in which the nuclear waste will be placed after being conditioned and solidified with a cement-mortar mix (A). For the second barrier, the steel drums will be piled together in concrete canisters and then embedded in mortar (B). The third barrier is the stable Opalinus clay geological formation in which the embedded canisters will be buried at a maximum depth of 600m (C).

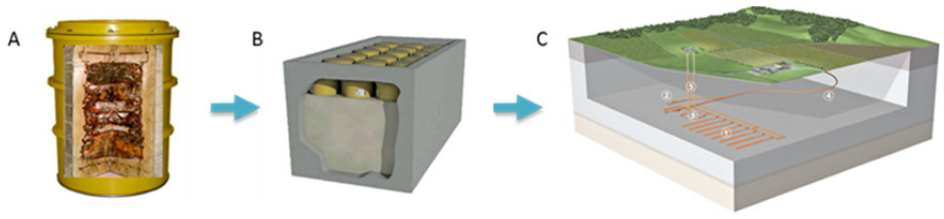

Figure 1: Deep geological repository concept for nuclear waste disposal in Switzerland

The present work is framed in the WP1 from the MIND project and it comprises two aims. The first aim is to determine the impact of H_2 release from steel drum corrosion on the planktonic community in Opalinus clay porewater. Concretely, we aim at determining whether biogenic production of methane ($CO_2 + H_2 \rightarrow CH_4$) can be achieved under hydrogen accumulation and sulfate depletion conditions in such a system. The second aim is related to the degradation of organic ion exchange resins that are used in nuclear power plants to remove metallic radionuclides (ex. $^{137}Cs^{+}$, $^{60}Co^{2+}$) from diverse liquid materials. Resins are the most abundant component (around 40%) of the Swiss organic nuclear waste inventory[2]. Their long-term degradation due to the absorbed radioactivity could result in smaller organic products that could be used by the microorganisms from the host rock. In this

respect, we aim at 1) characterizing in detail the degradation products from resin irradiation and 2) determining the metabolic and population changes in the Opalinus microbial community driven by the formation of such degradation products. In both cases, the final goal is to evaluate whether transformations of materials within the deep repositories (corrosion of steel drums and degradation of exchange resins in the waste) could result in a positive or negative impact of microorganisms on the safety of the repositories. Production of gas such as methane from other gases H_2 and CO_2 importantly would decrease the pressure in the rock which consequentially. Resin degradation products could promote microbial growth in the host rock and/or potentially stimulate unwanted metabolic processes such as gas production from aqueous organic compounds[2].

In the following paragraphs, I will explain in two separate parts the on-going work related to the first aim: methanogenesis under hydrogen accumulation and sulfate depletion and the planned work for the second aim: L/ILW resins degradation products as substrate for microbial growth.

Part A: Methanogenesis under H_2 accumulation and sulfate depletion

Introduction

Previous cultivation studies performed within the host rock (at the Mont Terri rock laboratory) succeeded in identifying methanogens in the porewater of Opalinus clay3. The most recent in situ experiment did not show any significant increase in methane or in number of methanogens over time when H_2 was regularly added to the water. During these experiments, it was observed a metabolic shift from oxygen oxidation to iron reduction and later to sulfate reduction along with the transition from increasingly more reducing conditions. The metabolic shifts were in agreement with changes in the microbial community composition in which sulfate-reducing bacteria dominated the system after 100 days. The authors speculated that the persistence of high levels of sulfate in the water (>12mM) would have impeded a further metabolic shift from sulfate reduction to methanogenesis because the reduction of sulfate renders significantly more energy than the reduction of carbon dioxide (Bagnoud et al., 2015). Hence, the research question arises: Would methanogenesis occur in the bioreactor if sulfate decreases far enough?

Materials and methods

On-going H_2 injection experiments are performed in the BCR-3 bioreactor in the Underground Rock Laboratory of Mont Terri (St-Ursanne, Switzerland); a facility located within an Opalinus clay geological formation at about 300m below the surface.

The bioreactor (Figure 2) has a descending orientation of 30º of inclination and it contains a 2.74m long interval separated from the gallery by a packer, and which is naturally fills with porewater from the rock at a rate of about 20ml/day. Two currently functional polyamides lines connects the borehole with the surface, allowing the recovery of the water from the first meter of the anaerobic chamber.

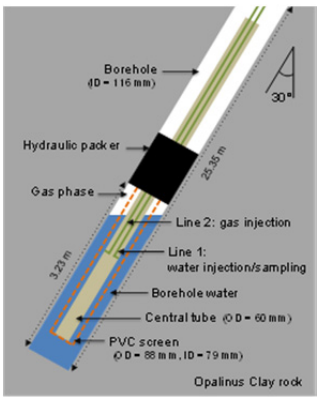

Figure 2: Schematic representation of BCR-3 bioreactor. Modified from Bagnoud (2015)

H_2 gas amendments are performed in batch since November 2015. Each 7-10 days about 500-900ml of water are recovered from the interval using line 1 of the bioreactor. Then, about 800ml of sterile artificial porewater without sulfate is injected in the chamber through the same line. Finally, 3L of H_2 are injected in the artificial porewater through line 2.

From the sampled water, the following chemical quantifications are performed: 1) Dissolved methane and hydrogen using gas chromatography with FID and TCD detectors; 2) Sulfate concentration using Ion chromatography; 3) Sulfide and iron (II) concentrations using colorimetry[4,5]; 4) pH using a pH-meter; and 5) Alkalinity by titration with HCl.

Additionally, microbial community data are collected: 1) Planktonic density by direct counting using fluorescence microscopy and FACS; and 2) Composition of the archaeal and bacterial community through 16S rRNA sequencing.

Results and discussion

The following results correspond to the first 102 days of the H_2 injection experiments. Focusing on the chemical behavior pictured in Figure 3, it can be observed that sulfate concentration decreased from around 15mM to 7.5mM along the sampling period.

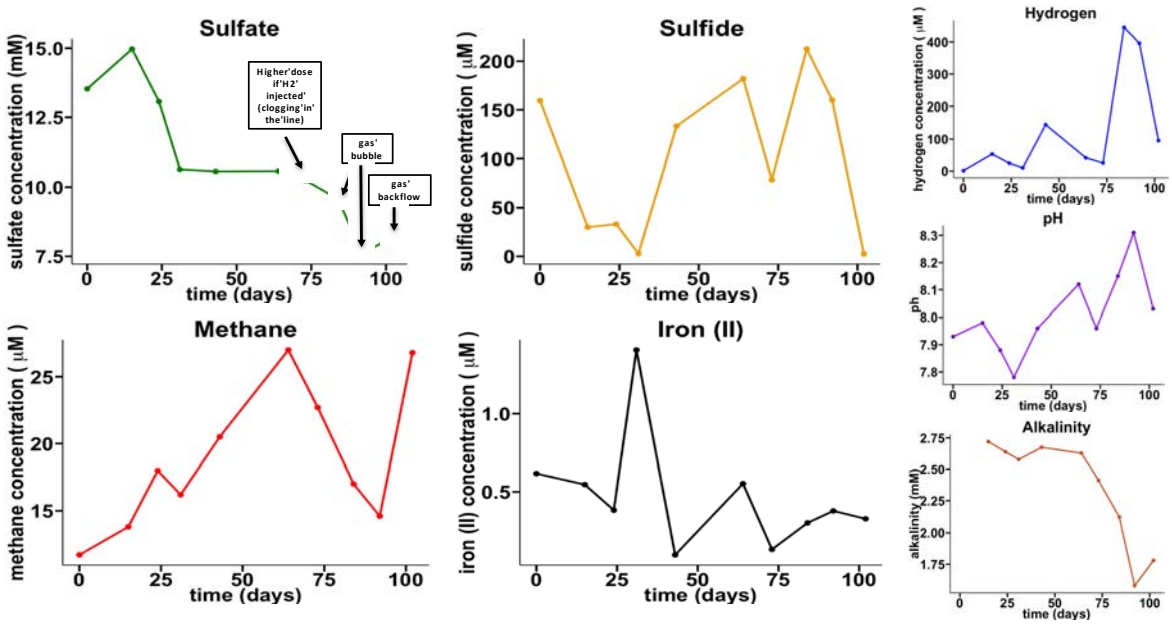

Figure 3. Chemical evolution of borehole water during the experiments

Sulfide remained high for most of the sampling period, reaching a highest value of 200µM. Methane concentration showed a slight increase for the first 60 days, but values remained below 30 µM. Iron (II) concentrations remained very low at less than 5µM. These chemical observations indicate that sulfate reduction is the predominant microbial metabolism. Meanwhile, hydrogen concentrations were high, which indicates efficient delivery of H2 to the borehole water. Concerning the microbial community analyses, planktonic density increased over time reaching a maximum at day 92 (figure 4). 16S rRNA analyses showed a dominance of sulfate-reducing bacteria with a high proportion of Firmicutes and Proteobacteria (e. g. *Desulfobulbaceae*, *Desulfobacteraceae*, *Peptococcaceae*, *Rhodobacteraceae* and *Rhodospirillaceae*) (figure 4B). These results are in line with the chemical results and with previous population observations from a similar H2 injection experiment performed by Bagnoud (2015) (figure 4C). We identified a methanogen (*Methanosarcinaceae*) but its abundance was remarkably low (<10 reads). Based on this, we measured $\delta^{13}C\text{-}CH_4$ in a gas phase sample from day 64, which was the day with the highest methane concentration and a high planktonic density. $\delta^{13}C\text{-}CH_4$ was -40‰, which indicates that the measured methane is likely not of biogenic origin

(background $\delta^{13}C\text{-}CH_4$ in Opalinus clay is -38.4 to -40.3‰ from Vinsot et al. (2014). All in all, we speculate that methanogenesis can still occur, but it is necessary to reach lower levels of sulfate.

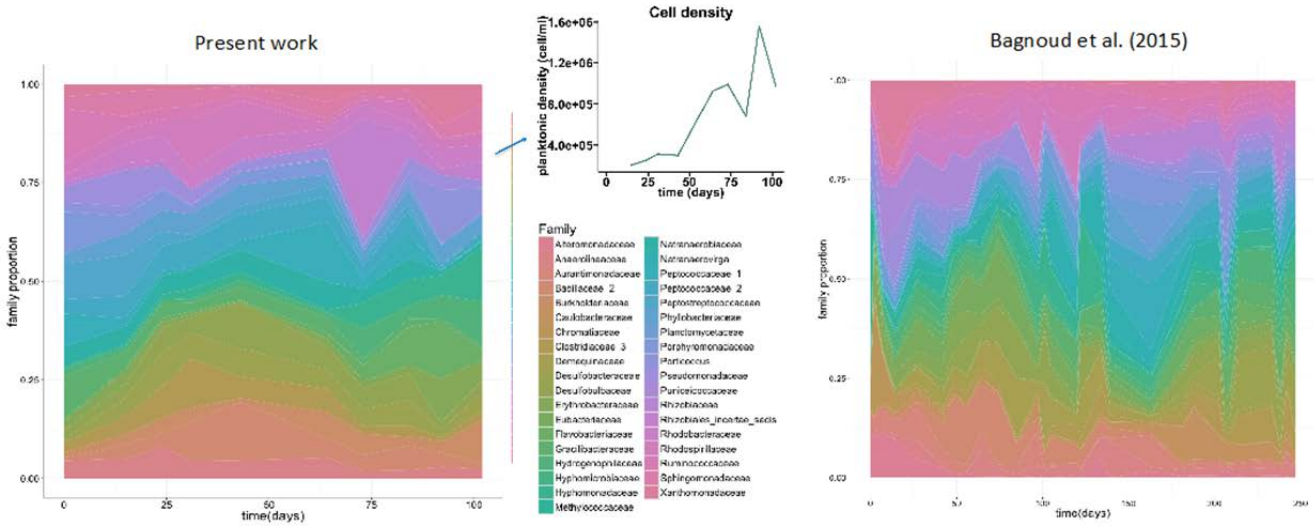

Figure 4. Family composition of bacteria present in the planktonic community from the current experiment (right) and previous work (right)

Conclusions and perspective

H_2 accumulation (up to 450µM) is not sufficient for methanogenesis to occur in significant levels in the borehole water of Opalinus clay when sulfate concentration is above 7.5mM. Hence, we will continue the experiment until we reach lower levels of sulfate. To determine how low sulfate should be in the bioreactor for methanogenesis to take place we will perform incubation experiments in which the sampled planktonic community will be grown under H_2 saturation conditions and media with varying sulfate concentrations. We will perform further $\delta^{13}C\text{-}CH_4$ analyses of sampled water and gas phase and we will incorporate $\delta^{34}S\text{-}SO_4^{2-}$ analyses to the routine water analyses to confirm microbial sulfate reduction.

Part B: L/ILW resins degradation products as substrate for microbial growth

Introduction

Ion exchange resins are copolymers made of polystyrene chains cross linked by divinyl benzene in which either sulfonic (cation exchange) or amine (anion exchange) groups are added[2]. Both types of resins can have either a bead or a powder structure and are normally present on the nuclear waste with the ionic groups associated to radionuclides (loaded resins).

It is estimated that I/LLW will receive a total irradiation dose of 1Mgy with varying dose rates within the first 1,000 years in the geological repository. Additionally, over this long period, the host rock might be saturated with porewater and a pH range might occur due to the interaction between leachates from the cementitious structures (strongly basic) and products from waste degradation and microbial metabolism (CO_2, HCl, organic acids which) (neutral to acidic)[2]. Previous studies on radiolytic degradation of resins showed the formation of sulfonic, amine and organic products (i.e., oxalate) as well as gases (e.g., H_2)[6,7]. However, these studies are generally confined to narrow

conditions of radiation and pH and more detailed information about the degradation products is missing. Likewise, the interaction between the degradation products and the porewater planktonic community remains an open issue.

Methods

Degradation experiments are performed in the loaded mixed ion exchange resins displayed in table 1 corresponding to the ones found in the Swiss nuclear waste inventory.

Table 1: Organic ion Exchange resins used in the current work

Resins	Composition		Loading
Bead resin (R1)	Lewatit M800 (anion exchange, amines)	Lewatit S200 (cation exchange, sulfonic acid)	Li^+, BO_3^{3-}, SiO_3^{2-}, SO_4^{2-} Cl^-, Na^+, Mg^{2+} K^+, Ca^{2+}
Powder resin (R2)	Powdex PAO (anion exchange, amines)	Powdex PCH (cation exchange, sulfonic acid)	Li^+, BO_3^{3-}, SiO_3^{2-}, SO_4^{2-} Cl^-, Na^+, Mg^{2+} K^+, Ca^{2+}

A small amount (0.5g) of each resin is placed in a Wheaton Ampule (Sigma-Aldrich) and saturated with 2mL of Opalinus clay porewater at pH 7.98 (original pH of the water) or pH 12.5 (expected pH from the leach of cement). The ampules are sealed and irradiated with a ^{60}Co ϒ radiation source. The gas phase and the aqueous phase of the irradiated resins are recovered and analyzed with a variety of mass spectrometry techniques. Non-irradiated controls are included in the study.

Results and discussion

The irradiation of R1 and R2 resins was performed at pH 7.98, with an average irradiation rate between 7 and 15kGy/h and a total dose of 1 MGy. GC-MS analyses showed puzzling results (figure 5). A first analysis performed in March 2016 showed a peak in the irradiated samples compared to the controls, which could correspond to radiolytic products. However, a second analysis performed one month later showed no difference between the irradiated and control samples. We have no definite explanation for these observations as of yet. It might be that the products were consumed by microorganisms as the samples were not handled in sterile conditions. It could also be that the radiolytic products degraded further by secondary chemical reactions. In this regard, the presence of oxygen might have influenced the system as it is a very reactive species[8].

Future perspective

We plan to do further spectrometric analyses of the currently irradiated resins (ex. Maldi-TOF ETS/MS). We also plan to use other kind of ampules that will allow to prepare and irradiated the resins in anoxic conditions. We plan to do irradiations at the rates of 0, 6, 24, 48, 102 and 204 KGy at 6kGy/h. The characterization of the degradation products will allow us to select potential metabolites for bacteria. We will prepare solutions of the selected metabolites that will be used in in situ biodegradation experiments on the planktonic community from Opalinus clay porewater in the Mont Terri laboratory. I will then monitor the chemical and population changes over time.

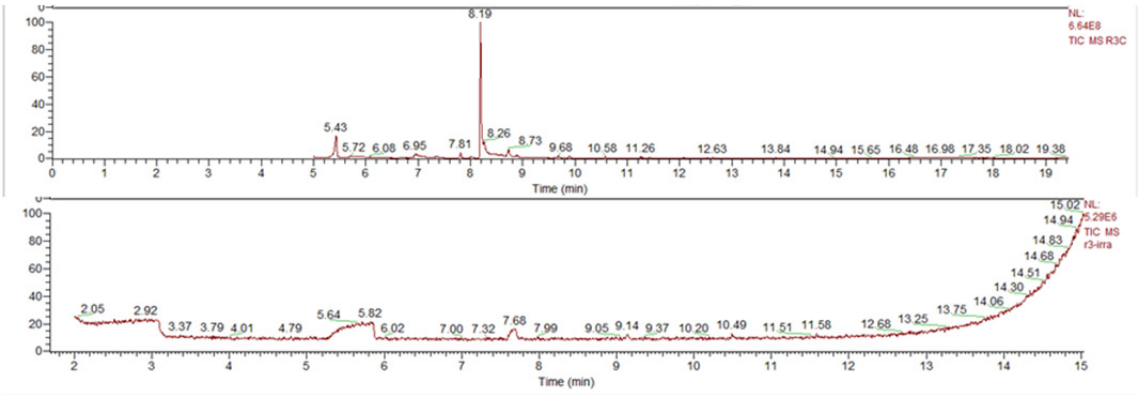

Figure 5: GC-MS results for R3 resin at pH 7.98, irradiated to a dose of 1 MGy using rates between 7 and 15kGy/h. A. Final temperature 250 ºC. B. Final temperature 300 ºC.

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BITUMEN LEACHATES AND DEGRADATION PRODUCTS: POTENTIAL STIMULATORS FOR GEO-MICROBIAL ACTIVITY

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Abstract

At least five European countries have to deal with bituminized Low/Intermediate-Level radioactive waste (bituminized L/ILW). As part of this type of waste can also contain long living radioisotopes (e.g. uranium, plutonium, americium, …), geological disposal of bituminized L/ILW is being considered as a potential safe long term solution. Clay deposits are being studied as host rocks for geological disposal of high- and intermediate-level long-lived radioactive waste. Bituminized L/ILW contains, besides bitumen and radionuclides, also large amounts of nitrates and sulphates. Over time, these salts will dissolve, leach and diffuse, together with water soluble organic substances which mainly originate from the degradation of the bitumen itself, into the surrounding clay host rock. The presence of these leachates might induce several physico-chemical processes (e.g. ionic strength changes, ion exchange reactions with Na^+, redox reactions with NO_3^-, …) that may affect the barrier properties of the clay host rock. To study the fate of such inorganic– and organic compounds, an *in situ* experiment, called Bitumen-Nitrate-Clay interaction (BN) experiment, was installed in the Opalinus Clay at the Mont Terri (Saint Ursanne, Switzerland) Underground Research Laboratory (URL) with the aim to clarify the (bio)chemical impact of a spreading nitrate and organic substances plume on the properties of a clay host rock. In the BN-experiment the transport and reactivity of nitrate is studied inside packed-off and anoxic intervals, constructed in a borehole drilled in the Opalinus Clay, that were filled and equilibrated with Artificial Pore Water (APW). The current BN-experiment set-up does not take any backfill or cement matrix into account but investigates first a purely aquatic environment in the form of a water-filled borehole. As such, the current BN-experimental setup allows free movement of dissolved macro- and micro-nutrients, electron donors and acceptors, and provides microorganisms a physically non-restricted environment (e.g. open space, maximal water activity (a_w)). The *in situ* microbial reduction of added nitrate and/or nitrite is being investigated, in the absence and/or presence of added electron donors relevant for the disposal concept of bituminized L/ILW. The results of the BN tests indicate that microbial nitrate reduction can occur with the naturally present electron donors in Opalinus Clay (e.g. pyrite, DOM, fermentation products, microbial necromass), but that the rate of nitrate reduction can increase by a factor of 10 to 20 when an extra electron donor (acetate or hydrogen) is added to the borehole solution.

The observed chemical evolution in the borehole water correlates well with the detected shifts in the microbial populations (analysed by 16S rDNA gene sequencing). The addition of nitrate inhibits the naturally slowly ongoing *in situ* microbial sulphate reduction and induces a shift in the microbial community, with nitrate- and nitrite-reducing microorganism becoming more dominant. These nitrate- and nitrite-reducing microorganism include strains from the genera *Pseudomonas, Cupriavidus, Pelomonas, Undibacterium, Acidovorax, Phenylobacterium, Brevundimonas* and *Corynebacterium*. Once nitrate (and/or nitrite) is completely reduced, the chemical composition and the microbial community of the interval solution gradually shift back towards their original state of slow sulphate reduction, showing strains from the genera *Pseudomonas, Desulfosporosinus* and *Gracilibacter*. This evolution is in agreement with the logic thermodynamic succession and preferential/prior usage of dissolved electron acceptors: nitrate is a more favourable electron

acceptor than sulphate, and when nitrate is present it will be used preferentially until depletion, after which sulphate is again next in line to be used as electron acceptor.

Introduction

In the past, bitumen has been used as matrix or stabilizer of Low/Intermediate-level long-lived radioactive waste. Already five European countries have a certain amount of bituminized Low/Intermediate-Level radioactive waste (bituminized L/ILW). Especially in Belgium a huge amount (more than 3000 tonnes) of bituminized Low/Intermediate Level Waste was produced. Several European countries study clay deposits as host rocks for geological disposal of high- and intermediate-level long-lived radioactive waste. Bituminized L/ILW contains, besides bitumen and radionuclides, also large amounts of nitrates and sulphates. Over time, these salts will dissolve, leach and diffuse, together with water soluble organic substances which mainly originate from the degradation of the bitumen itself, into the surrounding clay host rock. Furthermore, anaerobic metal corrosion and radiolysis of water and bitumen will produce substantial amounts of hydrogen gas. The introduction of all of these anthropogenic compounds can have an impact on several physico-chemical processes (e.g. ionic strength changes, ion exchange reactions with Na^+, redox reactions with NO_3^-, …) that may affect the barrier properties of the clay host rock. Besides, these foreign products may also alter microbial presence and/or activity. For instance, the release of nitrate could stimulate the growth and/or activity of nitrate reducing prokaryotes (NRP) leading to the formation of reduced nitrogen species like, nitrite, nitrogen gas or even ammonium and thereby altering the redox conditions of the clay host rock. As the redox conditions strongly impact the speciation, the solubility and the retention of redox-sensitive radionuclides (e.g. ^{79}Se, ^{99}Tc, ^{23X}U, ^{237}Np, ^{23X}Pu, …), also the transport properties of these radionuclides might be affected. Besides, three reduced nitrogen species (i.e. NO, N_2O and N_2) are gaseous under normal physico-chemical conditions and could thus form a separate gas phase when their concentration would exceed the solubility limit. This might render fissuring of the host rock and consequently result in the formation of preferential pathways for radionuclide migration.

To study the fate of possible leachates of Bituminized L/ILW on the biogeochemistry of the near-field host rock, an *in situ* experiment, called Bitumen-Nitrate-Clay interaction (BN) experiment, was installed in the Opalinus Clay at the Mont Terri underground research laboratory (St. Ursanne, Switzerland). The microbial analyses of the BN experiment intend to elucidate if and how currently present microbial communities are affected, and if and how microbes are involved in the observed biogeochemical processes. In this respect it is noteworthy to mention that the current BN set-up does not take into account any cement matrix or backfill, and offers the microbes only a pure aquatic environment, at almost neutral pH, in the form of a water-filled borehole. Such set-up allows free movement of nutrients, energy sources and dissolved electron donors and – acceptors, and does not impose any additional physical and/or chemical restriction on the microorganisms. In a real radioactive waste repository, the conditions will mostly deviate from the ones present in the BN experiment. Therefore, the *in situ* BN experiment can only be considered as a well engineered disposal 'test case'.

Description

The downhole equipment of the BN experiment consists of three packed-off intervals of ≅ 90 cm long, isolated from each other by inflatable rubber packers of which the outside is made of neoprene. Each interval is lined with a Stainless Steel filter screen and is hydraulically connected to a surface cabinet in which three separate individual pumps assure a close-circuit circulation of Artificial Pore Water (APW). An online monitoring system consisting out of an UV spectrophotometer and a redox and pH electrode (spectro::lyser; redo::lyser and pH::lyser from S::can Messtechnik GmbH, Austria) was installed on two of the three test intervals.

Each of the three intervals is also equipped with 5 batch sampling cylinders (40 ml or 150 ml) which allow undisturbed batch sampling of the interval solution, whenever required. These batch samples were used for additional chemical and/or microbial analyses.

The infrastructure of the surface cabinet allows to inject solutions or gasses of which the composition and concentration can be altered, to mimic physico-chemical conditions related with the disposal of bituminized-L/ILW. For instance, to study impact of a spreading nitrate plume, a concentrated solution of sodium nitrate was injected. Sodium acetate was used and injected as a representative substance for the organic water soluble fraction of bitumen degradation products. Hydrogen gas injections were performed to simulate its effect as a result of radiolysis and anaerobic metal corrosion. Already, several series of perturbation tests were performed, in which different concentrations and concentration ratios of nitrate, acetate or hydrogen gas were tested.

Microbial analyses were performed on the batch samples that were taken at pivotal moments (e.g. before, during and after initiating the perturbation tests). Microbial investigations, included Scanning Electron Microscopy (SEM), DNA-based molecular biology methods, ATP measurements, and cultivation-based techniques. Priority was given to DNA-based molecular biology analysis methods, as these methods can provide very accurate information on the composition and possible evolution of bacterial communities in response to the geochemical perturbations. During interval installation and the first series of perturbation tests, no precautions were taken to avoid external microbial contaminations.

Discussions

Figure 1 combines the most important chemical– and microbial analyses of a perturbation test in which only nitrate was added to the circulating APW solution. Microbial diversity was not constant as for this test no precautions were taken to avoid external microbial contaminations during APW equilibration and injecting of the nitrate containing solution.

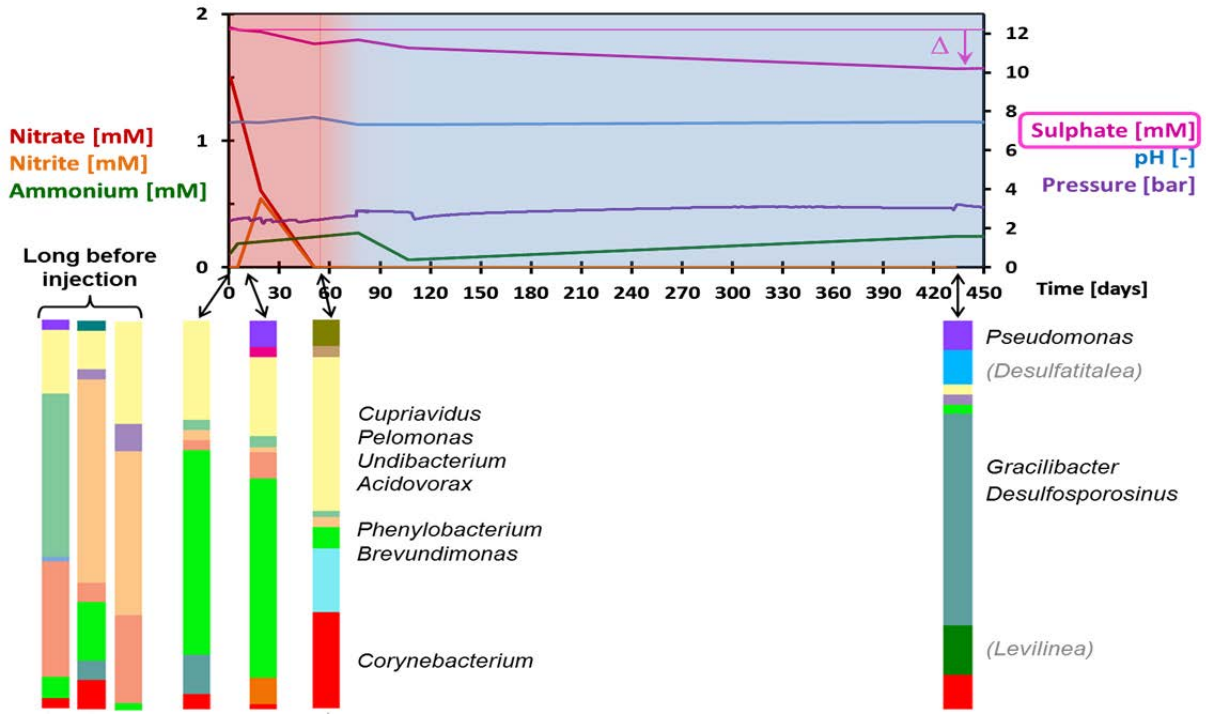

Figure 1: Perturbation test performed with pure nitrate ($NaNO_3$): Evolution of important physico-chemical parameters (top graph) combined with the results of the most important genera in microbial community of samples of the borehole solution, as analysed with 454-barcoded 16S rDNA metagenomics analysis.

Nevertheless, a microbial community shift can be observed in figure 1 which indicates that NRP's start to dominate the borehole solution when nitrate (and/or nitrite) is present. After the complete reduction of nitrate– and nitrite species, Sulphate Reducing Prokaryotes (SRP) are more prominent present indicating that sulphate becomes again the preferential electron acceptor. This tendency is also present in the chemical evolution of the sulphate concentration inside the borehole solution.

Figure 2 combines the most important chemical– and microbial analyses for a perturbation test in which first nitrate was added to the circulating APW and after 70 days a pulse of acetate was injected. No precautions were taken to avoid external microbial contaminations during APW equilibration after drilling the borehole. However, additional external contamination during the execution of this combined nitrate-acetate test was avoided by sterilizing all injection solutions prior to injection. Again, a clear microbial community shift occurred after the injection of nitrate. NRP's started to dominate the borehole community. The additional injection of a pulse of acetate accelerated the reduction rate of nitrate and consequently the increase of the nitrite concentration. When acetate was used up, the nitrate reduction rate declined until the same level as before the acetate injection. In the presence of acetate, nitrate and nitrite, *Acidovorax* outcompetes *Pseudomonas* and clearly dominates the borehole community. As soon as acetate is consumed the situation reversed in favour of *Pseudomonas*.

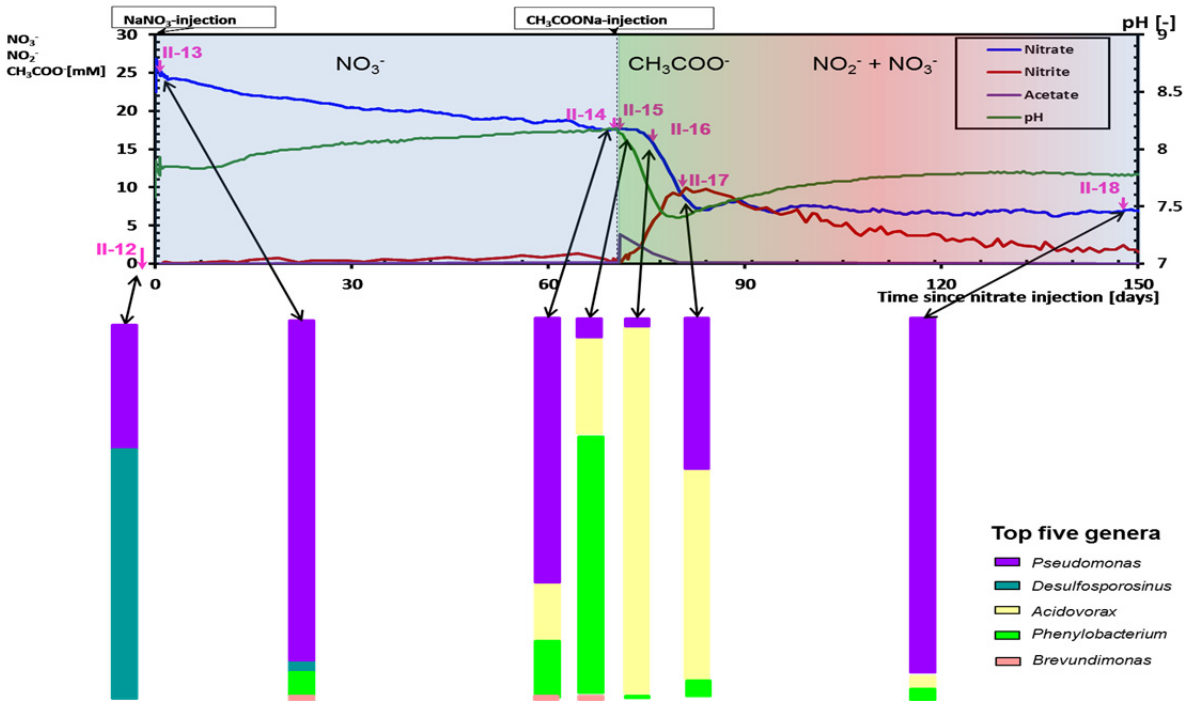

Figure 2: Perturbation test performed with successive injection of nitrate ($NaNO_3$) followed with an acetate (CH_3COONa) injection after 70 days: Evolution of important physico-chemical parameters (top graph) combined with the results of the most important genera in the microbial community of samples of the borehole solution, as analysed with 454-barcoded 16S rDNA metagenomics analysis.

Figure 3 combines the most important chemical– and microbial analyses for a perturbation test in which first nitrate was added to the circulating APW and after 50 days hydrogen gas was forced into the solution with a hydrogen exchange unit. No precautions were taken to avoid external microbial contaminations during APW equilibration after drilling the borehole. However, additional external contamination during the execution of this combined nitrate-hydrogen gas test was avoided by sterilizing all injection solutions and gasses prior to injection. Again, a clear microbial community shift occurred after the injection of nitrate. NRP's started to dominate the borehole community. The forced injection of hydrogen gas into the solution accelerated the reduction rate, although, after a

longer lag-phase than was the case for the acetate pulse injection (see figure 2). When hydrogen injection was stopped, the nitrate– and nitrite reduction rate declined until the same level as before the hydrogen injection. In contrast with acetate, no single genus dominated the borehole community during the hydrogen injection. More microbial genera remained active amongst which also the genus, *Clostridium*.

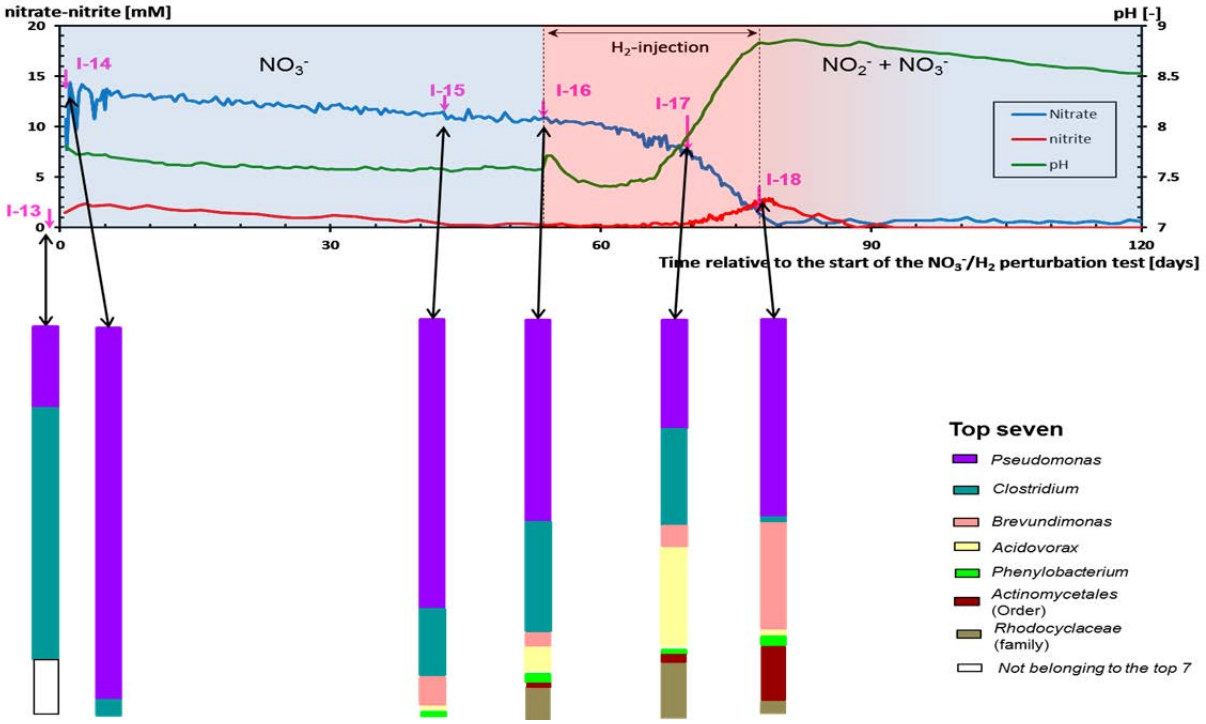

Figure 3: Perturbation test performed with successive injection of nitrate ($NaNO_3$) followed by forced hydrogen (H_2) gas injection after 50 days: Evolution of important physico-chemical parameters (top graph) combined with the results of the most important genera in the microbial community of samples of the borehole solution, as analysed with MiSeq barcoded 16S rDNA metagenomics analysis.

Conclusions

The *in situ* BN experimental infrastructure offers the possibility to mimic certain physico-chemical perturbations, related with the geological disposal of bituminized-L/ILW. The surface cabinet allows injection of prepared solutions or gasses into the three intervals. Several series of perturbation tests were performed, in which different concentrations and concentration ratios of nitrate, acetate or hydrogen gas were tested.

When nitrate was added, i.e. an electron acceptor that renders more chemical redox energy than the naturally present sulphate, a community shift appeared towards NRP's. If in parallel acetate, an easily oxidizable carbon containing electron donor, or hydrogen gas, a pure electron donor, was offered the reduction rate of nitrate was increased by a factor of 10 to 20. This high chemical rate was maintained as long as the additional electron donor was abundant. When the extra electron donor and nitrate were exhausted, the microbial communities present in the interval solutions gradually shift back towards a community in which again SRP's were abundant.

All perturbation tests demonstrate that microbial community shifts follow the thermodynamic succession of dissolved electron acceptor use by microorganisms. Nitrate (and/or nitrite) is a more favourable electron acceptor than sulphate, and so whenever nitrate is present in the borehole water, it will be preferentially used as electron acceptor until depletion. After nitrate depletion, sulphate becomes again the most favourable dissolved electron acceptor and as a consequence the SRP population of the microbial community becomes more dominant.

Acknowledgement

This study is performed in the frame of the Bitumen-Nitrate-clay interaction (BN) experiment, which is performed in collaboration with and co-funded by the Mont Terry consortium, in particular ANDRA, IRSN, NAGRA and SCK•CEN.

MICROBIOLOGICAL DEGRADATION OF LLW UNDER REPOSITORY CONDITIONS – CASE TVO

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Abstract

In Finland major part of the low level radioactive waste (LLW) is composed of protective plastic sheets, clothing, tools, and towels used during maintenance work. This miscellaneous maintenance waste is compacted in steel drums and deposited 60-110 meters below ground level. The Gas Generation Experiment GGE has been established in 1997 to examine gas generation from LLW in TVO's final disposal repository for operational LLW and ILW in Olkiluoto, Finland. Since 1999 gas generation has been rather constant and released gas was mainly composed of methane. The results obtained by qPCR also revealed that methanogens formed major part of the microbial population in GGE. In addition to anaerobic degradation of organic matter there are also other processes that influence the gas formation, such as corrosion of steel and sulphate reduction by bacteria. Considerable heterogeneity in chemical conditions has been detected in different locations of GGE which can be also seen as differences in microbiological activity and diversity.

Introduction

In Finland the power companies producing nuclear energy are responsible for the on-site storage, processing and disposal of low level radioactive waste (LLW) with the activity level not more than 1 Mbq/kg. In Olkiluoto (Teollisuuden Voima Oyj, TVO) drums are placed in concreate boxes and finally to rock silo with a capacity of about 5,000 m^3 at a depth of 60–100 metres below the ground surface. In Loviisa (Fortum) the drums are placed in an underground maintenance waste facility built at a depth of about 110 metres. [1]

The Gas Generation Experiment (GGE) is a large-scale simulation experiment that has been established in 1997 to examine gas generation from LLW in TVO's final disposal repository for operational LLW and ILW (intermediate level radioactive waste) in Olkiluoto. The main processes influencing the gas generation are biodegradation of cellulose-based biodegradable material and metal corrosion resulting to formation of H_2. This could lead to overpressure of repository and transport of radionuclides to the groundwater and surrounding environment. The aim of this study was to evaluate microbial diversity and activity in GGE in more detail using advanced molecular techniques.

Materials and methods

In GGE sixteen waste drums (à 200 dm^3) are arranged in a concrete box which is enclosed in a gas tight tank of acid proof steel with a volume of 20 m^2. The drums are filled with maintenance waste from nuclear power units including cellulosic material (paper, cardboard, cotton) and other organic materials (PE, PVC, glass fibre latex gloves, natural rubber). The GGE was filled with locally sourced untreated river water and maintained a temperature of 8-11°C.

Composition of the released gas and tank water are analysed at certain time intervals. Electrical conductivity, dissolved O_2, pH and redox potential were measured by on-line system. Samples for chemical analyses (e.g. SO_4^{2-}, Fe^{2+}, HS^-) were taken from the tank water. The experimental design and the results from the first 9 years have been described by Small et al. [2].

Microbiological samples have been taken from the tank water between the drums and from the drums. In addition small stainless steel capsules containing waste materials and small plate of steel have been loaded into that tank. The application of DNA-based high-throughput sequencing technology and real-time quantitative qPCR allowed the characterization of archaeal and bacterial communities in GGE.

Results

Since 1999 gas generation has been rather constant and released gas was mainly composed of methane. The results obtained by qPCR also confirmed that active population of methanogens exists in GGE. These results indicate methanogenesis and biodegradation of maintenance waste in anaerobic conditions. Both acetate (acetotrophic) and CO_2/H_2 (hydrogetrophic) utilizing methanogens were detected in GGE but their relative amounts are not known.

Sulphate reducing bacteria SRBs compete with methanogens for electron donors, such as organic compounds or H_2. SBRs belonging to the orders of Desulfobacterales, Desulfovibrionales, Desulfovibrionaceae and Desulfomonadales were detected in all GGE but the amount of *dsr*B transcripts was relatively small. This corresponds well with the decreasing amount of sulphate which is needed as terminal electron acceptor.

The conditions inside the tank have been heterogenic which can be seen in variation in pH and other chemical parameters. During the experiment the pH of the tank water has decreased from alkaline to neutral resulting from precipitation of $CaCO_3$. Inside the drums pH has been close to neutral thus providing more optimal conditions for microorganisms. qPCR analysis revealed that the number of bacteria, archaea, methanogens and sulphate reducers were higher in drum containing (hemi)cellulose-based material than in tank water. In addition, especially in the beginning of experiment the microbial activity has been higher at the bottom of the tank than at the surface lid level.

Conclusions

LLW contain approximately 41 w-% of cellulose- and hemicellulose-based material. During anaerobic biodegradation process hemi(cellulose) is transformed to CH_4 and CO_2 with concerted action of microbial consortium. Methanogens formed a major part of the archaeal population in GGE. In addition to anaerobic degradation of organic matter there are also other processes that influence the gas formation, such as corrosion of steel and sulphate reductions. Considerable heterogeneity in chemical and microbiological measurements has been detected in different locations of GGE.

References

(1) Posiva 2016., Nuclear waste management, Olkiluoto nuclear power plant, Loviisa nuclear power plant, summary 2012. www.posiva.fi

(2) J. Small et al. 2008. Experimental and modelling investigations of the biogeochemistry of gas production from low and intermediate level radioactive waste. Applied Geochemistry 23, 1383-1418.

Acknowledgement

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SPECIATION STUDIES OF RN/LN WITH SELECTED DEGRADATION PRODUCTS OF ORGANIC LILW – NEW SPECTROSCOPIC INSIGHTS INTO THE URANYL-ACETATE SYSTEM –

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Abstract

The interaction of acetic acid as the smallest carboxylic acid with a side chain with uranium(VI) is of importance because it occurs often as a degradation product of organic components of radioactive waste (e.g. cellulose, bitumen, polyvinyl chloride (PVC)). Our approach of using a combination of UV-vis, time-resolved laser induced fluorescence spectroscopy (TRLFS), and cryo-TRLFS allows the spectroscopic characterization of all three uranyl-acetate complexes including the determination of the corresponding stability constants. The speciation of uranium(VI) in the presence of acetate was successfully investigated at 50 µM by UV-vis spectroscopy and TRLFS. The calculation of the single component spectra and stability constants of the formed species based on factor analysis succeeded.

In addition the presentation will give an overview of our planned activities in cooperation with our partners (UNIMAN, SCK·CEN, and UGR) to contribute to task 1.2 of WP1. Here our focus and expertise lies on the application of different modern spectroscopic tools to directly characterize the speciation of RN/LN with organic degradation products and/or with selected microbes, to underpin the findings from UNIMAN and SCK·CEN.

Introduction

Organic polymers (e.g. cellulose, PVC, bitumen) present in low and intermediate level wastes (LILW) are exposed to ionizing radiation, alkaline pH, and organic degrading microorganisms. This may lead to the formation of smaller, water soluble organic compounds, affecting amongst others the chemical behavior and mobility of radionuclides (RN). In the worst case complexation will lead to an increased mobility and a decreased retention of RN in the barriers of a nuclear waste disposal. Therefore, the characterization of RN complexes with degradation products is necessary for the assessment of the safety and the long-term performance of a nuclear waste repository.

The interaction of acetate, being a degradation product of both cellulose (1) and bitumen (2), with uranium is well studied and a huge number of stability constants under different environmental conditions are given (e.g. 3-5). This small carboxylic acid is often declared as model for more complex organic compounds. Therefore, it is important to provide information for all changing properties resulting from the interaction with uranium. This also includes changes in spectroscopic properties. In 2014 Sladkov and Meinrath *et al.* published their results of luminescence and absorption spectroscopic studies of the uranium-acetate system (3, 4). However, they provided only spectroscopic data for the $UO_2(AcO)^+$-complex (1:1). The reason for this is probably the narrow range

where the 1:1 complex and the two other species $UO_2(AcO)_2$ (1:2) and $UO_2(AcO)_3^-$ (1:3) coexist (see Figure 1).

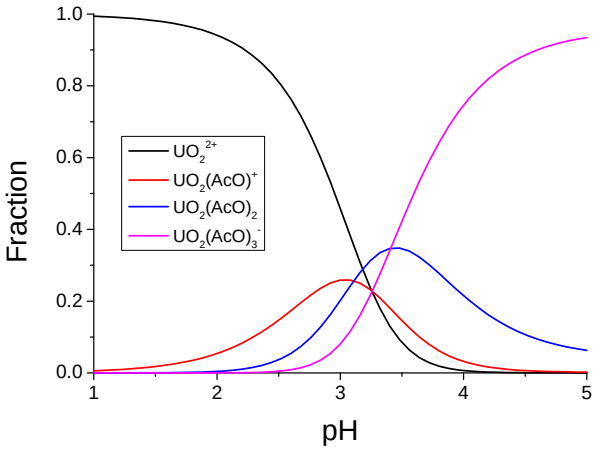

Figure 1: Speciation of the uranyl-acetate system at following conditions: [UO_2^{2+}]=50 µM, [AcOH]=100 mM.

The aim of this work is to fill the gap of spectroscopic data (e.g. especially for the 1:2 and 1:3 complex) of the uranium-acetate system using UV-vis spectroscopy and time-resolved laser induced fluorescence spectroscopy (TRLFS) at very low uranium concentrations in combination with factor analysis data treatment.

Description

Materials and Methods

General sample Preparation. For both UV-vis and TRLFS a 0.1 M uranyl perchlorate stock solution was used to prepare the samples, having a final concentration of $5 \cdot 10^{-5}$ M. The pH was adjusted with a Metrohm double junction electrode, using NaOH and $HClO_4$. The ionic strength was 1 M ($NaClO_4$).

UV-vis spectroscopy. For the first experiment a fixed acetic acid concentration of 4 mM was used and the pH was varied from 1 to 4. For the second experiment the pH of 2.8 was adjusted and the acetic acid concentration varied from 0 M to 0.8 M. The absorption of the samples was measured between 300 and 600 nm with a TIDAS 100 spectrophotometer (J&M Analytik GmbH). A 250 cm liquid waveguide capillary cell (LWCC, world precision instruments) was connected via optical fibres. Contrary to conventional UV-vis experiments, this capillary allows the detection of uranium in the micromolar range due to the enormous long path length. The inner surface of the capillary is coated with Teflon. An additional quartz layer protects the Teflon and avoids the retention of air bubbles. The aqueous core has a higher refractive index than the Teflon wall. Therefore, the light is confined within the liquid core by total internal reflection at the core-wall interface. Before the absorption was measured, the capillary cell was flushed with water and all air bubbles were removed from the system. Afterwards a dark and a reference spectrum were taken, which were subtracted from the sample spectra. The stability constants and single component spectra were calculated based on factor analysis with HypSpec (6).

TRLFS. In the first setup a pH of 1.5 was adjusted and the acetic acid concentration varied between 0 M and 0.6 M. In Series 1 of the second experiment the pH was varied between 1 and 4, whereas the acetic acid concentration was fixed at 4 mM. The acetic acid concentration in Series 2 was

100 mM and the pH varied again between 1 and 4. The luminescence of U(VI) and U(VI)-acetate complexes was measured after excitation with laser pulses at 266 nm (Inlite laser system, Continuum) and an averaged pulse energy of 0.3 mJ in a 1 cm quartz cuvette. The temperature was adjusted to 25°C. Time-resolved spectra were recorded with an ICC-camera (model 7467-0008, Princeton Instruments) in combination with a delay generator (model DG535 Digital Delay/Pulse Generator, Stanford Research) in the wavelength range from 370 nm to 670 nm. The collected spectra were evaluated using Origin 9.1G.

Results and Discussions

The evaluation of the spectra from the first UV-vis experiment resulted in a single component spectrum for the 1:1 complex, with an extinction coefficient ε = 16.0 l $mol^{-1}cm^{-1}$ at a λ_{max} = 419 nm (see Figure 2 left). The calculated stability constant is log β = 2.93 ± 0.01. These findings are in excellent agreement with the results from Meinrath *et al.* (ε = 17.8 l mol^{-1} cm^{-1} at λ_{max} = 418 nm, log β = 2.88 ± 0.03) (4).

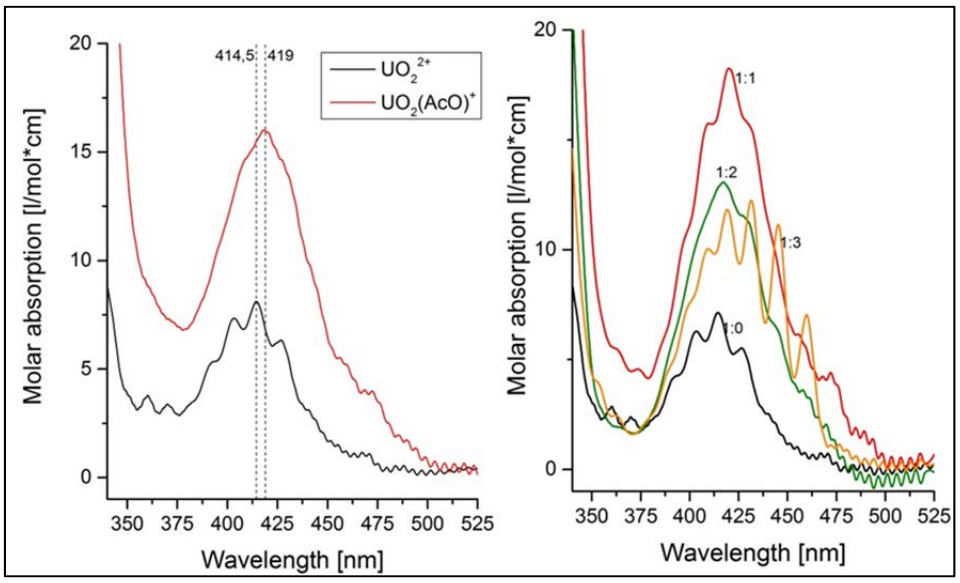

Figure 2: ***Left.*** *Calculated single component spectra of the free uranyl ion and the 1:1 complex.* ***Right.*** *Calculated single component spectra of the 1:1, 1:2, and 1:3 complex.*

Table 1: Parameters for the calculated uranium-acetate single component spectra.

Complex	λ_{max} (nm)	$\varepsilon_{\lambda max}$ (l mol^{-1} cm^{-1})	log β	
1:1	420.0	17.8	2.52 ± 0.04	this work
			2.58 ± 0.03	(7)
1:2	417.3	13.1	5.18 ± 0.03	this work
			4.37 ± 0.14	(7)
1:3	431.5	12.2	7.55 ± 0.07	this work
			6.86 ± 0.04	(7)

To the best of our knowledge it was possible for the first time with the set of 17 spectra of the second UV-vis experiment to calculate single component spectra for all uranium-acetate species (see Figure 2 right). The calculated values for ε, λ_{max}, and log β are summarized in Table 1. The spectroscopic parameters of the 1:1 complex match well with the previously discussed data. However, the calculated stability constants deviate slightly from the values given in the literature.

Figure 3 depicts the results from the first TRLFS experiment. The conditions were chosen in a way, that only the 1:1 complex was formed.

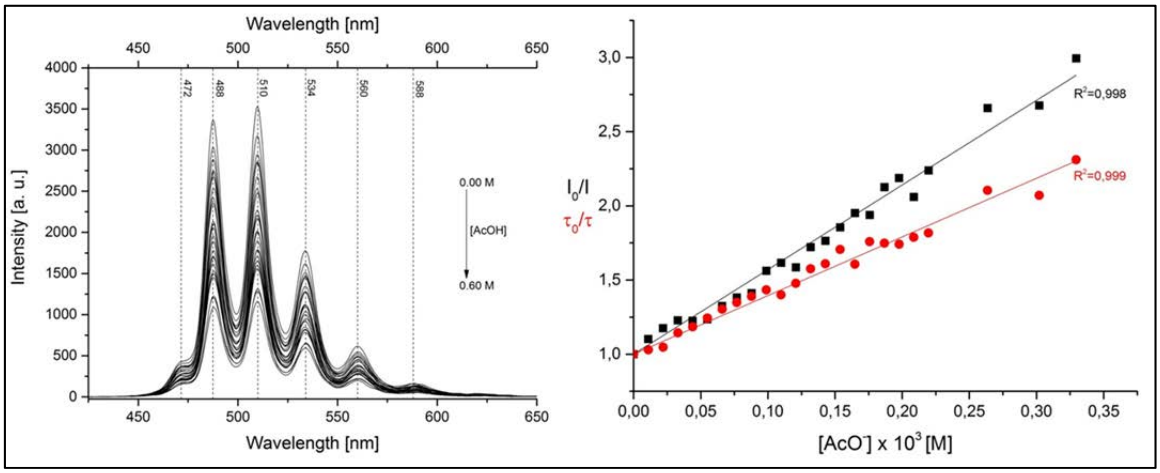

*Figure 3: **Left.** Decrease of luminescence intensity with increasing acetic acid concentration. **Right.** Stern-Volmer plot of the data from the time-resolved spectra ($[UO_2^{2+}] = 5 \cdot 10^{-5}$ M, pH= 1.5).*

Table 2: Parameters of Stern-Volmer plots and constants determined.

I [M]	pH	K_{SV} (Stern-Volmer constant) (L mol^{-1})	k_q (quenching rate) (l mol^{-1} s^{-1})	log $\beta_{1:1}$	
1	1.5	(3.98 ± 0.60) · 10^3	(2.06 ± 0.31) · 10^9	2.96 ± 0.37	this work
0.2	1.5	(1.75 ± 0.10) · 10^3	(7.40 ± 0.50) · 10^8	2.66 ± 0.07	(3)

The luminescence decreases with increasing acetate concentration indicated an increasing amount of 1:1 complex. The Stern-Volmer plot (cf. Figure 3 right) displayed a form characteristic for the occurrence of static and dynamic quenching due to complex formation. The Stern-Volmer constant, the quenching constant and the corresponding stability constant of the 1:1 complex are summarized in Table 2. These results are consistent with the findings from Sladkov (3). To determine whether the 1:1 complex is non fluorescent or not, TRLFS measurements at very low temperatures to avoid the dynamic quenching are in progress.

The emission maxima of the first spectrum in Figure 4 left (pH = 1.21: 470 nm, 487 nm, 509 nm, 534 nm, 560 nm, and 589 nm) can be assigned to the free uranyl ion. Series 1 of the second experiment showed the previous discussed quenching of the 1:1 complex. The higher amount of acetic acid used in Series 2 (cf. Figure 4 right) resulted in a speciation change accompanied with characteristic changes in the luminescence spectra. Until pH 2.8 the quenching occurs. The luminescence intensity increases subsequently with increasing pH. The bands were shifted to longer wavelengths and a new band arises at 461 nm. This can be assigned to the formation of the 1:2 and 1:3 complexes, matching with the speciation diagram in Figure 1. Thus, the 1:3 complex contributes mainly to the spectrum at pH 3.99. The time-resolved spectra of the first sample of Series 2 showed a mono-exponential decay with a lifetime of 1.92 ± 0.02 µs, which can be assigned to the free uranyl

ion. At pH 3.99 the corresponding time-resolved spectra showed a bi-exponential decay. The averaged lifetimes are 0.51 µs and 0.17 µs. These findings suggested that the 1:2 and the 1:3 complexes emit luminescence light.

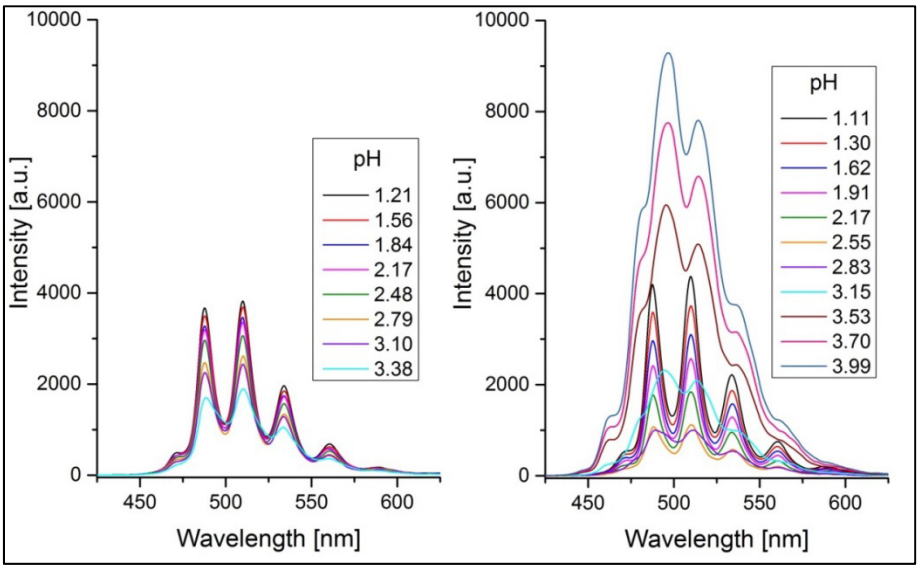

Figure 4: ***Left.*** *Decrease of the luminescence intensity due to formation of the 1:1 complex ([UO_2^{2+}] = 5·10^{-5} M, [AcOH] = 4 mM).* ***Right****. Increase of luminescence Intensity due to formation of the 1:2 and 1:3 complex ([UO_2^{2+}] = 5·10^{-5} M, [AcOH] = 100 mM).*

Conclusions

The approach of UV-vis and TRLFS in combination with factor analysis data treatment on order to describe the U(VI) speciation in the acetate system yielded consistent results with the literature. For the first time all single component spectra could be isolated from the collected absorption spectra of one experiment. UV-vis and TRLFS gave an average stability constant log $ß_{1:1}$ = 2.80 ± 0.25 (1 M $NaClO_4$). In addition to the existing knowledge a first description of the luminescence properties of the 1:2 and 1:3 complex was reported. Cryo-TRLFS is in progress for a comprehensive spectroscopic characterisation of U(VI)-acetato species. Moreover PARAFAC will be used to isolate the single component luminescence spectra. Spectral data of single components are essential prerequisites for the characterization of complex solutions with two and more ligands. In further experiments the pH range will be extended in the alkaline region. The described approach will be applied to more unknown but rather relevant systems like U(VI) - butyrate and - isosaccharinate.

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Acknowledgements

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ANAEROBIC REDUCTION OF SE(IV) BY BACTERIAL STRAIN ISOLATED FROM SPANISH BENTONITES

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Abstract

Microbes may play a major role in the biogeochemical cycle of radioactive elements within the concept of deep geological repository. For this purpose, the anaerobic reduction of selenite, Se(IV), by the bacterial strain *Stenotrophomonas* sp. BII-R7, isolated from bentonite formations of Cabo de Gata Natural Park (Almeria, Spain) was investigated. In Spain, bentonite formations were selected as a reference material for bentonite-engineered barriers in the disposal of radioactive wastes. Using acetate as electron donor and nitrates as electron acceptor, XRD analysis have clearly demonstrated that the cells of the studied strain are able to reduce Se(IV) to Se(0) forming Se nanoparticles (Se NPs). Using a combination of Scanning Transmission Electron Microcopy High-Angle Annular Dark-Field (STEM-HAADF) and Variable Pressure Scanning Electron Microscope (VP-SEM) techniques allowed for the first time to identify two different sized Se NPs (30 and 100 nm), located at the cell surface, the extracellular space and intracellularly. The results obtained indicated the role of bacteria isolated from bentonite clays in affecting the speciation of selenium under deep geological repository relevant conditions.

Introduction

Deep geological repository (DGR) has been considered as the best and safest disposal option for radioactive wastes, encapsulated in metal containers and surrounded by natural and artificial barriers. The microbial community of the selected host rocks and artificial barriers may influence the transformation of clay minerals, induce the metal corrosion of canisters and lead to the migration of radionuclides from the deposit to the environment through different processes like biomineralization, biosorption, reduction, etc. [1, 2]. Therefore, the effect of microbial processes on the safety of this disposal system should be taken in consideration.

Selenium is a common component of high level radioactive wastes (HLRW), specifically; the Se^{79} isotope formed by nuclear fission reactions. Several studies have indicated Se^{79} to be one of the critical radionuclides for the geological disposal of HLRW [3]. Se exist in four oxidation states i.e. +VI, +IV, 0, and –II in nature. The speciation of selenium depends on different factors including oxidation state, Eh, pH, etc. Under reducing conditions the speciation of this element is dominated by selenide [Se(-II)] and elemental Se[(0)]. On the other hand, selenate and selenite are the dominant species in mildly and strongly oxidizing conditions, respectively [3]. Several bacterial strains of the genus *Stenotrophomonas*, *Bacillus*, *Pseudomonas* has been described for their ability to reduce mobile Se(IV) to immobile Se(0) under aerobic conditions [4-5]. The reduction of Se(IV) is mediated by the activity of different enzymes such as nitrate reductase or glutathione reductase [6]. However, no Se reductions studies were conducted under DGR relevant conditions where different electron donors (e.g. acetate) and electron acceptors (e.g. nitrates) etc. are present. Therefore, the present work aimed to study the anaerobic Se(IV) reduction by the bacterial strain *Stenotrophomonas* sp. BII-R7,

isolated from bentonite formations of Cabo Gata Natural Park (Almería, Spain). BII-R7 strain is able to grow under anaerobic respiration conditions using acetate as electron donor and nitrate or Fe (III) compounds as electron acceptors. In addition, preliminary BII-R7 draft genome analysis, conducted in our research group, revealed the presence of enzymes described for their ability to reduce Se(IV) to Se(0) like glutathione-related enzymes and NADH-dependent enzymes.

Methods

1) Bacterial strain and growth conditions.

The bacterial strain used in the present work was isolated from Spanish bentonites collected from the Cabo de Gata Natural Park (Almeria, Spain) [7]. The cells were grown in LB medium (tryptone 10 g/l, yeast extract 5 g/l and NaCl 10 g/l, pH 7.0 ± 0.2) at 28 ºC and 120 rpm on a rotary shaker.

2) Bacterial Se(IV) reduction experiments.

Cells were harvested at mid to exponential phase by centrifugation from LB cultures, washed with 30 mM PIPES buffer to remove the medium ingredients. Afterword's, the cells were resupended in the same buffer and supplemented with 10 mM Na acetate as the electron donor and 20 mM $NaNO_3$ as the electron acceptor. Selenite at 2 mM was added to the mixture from sodium selenite stock solution, degassed using N_2 and incubated under anaerobic conditions for 2 weeks at 30 ºC.

3) X-ray diffraction analysis.

For the XRD analysis, the Se(IV)-treated cells was re-suspended in acetone and washed with Milli-Q water. The washed samples were dried at 28 ºC for 24h. The XRD data of the biogenic Se NPs produced were obtained with a Bruker D8 Advanced diffractometer associated to a LINXEYE detector available at the University of Granada. The obtained diffractograms have been analysed using the software X-powder (http:/www.xpowder.com) and DIFFRAC PLUS programme.

4) Microscopic characterization.

4.1 Variable Pressure Scanning Electron Microscopy (VP-SEM).

Variable Pressure Scanning Electron Microscopy (VP-SEM) was used to examine the Se NPs produced by the cells of the strain BII-R7. Samples were mounted and coated with carbon (Hitachi UHS evaporator) for variable pressure SEM (VPSEM) (Leo 1430-VP) (University of Granada).

4.2 Scanning Transmission Electron Microcopy High-Angle Annular Dark-Field (STEM-HAADF).

Se(IV)-treated bacterial cells were harvested by centrifugation at 15.000g for 15 min at 4 °C and washed twice with 0.9% NaCl. TEM sample were prepared as described in Merroun et al., 2005 [8]. Samples were examined under high-angle annular dark field scanning transmission electron microscope (HAADF-STEM), FEI TITAN G2 80-300. TEM specimen holders were cleaned by plasma prior to STEM analysis to minimize contamination. The high resolution STEM is equipped with HAADF detector and EDAX energy dispersive X-ray.

Results and Discussion

After 2 weeks of culture incubation a color change from colorless to red was observed in medium containing Se(IV). This indicated Se(IV) reduction to elemental form, Se(0), by the cells of the strain BII-R7. No red colorization of the medium was observed in abiotic control (without bacteria).

XDR analysis confirmed the anaerobic bacterial Se(IV) reduction to elemental selenium and the production of Se nanoparticles with a hexagonal structure and a size ranging between 10 and 30 nm (Figure 1).

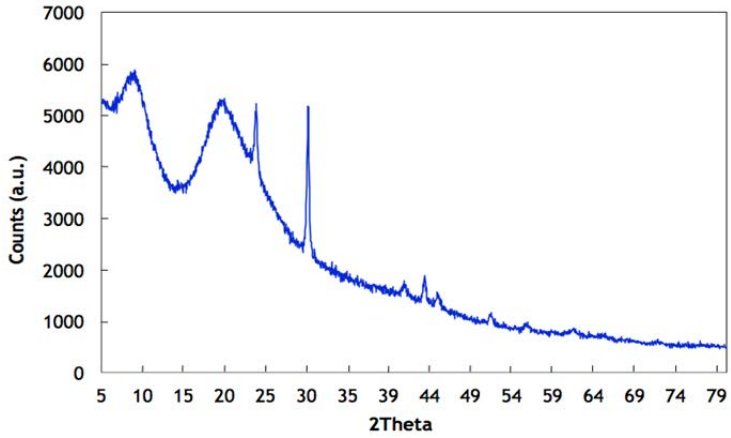

Figure 1: X- ray diffraction patterns of Se NPs formed by BII-R7 bacterial strain under anaerobic conditions.

VPSEM revealed the occurrence of extracellular 100-200 nm Se NPs around the cell surface and within the extracellular space as confirmed by EDX analysis (Figure 2)

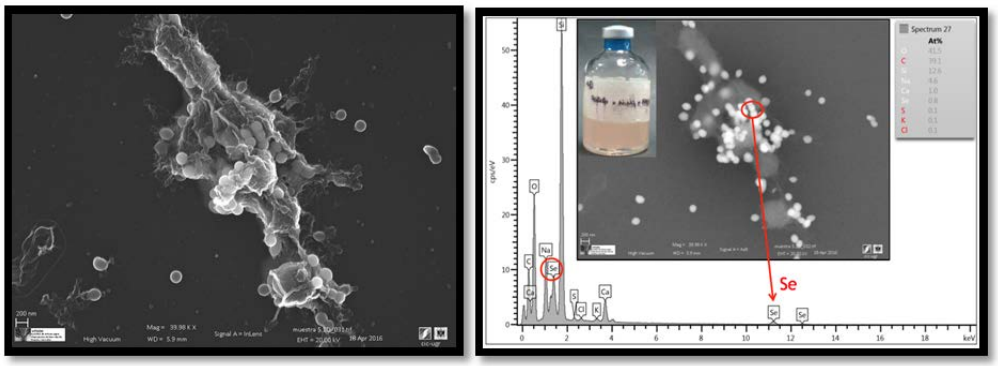

Figure 2: Variable Pressure Scanning Electron Microscopy of Se NPs around the cell surface and within the extracellular space formed by Stenotrophomonas sp. BII-R7 as revealed by EDX analysis.

STEM micrographs of thin sections of bacterial cells loaded with 2 mM Se(IV) in presence of Na acetate as electron donor and $NaNO_3$ as electron acceptor under anaerobic conditions are presented in Figure 3. In these micrographs, intracellular electron-dense accumulates were observed. EDX element-distribution maps derived from these accumulates showed that they are mainly composed of Se with a size ranging between 100-200 nm. Electron diffraction analysis revealed the amorphous nature of these accumulates.

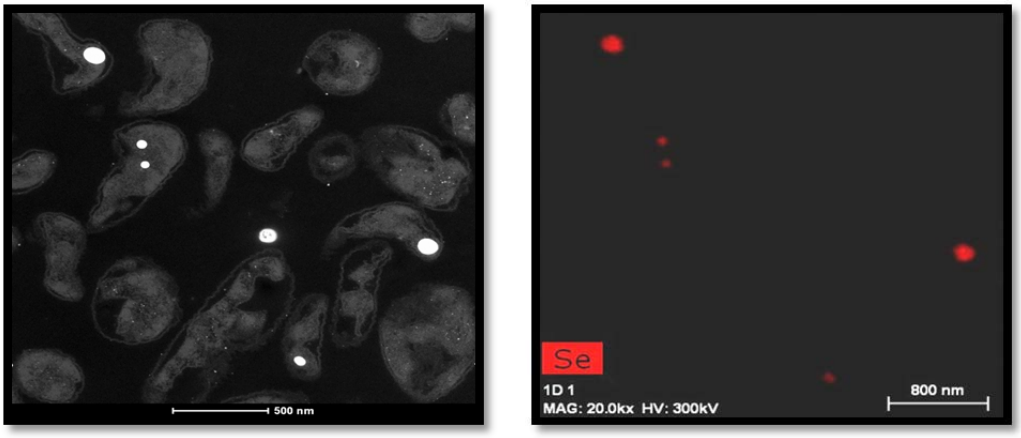

Figure 3: Scanning Transmission Electron Microscopy -High-Angle Annular Dark-Field (STEM-HAADF) micrographs of thin section of 2 mM Se(IV)-treated cells of BII-78 showing the presence of intracellular Se NPs. EDX composition maps of Se over the whole HAADF-STEM image.

Conclusions

The present study showed the ability of a bacterial strain isolated from Spanish bentonites, *Stenotrophomonas sp*. BII-R7, to reduce anaerobically mobile Se (IV) to immobile Se (0) producing Se NPs at the cell surface, within the cytoplasm and extracellularly. Different sizes and structures of Se NPs were detected: 10-30 nm hexagonal and 100-200 nm amorphous sized Se NPs. These results demonstrate that indigenous bentonite bacteria have a potential impact on the long-term safety of the DGR by changing the speciation of Se through its reduction and formation of Se NPs.

Acknowledgements

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COUPLING MICROBIAL PROCESSES IN GEOCHEMICAL MODELS

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Abstract

Workpackage 1 of The Microbiology in Nuclear waste Disposal (MIND) project addresses remaining uncertainties concerning the release of chemicals, gases and radionuclides from organic containing low and intermediate level waste (LILW). A modelling task (T1.4) will assist in the interpretation of laboratory and *in situ* experiments examining the microbial processes relevant to the degradation of organic ILW and interactions with radionuclides. The modelling will also enable wider application of the research to performance assessment. This paper summarises the background conceptual model and some example applications of the Generalised Repository Model (GRM) FORTRAN computer code that enables the chemical effects of microbial processes to be coupled with equilibrium chemical speciation and transport processes. The development of a new Application Program Interface (API) using a modular approach is described that will enable microbial processes to be coupled to geochemical speciation and 3-D reactive transport models.

Introduction

Workpackage 1 of The Microbiology in Nuclear waste Disposal (MIND) project addresses remaining uncertainties concerning the release of chemicals, gases and radionuclides from organic containing low and intermediate level waste (LILW). The principle effects that are being examined concern the degradation of organic polymer wastes, comprising cellulose, plastics, ion exchange resins and bitumen materials. These materials provide a potential energy and carbon source for microbial growth. Under anaerobic conditions degradation of the organic materials could result in methane gas generation that could enhance transport of gaseous radionuclides (e.g. ^{14}C), or could generate over pressures that could affect the engineered barrier system and affect the transport of radionuclides in groundwater.

Chemical hydrolysis and radiolytic degradation of the polymer materials will be an important step in releasing small bioavailable organic molecules. Some organic hydrolysis and radiolysis products may act as complexing ligands that could enhance the mobility of some radionuclides in groundwater, however microbial processes may attenuate such intermediate degradation products. In addition, bioreduction of Fe(III) and sulfate driven by organic and hydrogen electron donors should stabilise reduced forms of some important multivalent radionuclides (e.g. Se, Tc, U, Np, Pu).

Equilibrium chemical speciation models (e.g. PHREEQC [1]) are used widely to predict radionuclide solubility used in performance assessment calculations and to consider the effects of organic complexation on radionuclide solubility and sorption. Chemical models are also used routinely to describe and examine the pH buffering of repository concepts for ILW disposal utilising cementitious materials. Consideration of kinetic processes that determine the redox potential is less well developed and approaches are often scenario based and repository specific. e.g. [2]. In the case of the UK Low Level Waste Repository (LLWR) a near field model, the Generalised Repository Model (GRM), has been developed that specifically represent microbial mediated degradation of cellulose-rich LLW and the resulting effect on redox and pH evolution and gas generation. The GRM has been independently validated by blind-test modelling of a large scale gas generation experiment (GGE) that simulates gas generation processes in LLW disposed at the VLJ Repository, Olkiluoto, Finland [3].

This extended abstract provides an overview of the microbiological functionality of the GRM and summary case study applications that are relevant to its use in the MIND project. Within Task 1.4 of MIND the microbial subroutines in the GRM FORTRAN code will be ported to a new Application Programming Interface (API), which will enable the validated GRM conceptual model to be interfaced with state of the art chemical speciation and 3-D reactive transport models. The early stages of development of the MIND microbes API are discussed.

The Generalised Repository Model (GRM)

The GRM code was originally developed by British Nuclear Fuels Ltd (BNFL) [4,5] to model processes of Eh evolution and microbial gas generation at the LLWR located in Cumbria close to the Sellafield reprocessing site. For the environmental safety case (ESC) submitted to regulators in 2002 [4] the main focus of the near field modelling concerned the behaviour, over periods over 50,000 years, of uranium disposal to trench disposals excavated into local glacial sediments. Here the simulation of the redox potential (Eh) evolution was important to calculate the solubility of uranium and other multivalent radionuclides and metal contaminants. The GRM model also simulates the generation of methane-rich gas, which is important to the understanding the release of ^{14}C that is present mainly in metallic waste that are disposed in a cementitious wasteform placed in a surface concrete lined vault [4,6].

The GRM model couples in sequence kinetic microbiological and corrosion processes with equilibrium speciation and transport processes (Figure 1). The microbial and corrosion subroutines determine the evolution of the redox potential, which is used as an input to an equilibrium speciation calculation that utilises the FORTRAN PHREEQE solver. This chemical speciation module consider mineral and gas equilibrium (e.g. of cement minerals, CaCO3, Fe corrosion products, CO_2 and CH_4 gas solubility) and determines pH by charge balance. The speciation and solubility of radionuclides can also be included in the chemical model. The transport module uses a 2-D finite difference approach to discretise the model system and can be configured to represent a variety of experimental and repository applications using several hundred model cells. The transport module also solves sorption processes represented by a simple sorption distribution coefficient (Kd) approach, kinetic controlled radionuclide release processes and radioactive decay.

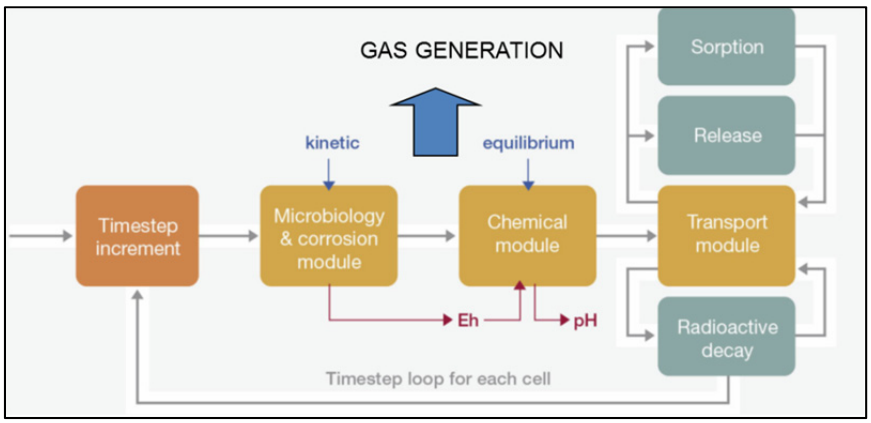

Figure 1: High level structure of the GRM

GRM Microbial module

The GRM microbial module includes consideration of the hydrolysis of organic substrates that provide an energy and carbon source for microbial activity. These include cellulose materials as well as proteins and fats and these hydrolysis processes are used to consider the recycling of biomass. Hydrolysis is modelled by a first order process that is dependent on pH:

$$\frac{dS_p}{dt} = -V_p S_p F_{pH}$$ Equation 1

Where V_p is the hydrolysis rate of the polymer, S_p is the concentration of the polymer and F_{pH} is a pH control factor. In the case of cellulose, the most important polymer for studies of LLW hydrolysis yields glucose monomer that is used in subsequent aerobic and anaerobic microbial processes.

The GRM represents the metabolism and growth of microbes using Michaelis-Menten (Monod) kinetics (Equations 2 and 3), which includes the growth and death of biomass:

$$\frac{dS}{dt} = -\frac{V \quad S \quad X}{K_m + S} F_{pH}$$ Equation 2

$$\frac{dX}{dt} = Y\frac{dS}{dt} - DX$$ Equation 3

Where; S is the substrate concentration, V is the maximum substrate removal rate, K_m is the half saturation constant, X is the biomass concentration which is defined by Y, the yield coefficient and D the death rate.

GRM includes separate FORTRAN subroutines that model the growth of the main groups of processes: aerobic, denitrification, Fe(III) reduction, sulfate reduction, acetogenesis and methanogenesis. Alkaliphilic and neutraphilic groups are represented to consider processes under the range pf pH conditions expected for cementitious LLW. Both H_2 and organic carbon are considered as electron donors for reduction of nitrate, nitrite, Fe(III) and sulfate electron acceptors. Either inorganic carbon (CO_2, HCO^{3-}) or organic species (e.g. acetate) can be used for biomass growth. Biomass has an assumed idealised composition of $C_5H_7O_2N$. Nitrogen can be obtained from nitrate or ammonia present. Alternatively, the model can be configured assuming that nitrogen supply is not limited, such as due to nitrogen fixation processes.

GRM representation of Eh evolution

GRM calculates a redox potential for individual finite difference cells by selecting a dominant redox couple (Table 1). A controlling couple is selected by considering the concentration of key species that are affected by the microbial process model considering from the most oxidised oxygen couple.

	Definition	**Criteria**
Oxygen	$\frac{1}{4}O_{2(aq)} + H^+ + e^- \Leftrightarrow \frac{1}{2}H_2O$	$O_{2(aq)} > 1x10^{-7}$mol/l
Nitrate	$\frac{1}{5}NO_3^- + \frac{6}{5}H^+ + e^- \Leftrightarrow \frac{3}{5}H_2O + \frac{1}{10}N_{2(g)}$	$NO_3^- > 1x10^{-6}$mol/l
Iron	(i) $Fe(OH)_3 + HCO_3^- + 2H^+ + e^- \Leftrightarrow FeCO_3 + 3H_2O$	$Fe(III)_{(s,\ aq)} > 1x10^{-6}$mol/l
	(ii) $Fe(OH)_3 + 3H^+ + e^- \Leftrightarrow Fe^{+2} + 3H_2O$	(i) in presence of $FeCO_3$ (ii) aqueous Fe^{+2}
Sulphate	$SO_4^{2-} + 10H^+ + 8e^- \Leftrightarrow H_2S_{(aq)} + 4H_2O$	$SO_4^{2-} > 1x10^{-6}$mol/l
Methane	$\frac{1}{8}CO_{2(g)} + H^+ + e^- \Leftrightarrow \frac{1}{8}CH_{4(g)} + \frac{1}{4}H_2O$	$CH_4 > 1x10^{-6}$mol/l
Fermentation	$C_6H_{12}O_6 + 4H_2O \Leftrightarrow CH_3COOH + 8H_2 + 4CO_2$	$CH_3COOH > 1x10^{-6}$mol/l

Table 1 Redox couples considered in the GRM

The Eh (or pe) is calculated from the Nernst equation for each redox couple. The selected Eh is then used as an input to an equilibrium speciation calculation that uses a thermodynamic database where the oxidation states of the major elements (N, Fe, S and C) are decoupled. The speciation of multivalent radionuclides such as uranium is calculated assuming redox equilibrium with the controlling redox couple.

Example application to the large scale gas generation experiment (GGE)

The GGE has previously been used to validate the GRM [3] and is included in the MIND project (see Vikman et al abstract). The GGE comprises 16 drums of waste together with a concrete container that are submerged in water within a gas tight vessel. The GRM model of the GGE (Figure 2 a) represents the inventory of cellulose and metal in each drum and considers mixing of reactive species in the waste drum and water regions. The model accurately represents the volume of methane gas generated (Figure 2b). In addition the model also simulates sulfate reduction processes that are observed during the first 3 years of the experiment (Figure 2c) The GRM also represents the heterogeneity in pH between the waste and water regions of the experiment (Figure 2d). Data collected since 2006 record the decline in pH of the tank water to neutral pH as a result of the microbial activity on the experiment. This larger data set covering 17 year operation of the GGE is currently being modelled as part of MIND task 1.4.

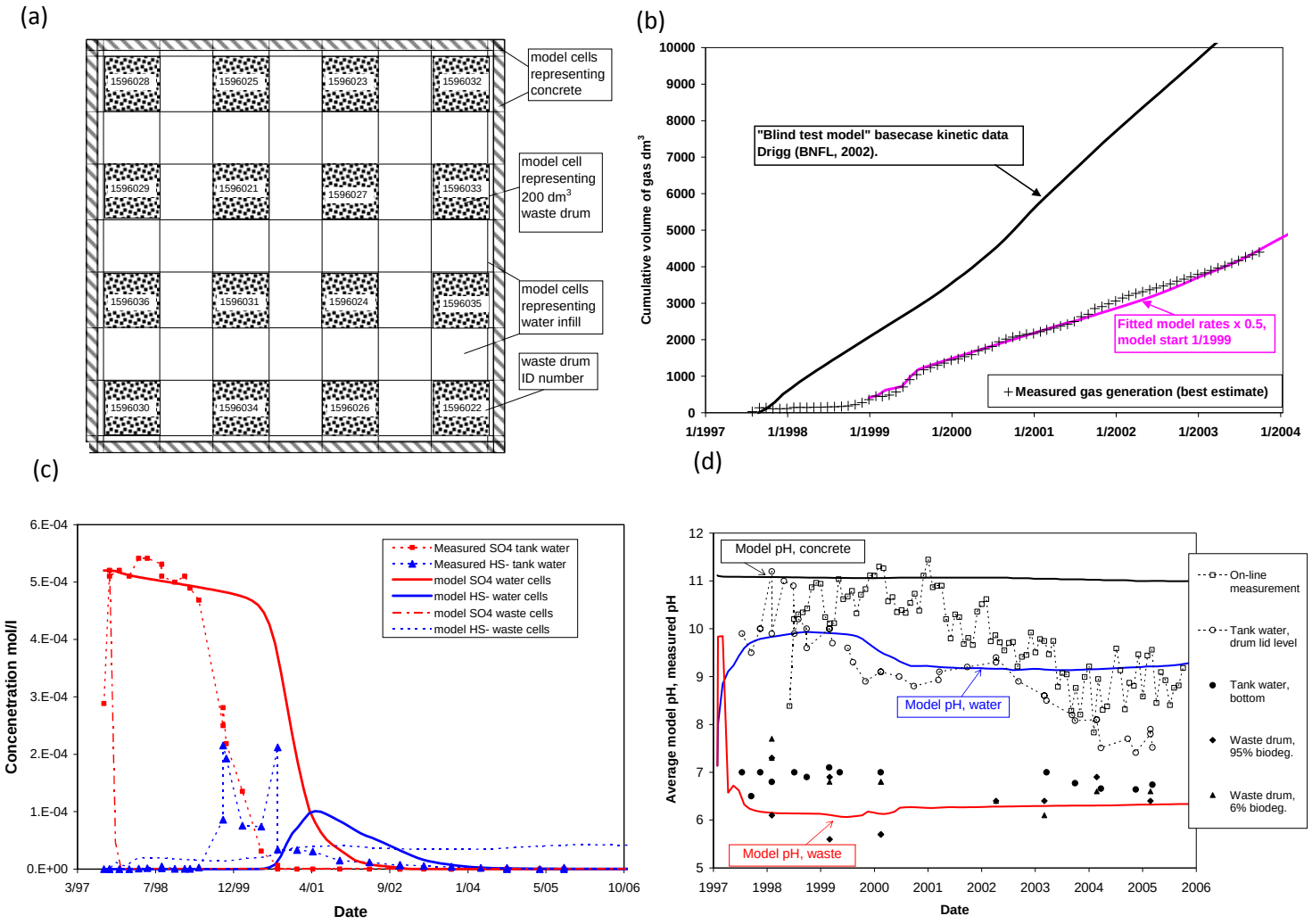

Figure 2 (a) discretisation of the GRM to represent the 16 waste drums present in the GGE, surrounding water and concrete (b) measured volume of gas (CH4) generated and blind test and fitted modelled volumes(c) measured and modelled sulfate reduction (d) measured and modelled pH of waste and water regions of the GGE (after ref 3)

Development of a microbial process API

In order to widen the application of the GRM conceptual model to geodisposal the MIND project is porting the microbial growth kinetic, hydrolysis, Eh and gas generation subroutines of the FORTRAN code to a new API that will enable the processes to be coupled with chemical speciation and transport in water and gas phases. The API is being developed using C++ 11 using Boost libraries (www.boost.org) for filesystem and property tree support. Other Open Source libraries are likely to be used later during the code development to provide graphical outputs and a user interface. The API is initially being developed on Microsoft Windows, but will be portable to Linux and other platforms.

In the first phase of development the following modules are being developed:

- A generic hydrolysis/radiolysis kinetic model for organic polymers that will simulate the formation of soluble organic species (e.g. glucose or isosaccharinic acid from cellulose, amines from ion exchange resins, phthalic acid and other characterised organics from PVC materials). The development of this module will take account of findings of the MIND Task 1.2 concerning organic polymer degradation.
- A generic Monod kinetic modelling approach allowing the definition of an unlimited number of microbe groups. Initially these will be those included in the GRM code, but will be expanded to include new processes of significance based on MIND Task 1.2 and 1.3.
- Coupling to pass chemical species (aqueous and gas) to PHREEQC to undertake chemical speciation and mineral and gas reaction. Here iPHREEQC [7] will be interfaced with the API. The current *findpe* GRM subroutine (Table 1) will be ported to the API and will be adaptable to include a wider range of redox couples.

Subsequent phases of development will focus on interfacing the API to transport models. Outputs from an initial prototype API are presented in Figure 3 and Figure 4. Figure 3 shows the growth of an aerobic microbe that utilises glucose for energy and biomass growth. In this example nitrogen limitation is considered and the availability of nitrate limits the complete consumption of glucose.

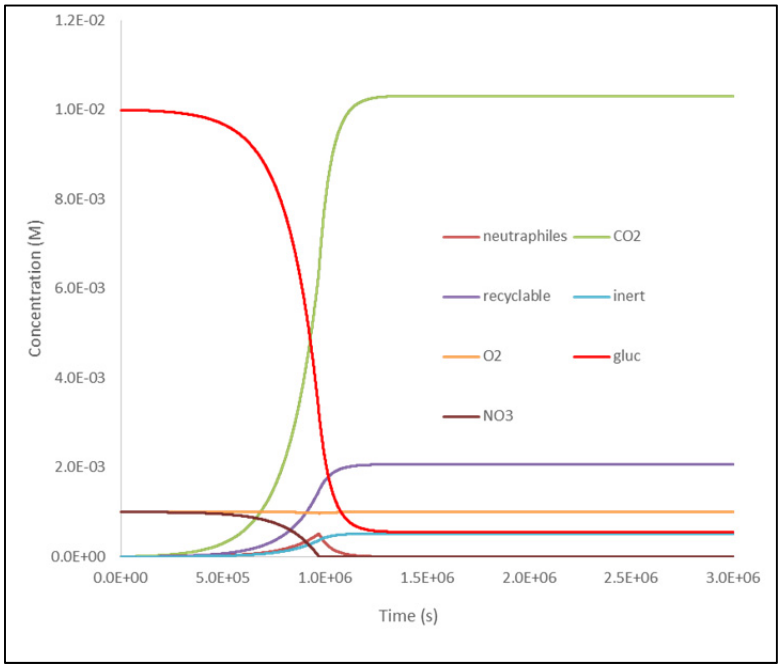

Figure 3: Example output from the prototype microbial process API, showing concentrations of biomass and chemical species

Figure 4. shows a further example where two separate populations of neutraphilic and alkaliphilic aerobic glucose oxidisers are represented. In this simple example pH is increased from pH 7 to pH 9.5 after 6.eE+6 seconds of the simulation, which results in a shift in growth from the neutraphilic group to the alkaliphilic group.

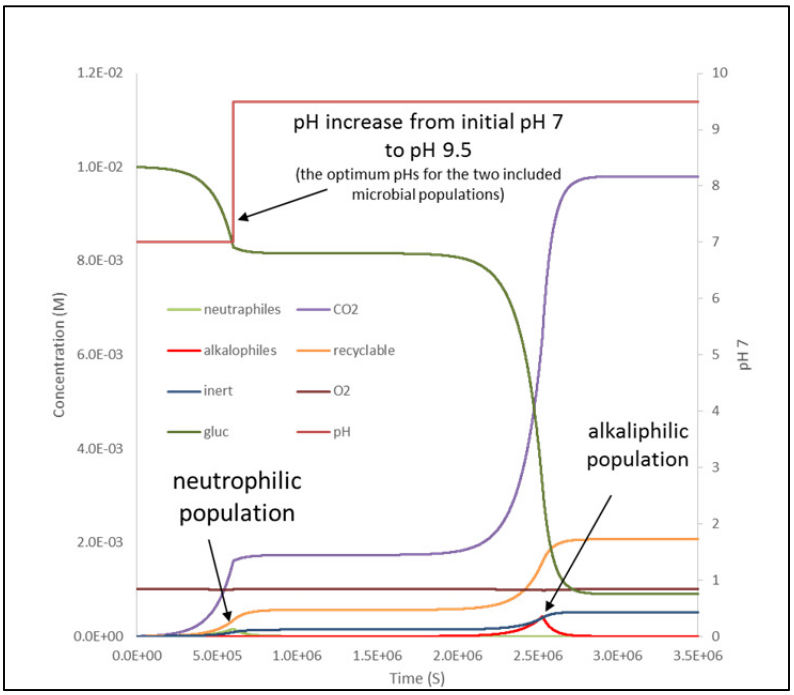

Figure 4 Example output from the prototype microbial process API, for a case with neutraphilic and alkaliphilic populations, whose growth is affected by an imposed change in pH.

Conclusions

The GRM code has been used in previous safety assessments for LLW disposal in the UK and its microbiological and gas generation functionality has been validated against the GGE experiment at the VLJ repository, Finland. The conceptual model represented in GRM has thus proved useful to investigate the coupled microbiological, chemical and has generation processes occurring in LLW and ILW. Work has commenced in the MIND project to port the FORTRAN subroutines in GRM that represent organic hydrolysis processes, microbial growth and control of Eh to a new API using a C++ object oriented approach. The API will enable wider coupling to chemical speciation and reactive transport models.

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MICROBIOLOGY IN RADIOACTIVE WASTE DISPOSAL

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Abstract

We conclude that the most important general constraints of deep life in continental rock systems are energy and space. A key question of the deep life is the supply of electron donors (mainly H2 and CH4) from some deep geological source. With respect to a HLW repository in crystalline rock, it is important to demonstrate the sealing capability of the buffer against the microbial activity. In general, clay rocks provide better sealing properties against microbial activity than crystalline rock. If copper canister is used, prerequisites and limitations of microbial sulphate reduction need to be demonstrated. Iron-based canister materials may produce excess hydrogen into the environment. Common process understanding on the role of gases in the microbiological systems is a one the key questions. What are sources, fluxes and how critical component of they constitute in the microbial metabolism.

Introduction

Construction of commercial nuclear reactors for energy production started during 1960's, and the next two decades were the time of rapid growth of the industry. Consequently, there was a growing need to develop concepts for the management of the highly radioactive spent fuel. ***Reprocessing*** applied in some European countries is an effective way to recycle useful components and to reduce the amount and activity of the waste, but many countries only have the option of ***direct disposal*** of spent fuel. Interim storage is required by all spent fuel after the removal from the reactors, and the period of the interim storage may be extended if required by the progress of the final waste management program. After all, there will be a need for geological disposal of some type of radioactive waste, regardless of the waste management strategy. During the operation nuclear facilities also produce waste fractions with low or intermediate radioactivity, which also have to be managed safely during and after the operation. The entire life cycle of a nuclear facility ends up with the decommission phase and disposal of the radioactive construction components.

General concepts of geological disposal have evolved parallel to the implementation of the nuclear power facilities. Primary requirements of safe geological disposal systems include general stability and good long-term predictability, good isolation and containment properties, as well as availability in terms of depth, volume and homogeneity of the geological formation. Continental crust of the Earth is a heterogeneous puzzle of rock units that have formed in different environmental conditions, evolved and migrated during the geological history, and finally aggregated and emplaced in their present positions. Consequently, different countries have dissimilar premises in developing their national programs for safe geological disposal.

Micro-organisms are known to be major players in the geochemical conditions since the early geological history of the planet Earth. The subject of geomicrobiology has for a long time been examining the role of micro-organisms in geological environments, but the focus has been in the near-surface conditions of soil and aquatic systems. Marine microbiology has been studying

biological processes even in very deep conditions, but the ***deep continental biosphere*** was very little studied before the introduction of the microbiology of nuclear waste disposal.

Disposal concepts

Salt deposits were the first geological formations considered for radioactive waste disposal already during the beginnings of the waste management of energy production. Currently the main site in operation is WIPP (Waste Isolation Pilot Plant) in New Mexico, where transuranic radioactive waste (TRU) from the research and production of nuclear weapons is to be disposed of. Other important sites based on the salt concept are Gorleben and Morsleben in Germany. Bedded salt deposits are free from fresh flowing water and practically impermeable, salt rock also seals all fractures and other openings relatively rapidly.

Even though free water activity is low in salt deposits, there are geomicrobiological aspects that have been discussed. Salt formations may be associated with entrapped brine formations, and the presence of halophile micro-organisms has been reported even in hypersaline environments. On the other hand, salt deposits are typically associated with organic-rich geological formations containing hydrocarbons and indigenous micro-organisms. Currently salt deposits are used for final storage of TRU, ILW and LLW mainly packed in steel containers.

Crystalline rock has been considered as the geological disposal option in many countries. Decisions made in Finland and Sweden are firmly based on this concept. Stripa mine in Sweden was one of the pioneering sites, where the geoscientific basis for nuclear waste disposal in crystalline rock was studied from different perspectives, including preliminary microbial observations. Since 1980's research has focused on specific underground research laboratories (URL's) providing more controlled environment for microbial sampling and research in crystalline rock: Grimsel in Switzerland, AECL in Pinawa, Canada, Äspö in Sweden, and Onkalo in Olkiluoto, Finland. Currently Olkiluoto is shifting from a research facility status to the construction site of the Finnish HLW repository. In Sweden the final repository site is planned to be Forsmark. Czech Republic is also considering deep geological repository in crystalline rock. The concept will be studied and demonstrated in Bukov Underground Research Facility (BURF) in metamorphic crystalline rock close to a uranium deposit.

Crystalline rocks, if available, are considered to provide stable host rock formations for nuclear waste repositories. Despite their hardness and very low porosity, crystalline rocks contain fractures possibly forming a more or less connected fracture network. Crystalline rock is often described by the concept dual porosity: fracture porosity providing space for free-moving microbes (> about 1 µm) and matrix porosity, where only dissolved chemical substances and small colloids may exist. Due to the flow heterogeneity, fracture waters of crystalline rocks show compositional variation in different scales, possibly affecting the nutritional conditions and diversity in the immediate surroundings of the cells.

More than 50 drill hole sites and underground research targets studied in Sweden and Finland have shown the presence of microbes in the fracture waters of crystalline rock. Additionally, many studies support the idea that the attachment of microorganisms on the fracture surfaces is a key phenomenon. On the other hand, stagnant matrix waters are subjected to long term water-rock interaction and they may be the primary source of the electron acceptors/sources and other nutritional chemical components.

Clay rock concept for the geological disposal has been one of the main options since 1970's. Clays are fine grained sedimentary material settling in undisturbed, often marine basins. Long-term sedimentation process and subsequent hardening to rock during millions of years have in many places resulted in the formation of deep and wide-spread homogenous formations, considered to be very potential as host rocks for nuclear waste disposal.

The Boom Clay in north-east Belgium has been intensively studied since 1974 because of its favourable properties to host a geological repository for high- and intermediate-level radioactive waste. In Switzerland, research focuses currently on Opalinus clay, and Bure clay deposit is likely the repository site in France. Underground research laboratories have been active in all these countries (HADES in Mol, Belgium, Mont Terri in Switzerland and Meuse/Haute Marne in France).

Clay rocks evidently have excellent properties for the isolation of radioactive wastes and other harmful substances from the biosphere. Dense, very fine-grained rock material is practically impermeable for water flow and advective transport. Clays also typically have the property of self-sealing, by which artificially generated open fractures or reactivated fault surfaces are closed again on saturation of the rock due to the swelling capability of certain clay minerals.

Porosity of a "fresh" clay is high (40-70 %), but decreases during compaction and diagenetic mineral growth (e.g. Opalinus clay about 20 %). Chemical and isotopic composition of clay pore waters indicate very long residence time, in fact signs of the chemical composition of original seawater may be still observed in pore waters of clay rocks. Thus clay rocks may contain microbes from the deposition environments. Typical particle size of clay minerals is less than a few micrometers, inhibiting the mobility of microbes. Molecular diffusion is the dominant solute transport process in the pore scale of clays.

Engineered barriers

All disposal concepts rely on different multi-barrier concepts, in which the technical barriers aim at isolating radionuclides. Spent nuclear fuel consists of insoluble uranium dioxide. In direct disposal spent fuel pellets inside corrosion resistant claddings are encapsulated in metallic canisters. In reprocessing options, useful isotopes are extracted for further use and the reprocessing waste is divided into different fractions. High-level reprocessing waste is about one order of magnitude less active than spent fuel, but still needs very long time of isolation from the biosphere. Consequently, vitrification into a glass or ceramic matrix is often considered as the principal immobilization technique for HLW. Bitumen-based materials have high reducing capacity, thus immobilizing well several radionuclides from liquid wastes. Similarly, high pH provided by cement based materials supports the containment of the radioactive materials in a solid matrix.

Metallic canisters aim at isolating the waste. Main options of metals are (various) steels and pure copper. Primary reason for choosing copper was the general resistance against corrosion in oxygen free conditions. However, the strong affinity of copper to form sulphides remained an issue to be considered carefully. Steel canisters may be subjected to different types of corrosion phenomena. On contrary to copper, metallic iron can be oxidized in water evolving hydrogen. Alloying iron by different components to make steel strongly increases the corrosion resistance of the material against oxidative corrosion, because a protective coating will form. Hydrogen generation due to steel corrosion is, however, considered as an important process to be considered in the performance assessment of steel containers.

Buffer material fills the open space between the deposition hole and the technical canister/waste form barrier. Bentonite is the typical buffer material in many concepts. By definition, bentonite is a clay material consisting predominantly of swelling smectite minerals (in practice montmorillonite). Ability to swell to a certain pressure is considered as a design criteria of the buffer. Grain size of individual smectite particles in commercial bentonites is less than 0.1 µm, so compacted bentonite is practically impermeable to water flow. Diffusion is considered as the only relevant transport mechanism in compacted bentonite.

Tunnels are an important part of the repository system, and they will stay open for long periods during the operation of the disposal facility. Excavation of the tunnels may produce some new fractures around the tunnels. Oxygenated conditions during the operation affect the excavated zone (EDZ) geochemically and microbiologically. Finally tunnels are filled, crushed host rock and swelling

clays are the main options for filling material. Concrete plugs are considered as additional barriers of transport. Oxygen-consuming microbes invaded during the construction phase can then only use up oxygen entrapped in the EDZ.

Microbial processes, constraints of life

Solar energy has been the main driving force of life since the emergence of first photosynthetic organisms more than 3500 million years ago. In the absence of light, the opposite process i.e., ***respiration*** consumes oxygen and provides energy for life in deeper layers of biosphere in the ground and oceans. Aerobic respiration uses typically carbohydrates as electron donor, but methane can also be oxidized in metabolic processes (aerobic methanotrophy). Aerobic chemolithotrophy extends the operational environment of the microbes to the rock: microbes can use certain inorganic constituents as electron source (sulphide minerals, ferrous iron etc.) and fix inorganic carbon (CO_2) for cellular chemosynthesis. Iron-sulphur system (often acidophilic) micro-organisms are known to be involved in metal corrosion processes and some species may also utilize ferric iron as electron acceptor instead of oxygen.

Anaerobic respiration processes determine a reduction sequence towards the deeper layers of the ground surface. Nitrate reducing microbes are primarily heterotrophs using mainly organic compounds as electron donors. Nitrate thus reduced to ammonium compounds is easily available for microbes, but certain microbes can also fix molecular N_2 directly for cellular synthesis. As a result, uncontaminated deep groundwaters are typical devoid of nitrogen compounds other than N_2.

Microbial reduction of Fe(III), Mn(IV) and U(VI) with concurrent oxidation of organic matter are important biogeochemical reactions in sub-oxic or anoxic aquatic sedimentary environments. Fermentative processes which need no external electron acceptor become predominant in deeper anoxic layers. Fermentative reaction chain is based on electron transfers between organic molecules and gradual consumption of organic matter, finally leading towards a mixture of methane and carbon dioxide. Hydrogen is an intermediate in the fermentative process chain, but –depending on the source material – may also remain as a constituent in the final product.

Sulphate and aqueous dinitrogen (N_2) are the two most resistant electron acceptors in deep groundwaters. The resistivity evidently depends both on their thermodynamic stability (low energy gain) and on the stable electron configuration of the molecules (covalence). Reduction of sulphate to sulphide requires microbial enzymatic catalysis, and takes place effectively within the uppermost tens of meters in strictly anaerobic sedimentary systems. However, in fractured crystalline rock the depth of the sulphate to sulphide transition zone varies substantially.

Microbial methane from fermentative reactions is frequently observed in anoxic near-surface organic sediments (e.g. peat, anoxic aquatic sediments). Deep methanic environment, however, typically shows dissimilar characteristics compared to methane from fermentative pathway. Deep methane typically correlates inversely with CO2, indicating that CO2 acts as an electron acceptor in the presence of a strong electron donor (e.g., H_2). Interestingly, N_2 is the last remaining electron acceptor, which seems not to be consumed effectively even if strong electron donors are present.

Finally, energetic prerequisites of life deep in the continental geosphere decrease, if no intrinsic and/or replenishing reducing power is available. The existence of a self-sustaining, hydrogen-driven deep biosphere has been discussed in the scientific literature, but the required primary gas source and renewal rate still need to be verified.

Discussion

Currently, there are no more doubts about the existence of a deep biosphere in the continental upper crust. Artefacts due to contamination have been excluded or at least reduced by various methodologies. Microbial communities have been observed to vary consistently with geochemical

environments, but there are indications that the population density decreases towards the depths of two kilometres and more. The origin and "reasons" of the unicellular life to occupy the harsh, low-energy conditions may depend on the geological conditions. In case of the clay rocks, a microbial community may even originate from the depositional environment of the rock, whereas in fractured crystalline rock the deep anoxic environment may just provide an appropriate refuge for anaerobic micro-organisms. Space requirement is a key question especially in dense clay rocks, where the average pore size is clearly smaller than the size of typical microbe. Consequently, it can be questioned, if there can be a viable indigenous community in clay rock or does the microbial activity come from a contamination.

The most important general constraints of deep life in continental rock systems are evidently energy and space. In terms of energy, continental deep systems cannot be paralleled with deep seated marine environments receiving a continuous energy flux transported by deep ocean currents. Continental deep biosphere may be based on the flux of near-surface electron acceptors (oxygen, nitrate, sulphate) and on the supply of organic matter acting as electron donor or maintaining fermentative processes. A key question of the deep life is the supply of electron donors (mainly H_2 and CH_4) from some deep geological source. In undisturbed bedrock conditions, natural decay series of uranium, thorium and potassium release energy in the form of alpha and beta particles, which may collide with water molecules or atoms in silicate structures producing oxidizing and reducing radicals (radiolysis). However, the net effect of the radiolysis is anything but straightforward to predict. Natural uranium and thorium decay series produce predominantly alpha particles, which are strong oxidizers immediately stripping the nearest available electrons to form a stable helium atom. On the other hand, K-40 decay emitting beta-particles (i.e. electron) has a net reducing effect, possibly producing hydrogen in surplus.

In radioactive waste disposal the scope must be wider than that of the natural deep biosphere. Construction and operation of the repository produces a transient oxic environment and an aerobic microbial population, which consume oxygen readily after the closure of the repository. However, sulphate and nitrogen compounds will be available for a longer period and the initial oxic and sub-oxic period must be accounted for, keeping in mind the early-phase high heat generation of the waste. A primary performance function of the buffer is to wet and swell to a target pressure protecting the canisters from early-phase oxic corrosion and later sulphidic corrosion. Anoxic corrosion of steel canister can theoretically take place as a reaction between iron metal and water, reaction products will be iron oxides and hydrogen. Microbes capable to use hydrogen would thus promote corrosion. Consequently, it is important to demonstrate the limits of viability of microbes both in buffer material and in the near field of the canister. In addition to the space limitation due to the swelling pressure, microbial activity can be suppressed by the energy limitation due to the availability of the electron acceptors.

Radiolytic processes are of fundamental importance for the deep life in general, but even more in the highly radioactive repository environment. Radiolytic disintegration of $NaNO_3$-bearing waste may increase the bioavailability of corrosive nitrate- and nitrite species. Compared to the natural radioactivity of the bedrock, repository is a hot spot emitting gamma radiation. In natural radioactive decay, gamma radiation is very weak and geochemically less important than the particle emission.

In case of radionuclide release from the repository, transport and retardation processes in the host rock become important. Many of the long-lived nuclides are effectively retarded and accumulated by biomass, both by reduction and precipitation and by binding in strong complexes with the some functional groups of biomass. However, there are some radioisotopes (e.g. C, Se) that are involved in metabolic processes. Understanding their transport behaviour requires solid understanding on the microbiology in terms of metabolism and overall activity.

Project MIND deals with two different waste management categories: 1) intermediate-level waste with high content of organics. Variable waste forms, activity and package concepts are included, 2)

high-level waste, predominantly spent fuel, capsulated in metal canisters and finally embedded in bentonite buffer in the deposition hole. On the other hand, the project is multidisciplinary covering essential fields of microbiology, geochemistry and numerical modelling. Final aim of the project is to combine the information from these different starting points and to find a common process understanding on the biogeochemical- microbiological processes relevant for nuclear waste management.

Conclusions

The most important general constraints of deep life in continental rock systems are evidently energy and space. With respect to a HLW repository in crystalline rock, it is important to demonstrate the sealing capability of the buffer against the microbial activity. More precisely, if copper canister is used, prerequisites and limitations of microbial sulphate reduction need to be demonstrated both experimentally and in the light of theories and models. Iron-based canister materials may produce excess hydrogen into the environment. A common process understanding on the role of gases in the microbiological systems is a one the key questions. What are sources, fluxes and how critical component of they constitute in the microbial metabolism. Lessons learned in MIND work packages 1 and 2 will be integrated in work package 3 to provide a solid safety case knowledge base about the influence of microbial processes on geological disposal of radioactive wastes.

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MICROBIAL PROCESSES IN THE CHEMICALLY DISTURBED ZONE DURING THE GEODISPOSAL OF INTERMEDIATE LEVEL WASTE: THE NERC BIGRAD PROJECT

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Abstract

The BIGRAD project was a multidisciplinary NERC-funded consortium that investigated "BIogeochemical Gradients and RADionuclide transport" processes in the alkaline and reducing conditions, expected to develop around the cement based concept for the (ILW / LLW) disposal areas of a geological disposal facility (GDF). Steep biogeochemical gradients are expected to result at the interface with the surrounding host geology in a "chemically disturbed zone" (CDZ), and the chemical, physical and biological evolution of the CDZ has the potential to influence the transport of radionuclides from the near-field to the far-field. The BIGRAD consortium ran from 2010-2015 and has generated in excess of 50 peer-reviewed papers encompassing its three work-packages and 2 cross-cutting themes (Figure 1). This review discusses the outputs from cross-cutting theme 1 (Biogeochemical Processes), conducted in Manchester with collaborators in the National Nuclear Laboratory, British Geological Survey and the Diamond Light Source. Collectively these BIGRAD experiments suggest that microbial processes in the alkaline CDZ have the potential to act as a "biological buffer" that can help prevent migration of priority radionuclides from a GDF.

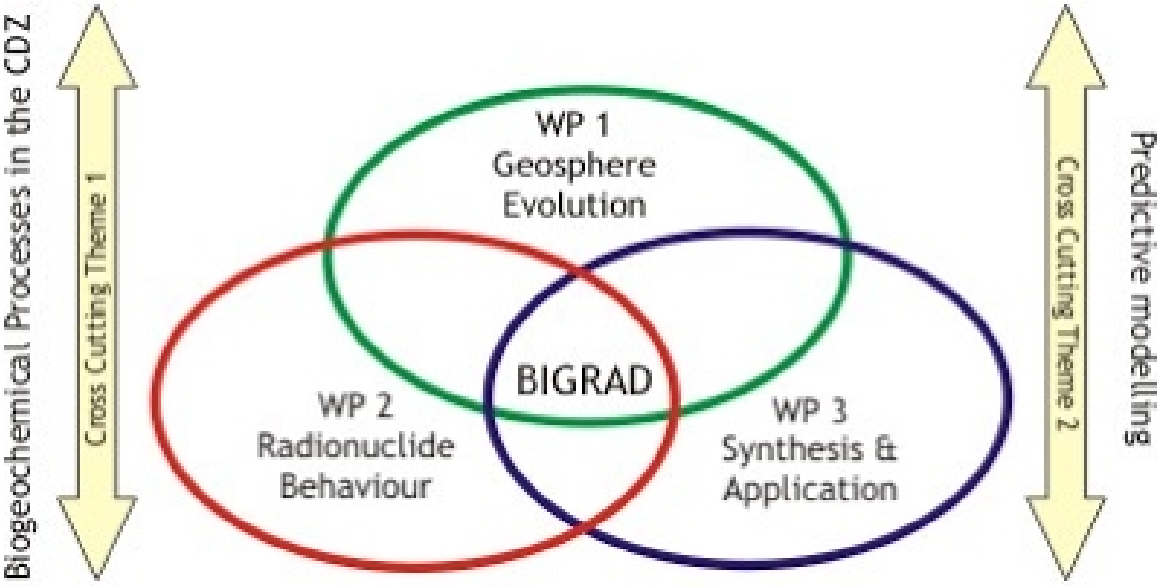

Figure 1: The BIGRAD consortium work packages and cross-cutting themes.

Microbiology of alkaline analogue sites

The microbial ecology of two alkaline analogue sites have been studied during BIGRAD, showing a rich diversity of microorganisms with the potential to alter the chemistry of the CDZ and influence the fate of priority radionuclides. The first study (Rizoulis et al, 2014) focused on Allas Springs (Troodos mountains, Cyprus), an ophiolitic serpentinisation active site that has been proposed as a natural analogue for cementitious radioactive waste repositories (Alexander and Milodowski, 2011). Samples (pH 9.4 to 11.7) were collected from a prominent (up to 4 m high) harzbergite outcrop and included rock, water and apparent biofilm/microbial mat samples. DNA was isolated from the samples, prior to 16S rRNA gene amplification followed by cloning and Sanger sequencing. The 16S rRNA sequencing identified phylogenetically diverse microbial communities in all samples, including *Hydrogenophaga* species. This is important as it indicates that alkali-tolerant hydrogen-oxidizing microorganisms could potentially colonize an alkaline geological repository, and metabolise molecular H_2, produced as a result of processes including steel corrosion and cellulose biodegradation within the wastes. Microbial metal reduction was also confirmed at alkaline pH in this study using enrichment microcosms and pure cultures of bacterial isolates affiliated to the *Paenibacillus* and *Alkaliphilus* genera. Overall, these data suggest that a diverse range of microbiological processes can occur in high pH environments, consistent with those expected during the geodisposal of cementitious intermediate level waste. Many of these, including gas metabolism and metal reduction, have clear implications for the long-term geological disposal of ILW.

The focus of most of the microbial work in BIGRAD was the Harpur Hill analogue site in Derbyshire, UK. Here a hyperalkaline plume discharging from industrial lime waste has deposited a substantial hyperalkaline deposit that overlies organic and clay-rich soils and Quaternary residual valley floor sediments (alluvium and possibly till). Studies at this site have indicated that the pH of the pore waters at depth (>1 m) within the hyperalkaline lagoon are stable (~pH12.5) and remain constant regardless of highly variable pH variations in the surface waters. Microbial diversity across the site has been surveyed and published in Smith et al. (2016). Microbial cell counts showed an inverse relationship to pH across water samples ranging from pH 7.5 to pH 13 collected on site (Figure 2), while high throughput DNA sequencing showed a rich diversity of microorganisms, including organisms affiliated with hydrogen-metabolising *Hydrogenophaga species* detected at the Cyprus site. At the fringes of the high pH plume, the interface with organic rich soil has provided a natural enrichment of alkaliphilic microorganisms that has been used as an inoculum for a series of BIGRAD studies aiming to define the upper pH limits for microbial life, and the impact of microbial metabolism on geochemistry, mineralogy and hydraulic properties of soils and sediments relevant to the CDZ.

The study of Rizoulis et al (2012) used pH 11.2 sediments from the margins of the Harpur Hill site to inoculate laboratory microcosms that were incubated anaerobically at a range of alkaline pH values with added electron donors and electron acceptors. These experiments were designed to identify the pH limits for nitrate, Fe(III) and sulfate reduction. There was a clear succession in the utilisation of electron acceptors (in the order of nitrate > Fe(III)-citrate > Fe(III) oxyhydroxide > sulfate) over the 10 week incubation period (Figure 3), in accordance with calculated free energy yields and Eh values over the pH range 10-12. The rate and extent of "bioreduction" decreased at higher pH, with an upper limit for the processes studied at pH 12. The reduction of Fe(III) oxyhydroxide and sulfate over the time period monitored (10 weeks) was very slow, and would need to be monitored for longer periods of time that are of more relevance to GDF timescales. Nevertheless these studies confirm that anaerobic microbial processes can proceed at moderately alkaline pH values, consistent with evolved cementitious systems, or in any lower pH microniches that may be present in heterogenous

ILW. Follow up studies (Williamson et al., 2013) focused on the fate of Fe(III) oxyhydroxides in microbially active reducing systems, again using a Harpur Hill pH 11.8 inoculum as a source of metal-reducing bacteria. The microcosms were incubated for 28 days and held at pH 10. In the presence of added electron donor (lactate) the Fe(III) mineral was enzymatically transformed to Fe(II)-bearing magnetite. The organisms that dominated in stable re-subcultures were novel Gram-positive bacteria affiliated with the Clostridia. The microbial reduction of amorphous Fe(III) oxyhydroxides to magnetite by lactate, a surrogate for cellulose degradation products (CDPs) that will accumulate within ILW, was considered significant, as it is a potent reductant for Tc(VII), Np(V) and other redox active priority radionuclides.

High pH radionuclide biogeochemistry

Early on in the BIGRAD studies it became obvious that there was a rich diversity of microorganisms growing in the soils and sediments of the alkaline analogue field sites selected for study, and many were capable of anaerobic metabolism (including metal reduction) when stimulated with simple carbon sources representative of CDPs. The impact of such forms of metabolism on radionuclide speciation and fate was a major focus within the biogeochemistry "cross-cutting" theme of BIGRAD. These studies addressed the impact of anaerobic metabolism on U(VI), Np(V) and Tc(VII). Additional studies were initiated to determine if the CDP isosaccharinic acid (ISA), a strong complexant of a range of radionuclides, could also be degraded under anaerobic conditions.

Multiple studies by the Manchester group (and many others working in the field) have demonstrated that neutraphilic bacteria are able to reduce soluble U(VI) to insoluble U(IV) at circumneutral pH (see Newsome et al. 2014 for examples). Williamson et al. (2014) showed that alkaliphilic bacteria from the Harpur Hill site were also able to catalyse U(VI) reduction in microcosms maintained at pH 10.5 when stimulated with electron donor (lactate), and incubated in the presence and absence of added Fe(III) as ferrihydrite. The reduction mechanism in these experiments was confirmed to be enzymatic, requiring live microbial biomass; autoclaved post reduction sediments were unable to mediate indirect U(VI) reduction via Fe(II) that had accumulated. 16S rRNA gene pyrosequencing again showed that Gram-positive dominated in the post reduction sediments. The precise enzymatic mechanism of U(VI) reduction by Gram-positive bacteria is poorly understood; previous studies have focused on Gram-negative bacteria at pH 7 (Newsome et al., 2014).

Np(V) is also susceptible to "bioreduction" at circumneutral pH, and BIGRAD experiments also addressed the fate of this priority radionuclide in high pH microcosms (Williamson et al., 2015). The conditions tested in the uranium experiments above were repeated, with low levels of Np(V) (20 Bq ml–1; 3.3 μM) to probe microbial impacts on solubility, whilst parallel higher concentration systems (2.5 KBq ml–1; 414 μM) facilitated X-ray absorption spectroscopy to study the fate of the neptunium. In these studies, Np(V) bioreduction was shown to be indirect, mediated by biogenic Fe(II) produced by the alkali-tolerant, Gram-positive bacteria present in the sediments. Parallel studies on Tc(VII) (manuscript in submission) have highlighted similar biogenic Fe(II)-mediated processes that result in the reductive precipitation of Tc(IV). Other BIGRAD-related studies have extended this work to other redox active radionuclides that may be susceptible to bioreduction via direct and indirect mechanisms, including iodine and selenium.

Microbial metabolism of cellulose and its degradation products

Finally, a productive new area of research was also initiated, that focused on the biodegradation of organic chelating agents associated with the alkaline hydrolysis of cellulose within ILW. Initial studies focused on the microbial degradation of ISA by enrichment cultures obtained from Harpur Hill inocula (Bassil et al., 2014). A wide range of cultures were able to degrade ISA at pH 10 and couple this process to the reduction of electron acceptors that will dominate as the GDF progresses from an

aerobic 'open phase' through nitrate- and Fe(III)-reducing conditions post closure. Furthermore, pyrosequencing analyses showed that bacterial diversity declined as the reduction potential of the electron acceptor decreased and that more specialised organisms dominated under anaerobic conditions (Figure 6). Examples of organisms responsible for these processes were isolated, and have been characterized in detail, and their impact on radionuclide speciation assessed (Bassil PhD thesis, 2015). Complementary long-term experiments were also conducted at higher pH values, in sealed flasks containing autoclaved tissue and cotton wool incubated in a saturated solution of $Ca(OH)_2$ (pH>12) (Bassil et al., 2015). These experiments confirmed previous reports that ISA is produced from cellulose polymers at high pH. However, when a small aliquot of Harpur Hill sediments was inoculated into these experiments, the CDP profiles that developed over the 30 month experiment were very different, with microbial activity implicated in the enzymatic hydrolysis of cellulose and the subsequent production of acetate. This in turn led to acidification of the microcosms and a marked decrease in ISA production from the abiotic alkali hydrolysis of cellulose. DNA analyses of microbial communities present in the microcosms further support the hypothesis that bacterial activities can have a controlling influence on the formation of organic acids, including ISA, via an interplay between direct and indirect mechanisms. These and the other results described here suggest strongly that microorganisms could have a role in attenuating the mobility of some radionuclides in and around a geological disposal facility, via either the direct biodegradation of ISA or by catalysing cellulose fermentation and therefore preventing the formation of ISA. These studies have also been extended to look at the metabolism of ISA by organisms growing at circumneutral pH values, more representative of the geosphere surrounding a GDF. In the first paper from this study (Kuippers et al., 2015), data were presented confirming the metabolism of ISA under these conditions, including the direct oxidation of the substrate under aerobic and nitrate-reducing conditions and the fermentation of ISA to acetate, propionate and butyrate prior to utilization of these acids during Fe(III) and sulfate reduction. Methane production was also quantified in these experiments, and the added electron acceptors were shown to play a significant role in minimizing methanogenesis from ISA and its breakdown products.

Conclusions

Collectively these BIGRAD experiments suggest that microbial processes in the alkaline CDZ have the potential to act as a "biological buffer" that can help prevent migration of priority radionuclides from a GDF

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PERFORMANCE ASSESSMENT IN BELGIUM AND THE POTENTIAL ROLE OF MICROBES: CASE STUDY FOR EUROBITUM DISPOSAL

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Abstract

In the near field of a radioactive waste repository, there may be opportunities for microbial life during the operational phase and for some time after repository closure. In case of geological disposal of Eurobitum bituminised waste in Boom Clay, there are possible effects due to nitrate reduction and oxidation of bitumen degradation products. After nitrate depletion (or in nitrate-free niches) secondary effects may occur due to sulphate reduction and/or methane production. It is believed that these microbial effects play a minor role compared to chemical perturbations induced by this waste and its barriers, mostly due to time and space restrictions. Nevertheless, substantial uncertainties remain concerning the persistence and potential role of microorganisms in repository conditions. Conservative hypotheses in performance assessment allow to deal with these uncertainties and to treat the direct or indirect impact of these processes on radionuclide release or mobility.

Introduction

In Belgium, geological disposal in a stable clay layer is considered as the reference solution for the long-term management of high and intermediate level radioactive waste. The safety approach relies on the defense-in-depth principle, consisting of a series of complementary and independent levels of protection. In a geological repository, multiple barriers, both engineered and natural, are foreseen between the waste and the biosphere. These barriers fulfil multiple safety functions, identified as follows: *(i)* isolation, provided by the host formation and the geological overburden, *(ii)* engineered containment, provided by the engineered barrier system (EBS) in case of heat-emitting waste, and *(iii)* delay and attenuation, provided by cement barriers and the clay host formation [1].

Currently, the Belgian waste management organisation ONDRAF/NIRAS is preparing its first Safety and Feasibility Case (SFC1), which is a decision-oriented document demonstrating the long-term safety of its deep disposal concept for Category B (long-lived) and C (heat-emitting) wastes in a poorly indurated clay formation [2]. Safety and performance assessments form a central element of the safety case. Performance assessments focus on the ability of the selected site and/or disposal design to fulfil their safety functions, while safety assessments evaluate the safety of the integrated disposal system by comparison with regulatory criteria. The safety assessment methodology adopted by ONDRAF/NIRAS distinguishes between a phase of preparatory safety assessment (PSA) calculations and the formal safety assessment (FSA) [3]. In the course of 2009-2012, SCK•CEN carried out performance assessment (PA) calculations for the reference scenario considering disposal in Boom Clay for the main waste families in Belgium, *i.e.* spent UOX and MOX fuel[1], vitrified high-level

[1] Currently, spent fuel is not declared waste. However, methodological studies on radioactive waste disposal consider both reprocessing and direct disposal options.

waste, compacted waste and bituminised waste [4]. These calculations take into account waste-specific radionuclide release mechanisms, characteristic radionuclide migration through engineered and natural barriers including precipitation, sorption and complexation processes, dilution in the aquifers surrounding the clay layer, and several biosphere pathways.

In recent years, the need to elucidate the impact of possible microbial processes in geological disposal systems is being acknowledged. There is general consensus that the overall potential for microbial activity in undisturbed Boom Clay is low due to its highly consolidated and low energy nature. However, especially in the period after waste emplacement and in the early post-closure phase, there may be opportunities for microbial life. Borehole water samples from different layers within the Boom Clay have shown to comprise a highly diverse bacterial community of which a large fraction is active [5]. The latter study shows that, although at least part of this community is probably introduced during the installation of the piezometers, the community can survive and be metabolically active.

The potential for microbial activity is the most prominent for organic waste forms, because of the presence of a variety of possible electron donors and acceptors. In Belgium, there is a substantial inventory of bituminised waste, of which about 80% is conditioned in a hard or "blown" bitumen matrix, known as Eurobitum waste. Eurobitum consists of ~60 wt% bitumen (Mexphalt R85/40) and ~40 wt% waste, mainly consisting of $NaNO_3$ (20-30 wt%) and $CaSO_4$ (4-6 wt%) salts. Radionuclides only make up 0.4-1.15 wt% of the conditioned waste form [6,7,8]. In repository conditions, the following microbial processes are identified as relevant: nitrate reduction, sulphate reduction, methane production, and oxidation of bitumen degradation products. This contribution discusses these processes, together with their potential impact on performance assessment. As a starting point, the most recent performance assessment calculations for the reference scenario of geological disposal of Eurobitum waste are briefly summarised. The description is not intended to be exhaustive, but sufficiently informative to get an understanding of the key assumptions in the assessment.

Performance assessment for EUROBITUM waste[2]

The integrated PA model consists of three compartment models, simulating radionuclide transport in respectively (i) the repository near field and clay, (ii) the aquifer, and (iii) the biosphere [4], as shown in Figure 1.

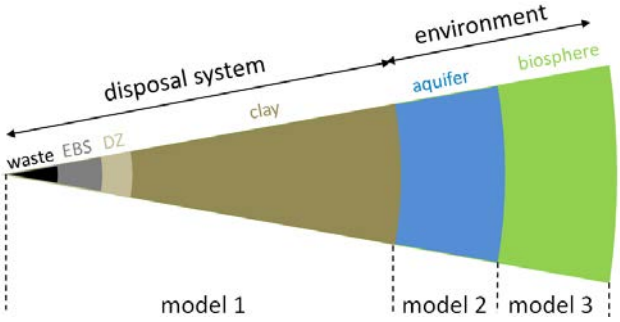

Figure 1: Compartment models used for Safety and Performance Assessment in Belgium. Model 1: near field and clay model, Model 2: aquifer transport model, Model 3: biosphere model.

Disposal concept

The radionuclide inventory was provided by ONDRAF/NIRAS, and considered maximum values for the "Eurobitum-1" production batch (~11,500 drums). Disposal was considered to occur in 2040 at the

[2] The PA calculations for EUROBITUM waste (Weetjens, 2015) were performed in the context of the PSA phase, meaning that they served as a basis for further discussion and were not part of a formal assessment.

earliest. The primary waste packages are emplaced in a prefabricated concrete container after which the voids are filled with mortar and the container will be closed with a concrete lid (Figure 2). This shielded waste package entity is referred to as a monolith-B. The number of primary waste packages inside is limited by osmosis-induced water uptake and swelling, which is not allowed to threaten the mechanical integrity of the Boom Clay. The monoliths will be placed in horizontal galleries lined with concrete wedge blocks and with an internal diameter of 3 m. The disposal galleries have a length of 1 km, and a spacing of 50 m. As a working hypothesis, the repository is assumed to be situated at a depth of about 240 m, at the centre of the Boom Clay layer. The Boom Clay thickness considered in the performance assessment is 90 m, representing the most homogeneous low-permeable part of Boom Clay between 191 m and 281 m BDT[3].

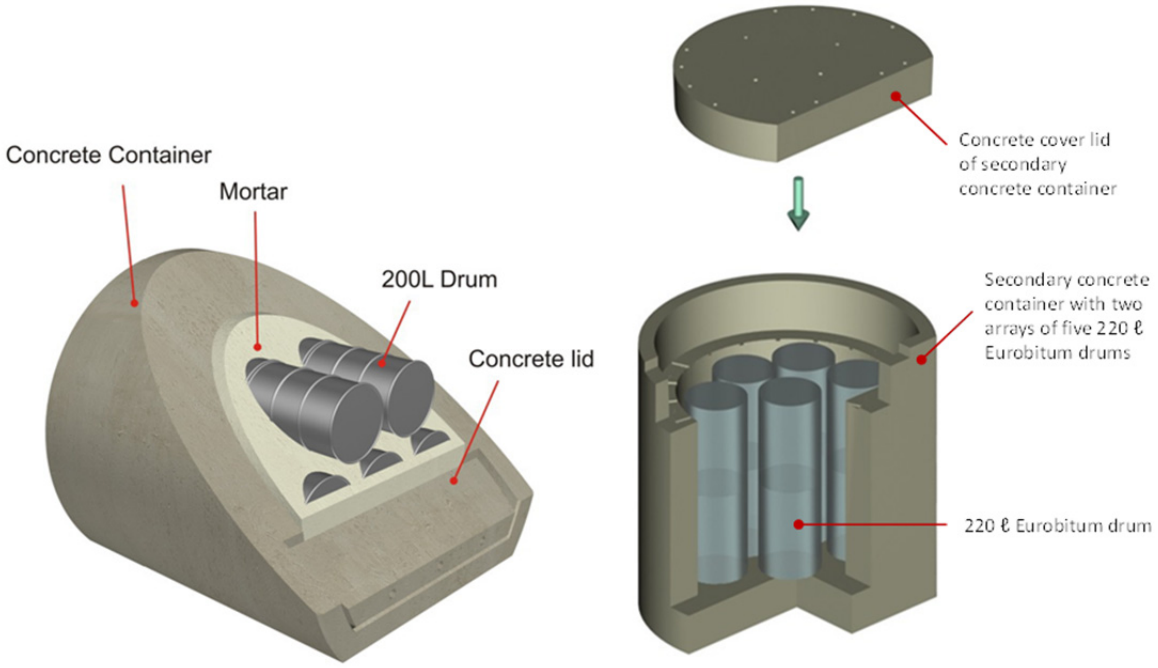

Figure 2: Waste package design for the disposal of Eurobitum bituminised waste: concrete monolith-B with ten 220 ℓ drums.

Radionuclide release and transport

Radionuclides are assumed to be congruently released with salt leaching, although there is qualitative experimental evidence that radionuclide release is much slower, which is attributed to sorption on bitumen and on metallic components [9]. Extrapolation of leaching test results indicates a total salts leaching time between a few hundred to a few thousand years. The uncertainty on radionuclide release mechanisms and timescale has been taken into account by applying a conservative constant release rate of 10^{-3} a^{-1} for both the early free swelling phase and the subsequent restricted swelling phase [10]. After release, radionuclides will diffuse through concrete barriers and the Boom Clay layer towards the Neogene aquifer. With the exception of a disturbed zone around the repository, sorption on clay minerals, complexation by dissolved organic matter and precipitation processes are implemented in the clay zone. Solubility limitations are also implemented at the waste-concrete interface.

Considered biosphere pathways

In the biosphere model, water is extracted from a draining river (river pathway) or a well in the Neogene aquifer located at an adverse location with respect to the contamination plume (well

[3] Below Drilling Table

pathway). This water is used as drinking water for humans and cattle, and irrigation of crop fields and pastures. The model considers exposure through ingestion of water and contaminated foods, inhalation of resuspended dust and radon, and external irradiation. Three age groups (infants, children and adults) are considered for estimating the dose impact, as recommended by ICRP publication 101 [11].

Results

Performance assessment results are expressed as a set of complementary safety and performance indicators, where safety indicators aim to allow statements on repository safety through comparison with appropriate reference values, and performance indicators aim to explain the functioning of the repository system by quantifying the contribution of its main barriers or safety functions. The main safety indicator is the annual effective dose, which is to be compared to a regulatory dose constraint. Figure 3 presents the annual effective dose to infants for the well pathway in the hypothetical case of Eurobitum disposal in Boom Clay [10]. The plot shows a typical bimodal shape, for which the first peak is due to mobile fission and activation products, such as ^{79}Se, ^{94}Nb, and ^{129}I, and a second peak due to actinides, which are confined much longer in the disposal system and are only released to the environment after 1 Ma due their low solubility and mobility. Note that for ^{79}Se, both oxidised (Se(VI)) and reduced forms (Se(0), Se(-II)), with different migration characteristics are considered possible in Boom Clay redox conditions, and both variants are taken into account in the PA calculation. After 1 Ma, ^{226}Ra, a progeny of the ^{238}U decay chain and including the radiobiological effects of ^{222}Rn, determines the dose rate. The overall dose rate remains 2 to 3 orders of magnitude below the dose constraint, which is set to 0.1 mSv/a by the Belgian regulatory body FANC.

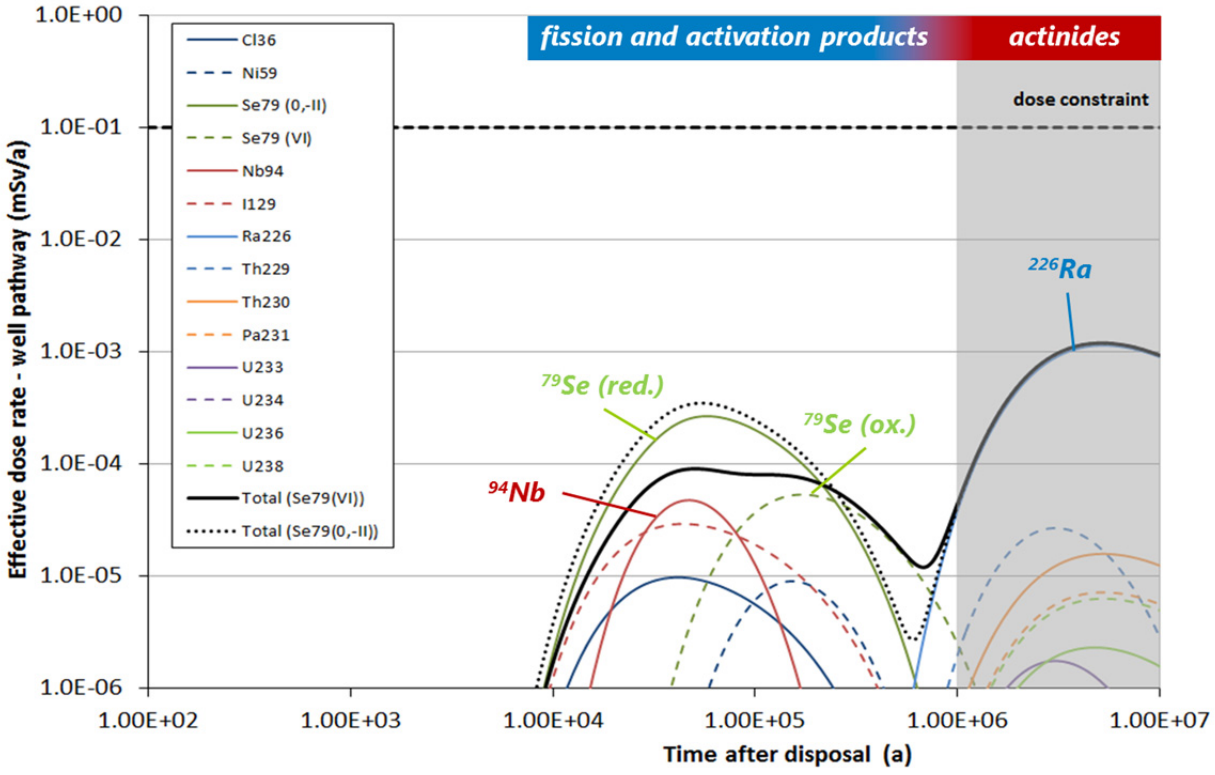

Figure 3: Hypothetical annual effective dose (age group: infants) for the well pathway due to disposal of Eurobitum waste in Boom Clay. The assessment period is restricted to 1 Ma, afterwards geological confinement becomes highly speculative and the results (grey zone) are no longer considered part of the assessment.

Abstraction of microbial processes in PA

As mentioned above, the overall potential for microbial activity in undisturbed Boom Clay is considered low, mostly due to its highly consolidated and low energy nature. Upon excavation and waste disposal, both opportunities and inhibitory factors arise, shifting this presumably dormant community towards a speculative state of heightened activity at first, due to increased space and water availability, oxygen penetration, and most importantly, due to the introduction of viable, allochtonous microbes. After waste emplacement, and backfilling and closure of the disposal galleries, rapid oxygen consumption and near extermination of the near-field microbial community is expected due to the nature of the EBS and radioactive waste (*e.g.* high pH), and the sealing of any voids. Nevertheless, even this short period of presumed microbial activity could change the initial state of the repository or some other hypotheses on which the long term safety assessment rests. The table below summarises the potential microbial effects in case of Eurobitum disposal and the way these effects are abstracted in performance assessment, *i.e.* usually through conservative hypotheses concerning their impact on radionuclide release and transport.

Table 1: Abstraction of microbial processes into performance assessment for Eurobitum waste disposal in Boom Clay.

Microbial process	Potential effects	Impact / PA abstraction
Nitrate reduction (NRP)	Redox shift in near field	RN speciation & mobility (*e.g.* ^{79}Se)
	Presence of NH_4^+	Ligand, solubility ↑ (*e.g.* Ni, Pd)
	Generation of nitrous gases $NO_3^- \rightarrow NO_2^- \rightarrow NO \rightarrow \mathbf{N_2O \rightarrow N_2}$ Gas volume increase, or decrease if according to following reaction: $5\ H_2 + 2\ NO_3^- + 2H^+ \Rightarrow N_2 + 6\ H_2O$	- Supporting calculations performed - Conservative thickness DZ considered - Pressure decrease conservatively neglected
Sulphate reduction (SRP)	Generation of corrosion-aggressive species $S_2O_3^{2-}$, HS^-, S^{2-} in DZ	Safety concept for Eurobitum disposal does not consider a watertight carbon steel overpack
Methanogenesis	Gas volume decrease according to following stoechiometry: $CO_2 + 4\ H_2 \leftrightarrow CH_4 + 2\ H_2O$	- Expected only after depletion of nitrate - Pressure decrease conservatively neglected
Microbial degradation of bitumen and of its chemical and radiolytic degradation products	Generation of small soluble organic molecules	- Negligible compared to (high concentration of) DOM within Boom Clay - Complexation with DOM explicitly taken into account

Activity of nitrate reducing prokaryotes (NRP) could have several effects:

- Changes in redox potential could increase the uncertainty on the speciation of some radionuclides. This can explicitly be taken into account in the PA (e.g. ^{79}Se)
- In case NH_4^+ is produced due to (microbially mediated) direct/dissimilative nitrate reduction to ammonium (DNRA) and/or due to interaction with steel [12], this can act as a ligand and increase the solubility of some radionuclides. A significant effect could be expected for Ni and Pd [13]. The PA considered a variant case with a four orders of magnitude higher solubility, yet without any effect on the long-term radiological impact.
- Microbial denitrification may lead to the production of nitrous gases. NO is toxic for microbes, so it is not expected to accumulate, but it will rather convert into N_2O and N_2 [14]. Supporting calculations have been performed to assess the risk of gas pressure development. Besides, it is highly likely that

hydrogen is consumed in the denitrification process, leading to an overall gas pressure decrease with factor 5. These beneficial effects are usually neglected in PA.

In low nitrate concentrations, sulphate reducing prokaryotes (SRP) could convert sulphate, present in the waste, but also in the Boom Clay oxidized zone due to pyrite oxidation, into thiosulphates or sulphides. These aggressive species could induce localised metal corrosion, a specific concern for disposal of heat-emitting waste because of the presence of high performance steel barriers (*i.e.* watertight overpack). Since there is no containment safety function (in the sense of watertightness) defined in the safety concept for Eurobitum waste, this process is considered not relevant.

In low nitrate concentrations, methanogenic bacteria may produce CH_4 from CO_2 and H_2 potentially present in the repository near field. Again, this will reduce the overall gas pressure by factor 4 due to stoechiometry, but the effect is conservatively neglected in PA.

Chemical and radiolytic degradation products of bitumen could serve as carbon source and/or electron donor for microbes, thereby potentially generating small soluble organics which could enhance radionuclide migration by complexation. The additional fraction produced through microbial activity is considered negligible compared to the relatively high content of dissolved natural organic matter (DOM) in Boom Clay. Complexation to Boom Clay DOM is explicitly taken into account in PA.

Conclusions

This contribution explained the key assumptions in the performance assessment for Eurobitum waste disposal in Boom Clay and the abstraction of potentially relevant microbial processes and associated uncertainties in the assessment through appropriate model parametrisation and/or conservative hypotheses. This approach suggests that no adverse long-term effects are to be expected, and that some processes might even have a beneficial impact on repository safety.

Acknowledgement

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EDUCATION AND TRAINING
A BASIC COURSE IN MICROBIOLOGY AND NUCLEAR WASTE DISPOSAL

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Abstract

Here we present motivation for a basic course in microbiology and a scientific background to why microbiology is important in the disposal of radioactive wastes. This course in microbiology will be announced to a wide target of the professional community such as geologists, chemists, hydrologists, engineers, nuclear material specialists, safety case specialists and modelers.

Introduction

Education and training (E&T) initiatives are foreseen in MIND order to raise awareness of the relevance of microbial issues in otherwise typically abiotic fields of expertise, and to dissipate the knowledge gained in the MIND project to students and professionals within and beyond the known geomicrobiology expert circles. To bring the scientific findings of the MIND project directly available to young students, course modules will be developed that can be delivered as stand-alone courses or that can be integrated in an already existing academic program. In addition, training modules will be developed for professionals active in the nuclear and/or waste management area. These professionals will benefit from the MIND findings in their daily practice. The overall aim of both the education and training initiatives is to cultivate awareness of the relevance of microbial issues in otherwise typically abiotic fields of expertise, and dissipate the knowledge gained in the MIND project beyond the known geomicrobiology expert circles.

In order to bring the scientific findings of this project directly available to young students, course modules will be developed that can be delivered as stand-alone courses or that can be integrated in an already existing academic program. Relevant networks, groups and programs dealing with related education (such as PETRUS III, CMET, ENEN Association, European Master in Radiation Biology, European Master in Radiation Protection, etc.) will be contacted in order to explore possibilities for efficient collaboration, and to ensure complementarity to already existing courses. These modules will be developed according to the Bologna principles. ECTS (European Credit Transfer and Accumulation System, a tool that helps to design, describe, and deliver study programs and award higher education qualifications) credits will be allocated to the courses, that will hold theoretical classes and where feasible, field excursions.

In addition, training modules will be developed for professionals active in the nuclear and/or waste management area. These professionals will benefit from the MIND findings in their daily practice. Integration of this (these) module(s) in their lifelong learning pathway broadens their perspective and increases their overall competences. This is fully in line with the aims of the Copenhagen process. According to the European framework, all course content will be developed following the state of the art of the science and the didactic means. Course content will be adapted to the target audience, an EQF (European Qualification Framework) level will be determined and learning outcomes will be developed according to the ECVET (European Credit system for Vocational Education and Training) principles. Here we present motivation for a basic course in microbiology and a scientific background to why microbiology is important in the disposal of radioactive wastes.

Motivation for the course

Microbial processes

Biological life processes should be separated from chemical inorganic processes. This is because most life processes are running along biochemical process pathways that are very different from those of inorganic chemical processes. Many chemical processes are restricted by various reaction barriers e.g. the reduction process of sulphate to sulphide [8, 17]. They are, therefore, very slow or not possible at all under conditions typical for repositories for interim storage and long-term storage of radioactive wastes. Biological processes, consequently, include many reactions that do not occur in sterile lifeless chemical systems. This is because life has the ability to over-run activation energy barriers and other energetic circumstances that block spontaneous chemical reactions. Life is possible from −20 ºC up to around 113 ºC, where all life processes stop. Life is also possible within a large pH range, from pH 1 up to at least pH 12.5 [43, 54]. Finally, many processes overlap biology and chemistry such as sulphide and ferrous iron oxidation which occur both as chemical and biological processes. Life processes in radioactive waste interim and long-term repositories will almost exclusively be under the control of microorganisms.

Microbial processes comprise red-ox reactions with decomposition and the production of organic molecules with different electron donors, energy sources and electron acceptors. Organic carbon in wastes, buffer materials and reduced inorganic molecules such as H_2 from anaerobic corrosion processes and methane from geological sources are possible electron donors and energy sources for microbial processes. During the microbial oxidation of these energy sources, microorganisms preferentially reduce electron acceptors in a particular order. First O_2, and thereafter nitrate, manganese(IV), ferric iron, sulphate, sulphur and carbon dioxide are reduced. Simultaneously, fermentative processes lower pH and supply the metabolizing microorganisms with, for example, H_2, methane and short-chain organic acids such as acetate. Fermentation, in contrast to respiration, does not require an external electron acceptor, the oxidation-reduction process comprises rearrangement of electrons in exogenic modes, thereby releasing energy for life processes.

It is well known that microbes can mobilise trace elements [Ref. 2 and references therein]. Firstly, unattached microbes may act as large colloids, transporting radionuclides on their cell surfaces with the groundwater flow. Secondly, microbes are known to produce ligands that can mobilise soluble trace elements and that can inhibit trace element sorption to solid phases. Thirdly, viruses are commonly present in large numbers in groundwater and most of them are the size of colloids and their proteinaceous shells readily sorb trace elements [28, 29]. Fourhtly, a large group of microbes catalyse the formation of iron oxides from dissolved ferrous iron in groundwater that reaches an oxidising environment [5]. Biofilms in aquifers will influence the retention processes of radionuclides in groundwater [1].

Radioactive waste repositories will not be sterile, and microbial processes will occur at rates determined by the prevailing chemical and physical conditions of the respective repository (Figure 1). Low and intermediate waste repositories (LIWR) will contain large amounts of organic material that can be degraded by microorganisms with formation of gas and compounds that may mobilise radionuclides. Interim storage in pools with pure water must be applied to cool down high level radioactive wastes (HLRW) before final disposal. Some microorganisms have adapted to live at very low concentrations of organic carbon (<2-3 μg organic carbon per litre). These microbes often live attached in biofilms and they increase the risk for clogging, accumulation of radionuclides on surfaces and biocorrosion of stainless steel. In deep repositories for HLRW, microorganisms in groundwater and buffer materials can produce sulphide and other substances that are corrosive to metal canisters. Some microbes may mobilise radionuclides. Below follows a brief compilation of various aspects regarding microbial processes than must be addressed in safety cases.

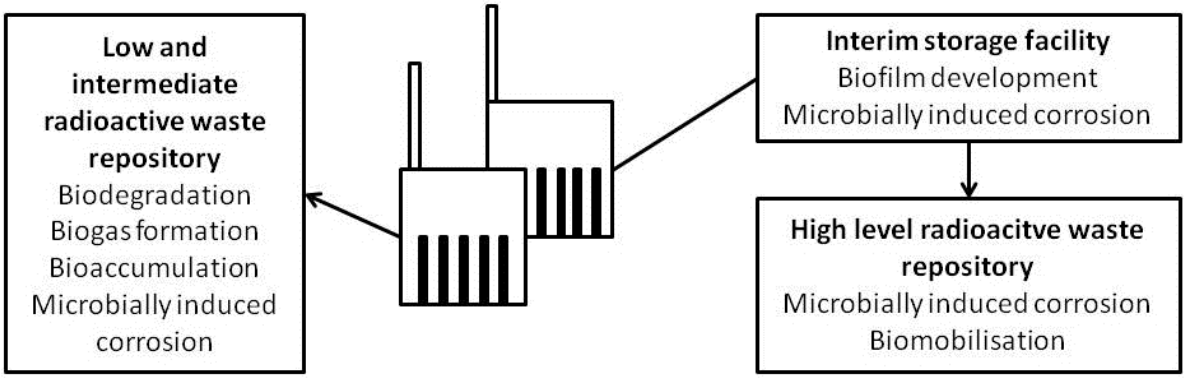

Figure 1. Major microbiological processes that can occur in radioactive waste storages and repositories.

Interim storage facilities

Growth of microorganisms are often observed in interim storage facilities (ISF) [30, 58]. Microbial growth in ISF is a concern for ion exchanger and rod filter clogging. Biofilm can also bind radioactive substances and particles, thereby accumulating radioactivity at positions in the systems supposed to be radiation free. Biofilms are known to increase the risk for microbially induced corrosion (MIC) which eventually may damage stainless steel construction in storage pools and tubes for circulation of cooling water.

Low and intermediate radioactive waste repositories

The pH in low and intermediate radioactive waste repositories (LIRW) will decrease over time [52]. Therefore, the influence of microbial processes will increase in magnitude concomitantly. In an aerobic repository environment microbial degradation of plastic polymers via de-polymerization will occur. The initial degradation is mediated by de-polymerases which break down the long polymer into oligo-, di- and monomers [Ref. 20 and references therein]. The closer to a natural polymer the structure of the polymer is, the faster the microbial degradation proceeds. The aerobic degradation end products are carbon dioxide and water. Microbial degradation of polymers occurs in anaerobic environments as well. The degradation products in anaerobic processes are organic acids, carbon dioxide, methane and water [Ref. 20 and references therein]. The degradation of polymers is often a slow process but initially when O_2 is present microbial degradation will produce smaller units that can be utilized in anaerobic microbial degradation processes. Polyethene is degraded by lignin degrading white rot fungi by the action of a manganese peroxidase [23]. Especially in nutrient limited conditions the elongation capacity and tensile strength of the polyethylene were found drastically decreased by degradation of the fungi tested.

Bitumen is a complex colloidal system consisting of a mixture of mainly high molecular aliphatic and aromatic hydrocarbons. It consists mainly of four different compound groups; saturated hydrocarbons, cyclic hydrocarbons, resins and dispersed particles named asphaltenes. Roffey & Nordqvist [49] concluded that biofilm formation on bitumen occurred both under aerobic and anaerobic conditions. A pH of 9.8 did not inhibit growth on bitumen by aerobic microorganisms. Degradation studies of bitumen have shown that parts of the hydrocarbons in bitumen are biodegradable. Potter and Duval [45] could measure a 50 % decrease in the aromatic and aliphatic fractions in a bitumen-based fuel sample (trade-name Orimulsion). The bitumen had a large surface area, glass beads were covered with the substance, and the degradation was aerobic. Factors that affect the degradation rate are surface area, temperature, availability of additional nutrients like nitrogen and phosphorous and of course the access to O_2. Bitumen mixed with waste will have an uptake of water. This happens both when bitumen is immersed in water and placed in humid air. Tests showed that samples with a mixture of bitumen, salt and sludge particles swelled about 10 – 15 % in one year and samples with radioactive waste swelled to twice its original volume. Other test

showed no difference between non-radioactive and radioactive waste samples [44]. Water in the bitumen-waste mixture will increase the possibility for microbial degradation of the hydrocarbons in the bitumen concomitant with degradation of the waste product, because water is needed for microbial processes to proceed.

Cellulose is a compound that is easily degraded by microbial processes. It is also chemically degraded under alkaline conditions [16, 55, 56] to compounds that can be further degraded by microbial processes [6]. There will be some moisture in the material which will facilitate heterotrophic degradation by mould and bacteria especially as long as there is O_2 present in the repository. During aerobic respiration water is formed which enhances further degradation of the cellulose material. When O_2 has been consumed, fermenting processes are likely to start [6]. These processes acidifies the environment by production of acids like acetic, citric, oxalic, together with carbon dioxide. In addition, anaerobic respiration processes can occur such as nitrate-reduction, iron-reduction and sulphate-reduction depending on how the ion-exchange resin was prepared, for instance with Na-salts like nitrate or sulphate. Lignin is one part of the carbohydrates in wood. This compound is mostly degraded by fungi named “white rot”.

The ion-exchangers used in nuclear power plants are usually strongly acidic with styrene resin. One type of ion-exchange is Amberlite IR-120 which is a strong acidic ion-exchanger with sulphonic acid groups on a styrene resin [44]. Styrene (vinyl-benzene) is a polymer and it is a natural component in plants. It is aerobically degradable by different types of bacteria [19, 36, 37, 50, 51]. It has also been shown to be degraded by an anaerobic consortium of microorganisms [19]. Ion exchange resins can swell up to 200% in water [53]. The ionic state of the resin affects the swelling capacity, H^+ and OH^- results in the largest swelling. Resins are therefore treated with sodium sulphate to reduce the swelling. Glass-wool and mineral-wool are examples of insulation materials that are deposited in LILW repositories. During the production of the insulation phenol-plastic (Bakelite) is used as binding agent. From this addition phenol and formaldehyde is produced in the insulation products. These compounds are organics that can be degraded by microorganisms both aerobically and anaerobically [15].

Microbial growth is possible in a waste matrix that has been solidified with cement or concrete. The main microbial process that can degrade concrete is acid production by microorganisms. Concrete degradation by microorganisms is a well-documented problem in sewage pipes made of concrete and it has also been found in LIRW [18]. Because of the high load of organic material in many LILWs, the O_2 in the water is rapidly depleted by aerobic microorganisms and anaerobic degradation by sulphate-reducing bacteria (SRB) takes over with production of hydrogen sulphide. The hydrogen sulphide is oxidized if it contacts O_2 and sulfuric acid is formed. The hydrogen sulphide oxidation can also be anaerobic by sulfur-oxidizing bacteria using nitrate as electron acceptor [46]. This occurs at lake or sea sediment surfaces and i.e. by the sulfur-oxidizing bacteria *Beggiatoa* that is responsible for the oxidation of hydrogen sulphide in marine sediments.

There are studies performed on microbially induced degradation of concrete. Small et al. [52] investigated the biogeochemistry and gas production from a low and intermediate level waste in concrete and steel tanks. During the seven years of the experiment, the measured pH in the storage decreased from between 10 and 11 to 7.5 in the water above the concrete tanks used for storage of different kind of waste. In tanks with biodegradable material, the pH was as low as 5.5. Vincke et al. [57] studied the influence of different additives on the hydrogen sulphide oxidation and sulfuric acid production. In their study, in laboratory scale experiments, the pH decreased from 8 to 1 in microbially active systems. In sterile systems the pH did not change. Microorganisms capable to cause degradation of silicate materials were found in a LIRW that had been in operation for 15 – 45 years [18].

High level waste repositories

A large and diverse array of investigations and experiments has been conducted to increase the understanding of microbial processes in deep groundwater and HLRW repositories. Sampling procedures have been developed and thoroughly tested as have underground facilities for model studies [35, 38, 40]. The first important parameter to analyse in repository environments is biomass. Three different methods have been developed and found to correlate. Microscopic counts and biochemical analysis of adenosine-three-phosphate (ATP) agreed well [13]. The determination of cultivable microorganisms and ATP also agreed well when analysed [42]. Many different phenotypes of microorganisms have been found via cultivation [21, 22], including fungi [10, 48]. The influence of viruses on microbial processes has been identified as an important factor to include in model studies. In particular, they seem to have an important mitigation effect on sulphide production by SRB [12, 28, 40].

Microbial biofilms were found to significantly influence the sorption of radionuclides on glass and rock surfaces [1, 3]. In addition, it has been demonstrated that microbial iron oxidising biofilms are strong sorbents for trace elements [4, 5]. Microorganisms from deep groundwater produce complexing agents that mobilise radionuclides [11, 24]. Such complexing agents have a strong influence on radionuclide mobility. They can mobilise uranium [25, 26] and strongly bind curium [32], uranium [31] and neptunium [34]. Interactions between SRB and curium have also been identified [33]. Finally, it has been found that microorganisms can sorb radionuclides on their cell surfaces [41], thereby facilitating mobilisation.

H_2 is readily used by many different phylogenetic traits of anaerobic microorganisms, such as SRB [7], acetogens [9] and methanogens [14]. Of particular interest is the H_2 produced during the anaerobic corrosion of iron components in a repository, for example, the rock bolts installed to secure the tunnel from falling rocks. Such corrosion, evolving H_2, may lead to the local production of large amounts of sulphide [38-40]. In HLRW repositories with sulphate-rich groundwater there is a strong potential for microbial sulphate reduction to sulphide via two metabolic processes. First, in addition to the naturally occurring H_2 in groundwater, iron in water-filled deep underground repositories construction is bound to corrode anaerobically with the concomitant production of H_2 [47], which may induce SRB growth and sulphide production. Secondly, in the case where sulphate-rich groundwater mixes with deep methane-rich groundwater SRB growth and activity can be induced [40]. Regarding the anaerobic metabolism of methane, the situation is obscure. Although anaerobic oxidation of methane (AOM) with sulphate as final electron acceptor is a well-documented process, detailed information about the metabolic pathways of AOM awaits successful cultures [27]. The electron donor is likely to be methane, but more research is required before conclusions can be drawn regarding the detailed nature and extent of AOM processes in HLRW environments.

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WHAT'S ON YOUR MIND? FOLLOWING MICROBES, MICROBIOLOGISTS, AND MICROBIOLOGY INTO NUCLEAR WASTE MANAGEMENT

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Abstract

Geological disposal of radioactive waste (GD) is a challenging topic where social and technical aspects blend and where input from social sciences is required with regard to the design, implementation and post-construction management of the installation.

Within the MIND project WP3, "Communication, Integration and Dissemination", task 3.2 evaluates the impact of the inclusion of microbiology on expert conceptualization and public perception of geological disposal. It does so by addressing two main research queries: i) how radioactive waste disposal is (re)configured through the MIND project, i.e. through the inclusion/exclusion of microbial processes in the evaluation of safety for geological repositories and ii) how lay citizens / publics' risk perception of geological disposal changes depending on their knowledge about the host environment and microbial processes. Based on the findings, a strategy will be developed for risk communication with the general public and informed civil society, taking into account scientific uncertainties, potentially differing perceptions of risk and the specifics related to the biotic vs. abiotic environment of the repository. The description of the research plan can be found in (1).

Introduction

Geological disposal is typically presented as a passive isolation strategy for long term radioactive waste management. Since microbial processes are of dynamic nature, the inclusion or exclusion of microbiology in the conceptualization and performance assessment of geological repositories may bring about differences in knowledge production and evaluation of GD, for both experts and the lay public.

To evaluate the impact of the inclusion of microbiology on expert conceptualization and public perception of geological disposal, the three main research objectives of task 3.2 within the MIND project are as follows:

1. Ascertain how radioactive waste disposal is (re)configured through the MIND project, i.e. through the inclusion/exclusion of microbial processes in the evaluation of safety for geological repositories;
2. Assess how experts and non-experts (informed civil society and lay public) perceive and make sense of nuclear waste disposal when confronted with mutual inputs, and suggest ways of integrating public values and considerations into ongoing research and development (R&D) processes in the field of nuclear waste disposal;
3. Identify characteristics and differences in risk perception by experts, informed civil society and lay publics of GD in the presence of microbiological processes and develop a communication approach supporting the stakeholders in making informed decisions.

The remainder of this paper will elaborate on the first objective, summarising the methodological approach undertaken, as well as the first results.

Expert conceptualization

Description

This subtask will follow how microbes, microbiologists and microbiology are enrolled into the MIND research project, and nuclear waste management more generally. It draws on Science and Technology studies (STS), which highlights the study of the mobilization of resources as a key area in revealing how scientific practice operates. As Bruno Latour (2) argues (pp. 41), by observing what occurs “inside the links” between actors and their networks, we can better grasp how science, technology and “the social” shape the world. Such networks are in constant change, temporarily stabilizing when multiple points of view are aligned toward a particular outcome.

Since the mobilization of microbiology in nuclear waste management is a fairly new phenomenon, a grounded, inductive approach is adopted, which observes how actors process new data and insights, and subsequently resolve challenges. Drawing on semi-structured interviews with actors in the MIND project (MIND researchers, researchers, non-microbiologist experts, members of the MIND Implementer Review Board), on techniques of participant observation, and documentary analysis, we will ascertain how the integration of microbiology in nuclear waste management elicits or affects: (i) new topics and processes, (ii) knowledge gaps and uncertainties; (iii) priorities and opportunities; (iv) future outlooks. Examples of pending or new debates may pertain to the safety and (ir)retrievability of radioactive waste, the requirement for post-closure monitoring, anticipation of possible sociotechnical scenarios, new ways of managing risks, etc.

The semi-structured interviews with experts the notes from participatory observation will allow us to identify the abovementioned issues (i-iv) and the strategies actors deploy to discuss and manage these issues (e.g. the development of intermediary languages using models, scenarios, calculation cases). We will also draw on relevant public statements, authorizing statutes, MIND documents, etc. to facilitate interviewing and draw out responses from experts. Data of the communicative interactions (interviews, group meetings, conference presentations) will be collected using a digital audio recorder and subsequently transcribed and analyzed using NVIVO software. All data will be fully anonymized to ensure research subjects' privacy.

Findings from this subtask will be fed back to research respondents, i.e. the MIND expert community, to enhance reflexive awareness of how experts conceptualize and negotiate the role of microbiology and microbiologists in nuclear waste management, and to initiate debate among them on outstanding issues, challenges, and problem areas within their research field. Given the specific attention accorded in the project to managing risk, safety, and uncertainty, we will single out these particular issues for further discussion with broader publics (see below).

Results

The aim of our participation to the MIND Project Annual meeting in Granada (May 2-4, 2016) was twofold.

First, it allowed us to do participant observation in this network of microbiologists who were *in action*. During this meeting, they questioned and justified their role in the governance of nuclear waste by presenting empirical data collected during experiments. This allowed us to see how microbiologists explore the actions of micro-organisms in waste disposals. In this respect, we understood that conditions of some experiments “are totally not realistic”, but this is not the most important issue for most researchers, the aim being to see “can we even go there, do we have anything to do in this area of the research”. Microbiologists also highlighted disciplinary bordersissues like the one emerging when defining what is a question for microbiology and not a question for geology or engineering. Framing effects are thus raised by the microbiologists who argue that they can influence who is legitimately in charge of investigating it. That is at the heart of the debate, since framing radioactive waste issues in the terms used in microbiology can make microbiologists legitimate actors in the governance of nuclear waste.

Second, our participation to this meeting also aimed at presenting the role of social scientists in the project and organizing a debate (at the end of the day) in order to discuss the role and struggles of microbiologists in the governance of nuclear waste. The questions we asked participants were the following:

- Why is it important to study microbial processes in relation to nuclear waste disposal?
- How will microbiology make a contribution to treating radioactive waste?
- Does taking microbiology into account contribute to a more complete and realistic safety case, as the MIND website suggests? In which ways? Why is this important?
- What is needed to make the MIND project a success?
- What are the biggest challenges you and your colleagues face in this research field?

During the discussion, the microbiologists raised the following issues. For them, microbiology is "of course" very important for RWM. But "they are aware" that not everybody thinks the same. They are aware that they have to make great efforts to get on board the people who count in RWM. In this respect, microbiologists said that the most important is not really to publish a lot of scientific papers on the topic because nowadays it is very easy to publish papers. The most important is to be taken seriously by the people in charge of RWM, which seems more difficult. On reason for this, as pointed out by a microbiologist, is that microbiology makes RWM more complex, since in microbiology, there is no clear answer. "It always depends" she said, because lots of factors are at play. From a scientific point of view, she argued, there is no doubt that microbiological phenomena have to be taken into account, when designing a repository for example. But by bringing microbiologists into play, one also brings *complexity*. That is an issue, since the actors currently involved in RWM prefer models, clear-cut answers and calculable (un)certainties, while microbiology puts into question issues for which answers are stabilized. In response to this, participants argued that they are sometimes tempted to translate their research questions or results into a way that can please current RWM actors. Or, as said by a participant, they could also avoid proposing research in some areas/topics because they know the proposal would be refused or not funded. Finally, a microbiologist argued that the introduction of microbiology in RWM also involves financial issues. For example, microbiology may show that microbes can have an impact on the canister. He argued that it could partly be solved (in theory) by thickening the canister with 1mm which would technically be enough, but which financially would be undoable because it is too expensive.

Conclusions

We studied the role of microbes and microbiology in radioactive waste disposal through the lens of sociology of science (Actor-Network Theory). This lens allows us to assess how microbiologists in the MIND project state the case for the inclusion of microbiology in geological disposal, and which arguments they draw on to justify their research. We will build on the preliminary findings above (e.g. the question of how to manage uncertainty and complexity) to direct our semi-structured interviews with MIND researchers and third parties (i.e., radioactive waste experts not directly implicated in the project) in the ensuing months. We will also draw on these data in subsequent research stages, when we engage other stakeholders (e.g. informed civil society and lay people) and initiate a deliberative workshop with lay publics.

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DEEP GROUNDWATERS IN FINLAND - TOWARDS THE INVENTORY OF GAS

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Abstract

In order to provide data needed to address the question on geochemical constraints of biological activity at nuclear waste repository sites, geochemical data of dissolved gases from deep drill holes and mines in Finland were collected based on a literature survey. Gas data were found from 20 separate localities covering a depth range of 230 –2500 m below surface. The gas phase is dominated by nitrogen and methane, although significant variation exists between different sites and with depth. At least partly this variation can be related to differences in lithology (rock types) and residence time of water within the bedrock.

Introduction

Within fractured bedrock, the fluid phase can both mobilize and disperse potentially hazardous or corrosive compounds such as ^{14}C or sulphide and to provide energy and nutrients to microorganisms. Some of the most important electron donors in anoxic deep biosphere, namely hydrogen (H_2) and methane (CH_4) are gases. Furthermore, dissolved gases in groundwaters can be used to determine diffusive transportation rates and groundwater residence times which can give valuable information on the gradients, production rates and isolation of groundwater from the surface water cycle. Therefore, geochemistry of gases plays an important role in the safety assessment of geological disposal of nuclear wastes.

Collection of data and results

Based on a literature review 20 different deep groundwater study sites, which have sampling depth of more than 200 m and where the determination of gases has been done, were indentified across Finland (Fig. 1). Of these, the absolute concentrations of gases were available from 11 locations. The sites include both drill holes and deep mines in central and southern Finland with the deepest samples from 2480 m depth.

The earliest studies were carried out already in the 1960's [1,2]. Most of the studies date back to 1980's and 1990's [3,4,5,6,7] with many of them already related to nuclear waste disposal research. Unfortunately many of these studies only report average compositions of specific groundwater layers and numerical data essential for modelling purposes is not always included in the publications. The more recent investigations include the studies at the Olkiluoto nuclear waste repository site (e.g. [8]), Outokumpu Deep Drill Hole geolaboratory [9,10] and the Pyhäsalmi Cu-Zn mine [11]. These sites comprise a wide spectrum of geochemical data including gas compositions with depth.

Figure 1: Sites of geochemical investigations of deep groundwaters (>200 m) in Finland including determination of gas composition.

The most commonly detected gases in deep groundwaters are nitrogen, methane, hydrogen, helium, argon and occasionally carbon dioxide. Oxygen is also often detected, although mainly result from contamination with air [10]. Longer chained hydrocarbons have rarely been investigated in the earlier works but even though hydrocarbons up to pentane have been found in the more recent studies [11] their concentrations are minor compared to methane.

Significant variation in the gas composition and concentrations exists from site to site and also with depth in a single site. Saline groundwaters tend to contain the highest amount of gas with the concentration of H_2 as high as 2.2 mM in Outokumpu with total dissolved solids (TDS) up to 70 g L^{-1} [9] and the concentration of CH_4 more than 40 mM at saline groundwater (TDS up to 84 g L^{-1}) in Olkiluoto [8].

At least in some cases variation in geochemical compositions (including gases) could be related to changes in lithology and this variation has been found to correlate also with microbial community structure [9]. Residence time of water within the bedrock also affects the gas composition and concentrations. Especially noble gases, which form within the crust as a result of natural radioactive decay reactions, do not take part into chemical reactions and accumulate with time.

Conclusions

In the context of microbiological risks related to nuclear waste disposal, the geochemistry of gases in deep groundwaters is an integral part of the determination of geochemical constraints of biological activity at disposal depths. Data on gas compositions and concentrations do exist from several separate locations in Finland, although sometimes numerical data is not readily available. Site to site as well as depth dependent variation should be taken into account and could possibly be used to predict changes related to different lithologies.

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Acknowledgments

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MICROBIALLY INDUCED CORROSION OF STAINLESS STEEL 316L UNDER ANAEROBIC CONDITION

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Abstract

The steel canister design in the Czech disposal concept is composed of two layers of carbon steel and stainless steel. This work was focused on microbially induced corrosion (MIC) of 316L stainless steel specimen under anaerobic conditions. The tested environment was a granitic water from Josef URC (Underground Research Centre) containing sulphate-reducing bacteria (SRB). A parallel experiment was performed in the sterile granitic water. EIS (Electrochemical Impedance Spectroscopy) measurement was used for investigation of MIC in the laboratory under anaerobic environment. For the identification and evaluation of corrosion products was used Raman micro-spectroscopy and metallography.

Introduction

The reference canister design in the Czech DGR (Deep Geological Repository) concept assumes to be composed of two layers with one outer layer of carbon steel and a second inner layer of stainless steel (fig.1).

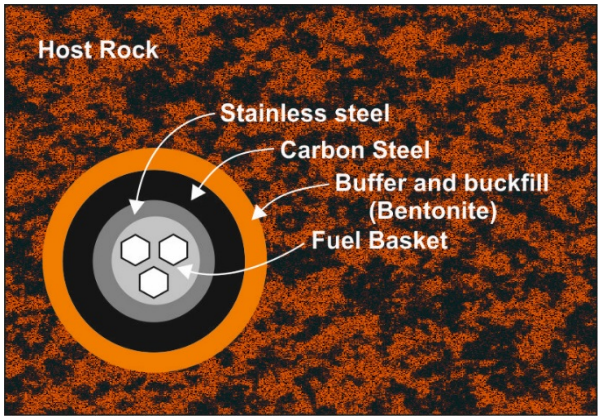

Figure 1: Multibarrier system of Czech DGR concept

This work was focused on microbially induced corrosion (MIC) of 316L stainless steel specimen under anaerobic conditions. The tested environment was a granitic water from Josef URC (Underground Research Centre) containing sulphate-reducing bacteria (SRB). A parallel experiment was performed in the sterile granitic water from Josef URC with different concentration of oxygen at the beginning of the experiment. EIS (Electrochemical Impedance Spectroscopy) measurement was used for investigation of MIC in the laboratory under anaerobic environment. This method is non-destructive and enables long-term measurements. For the identification and evaluation of corrosion products was used Raman micro-spectroscopy and metallography.

Material and methods

The experiment was performed under an inert atmosphere of argon in the glove box, which has maintained the oxygen concentration below 1 ppm. The tested environment was a granitic water (Tab. 1) from Josef URC (Underground Research Centre) containing sulphate-reducing bacteria (SRB). Parallel experiments were performed in the presence of micro-organisms and the sterile granitic water from Josef URC with different concentration of oxygen at the beginning of the experiment. Beginning concentration of dissolved oxygen in sterile solution was $c(O_2)$ = 5.1 mg/l and non-sterile solution was $c(O_2)$ = 0.9 mg/l.

analyte	concentration [mg·l^{-1}]	limit of quantification
Mg	12,6	< 0,1
Ca	60	< 0,1
Na	54,7	< 1
K	1,79	< 0,1
Fe	1,01	< 0,02
Mn	0,11	< 0,005
Cr	< 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
parameter	**value**	
conductivity [mS·cm^{-1}]	61,1	
pH	7,2	

Table 1: Water chemistry after withdrawal

The test material was 316 L stainless steel. The electrode surface was polished with progressively SiC paper up to 600 grit size and sterilized in 96% ethanol inside of the glove box. The granitic water from Josef URC was sterilized with UV lamp. Electrochemical impedance measurement was carried out in the three-electrode system. The auxiliary electrodes were two graphite rods. The reference electrode was a calomel electrode (SCE) and the standard potential is E_{SCE} = 0.242 V. The size of the exposed area of working electrode was 1 cm^2. The EIS measurements were carried out at the open circuit potential in the frequency range 100 000 – 0,005 Hz and amplitude of the perturbation signal was set to 10 mV. Evaluation of impedance data was performed by the using equivalent circuit $R_s(C_{dl}R_p)$ shown in Figure 2. Based on this equivalent circuit values will be determined polarization resistance (Rp), the capacity of electric double layer (C_{DL}) and the electrolyte resistance (Rs).

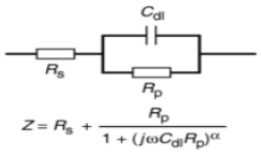

Figure 2 : *E*quivalent circuit $R_s(C_{dl}R_p)$ applied for EIS data evaluation.

After finishing of experiments, the samples were analyzed in the glove box with Raman micro-spectrometer with thermoelectrically cooled CCD detector and resolution ~3.5 cm^{-1}. The excitation wavelength was 532 nm with laser power control in range 0 – 50 mW and measuring range 50 – 1800 cm^{-1}. The molecular biological analysis (quantitative PCR) included relative quantification of functional genes dsrA (dissimilatory sulphate reductase) and apsA (adenosine-5'-phosphosulfate reductase) of sulphate-reducing bacteria.

Results and Discussion

Measurements of values of polarization resistance (Rp) and double layer capacitance (Cdl) in the systems were performed by the electrochemical impedance spectroscopy (EIS). The initial decrease in the polarization resistance (Fig. 3) in the both exposed environments were due to a decrease of oxygen concentration leading to the formation of the anaerobic environment. At the beginning of experiment sterile solution contained a higher concentration of oxygen than the non-sterile solution. The anaerobic environment was created by the gradual release of oxygen from the solutions due to diffusion and electrochemical reactions.

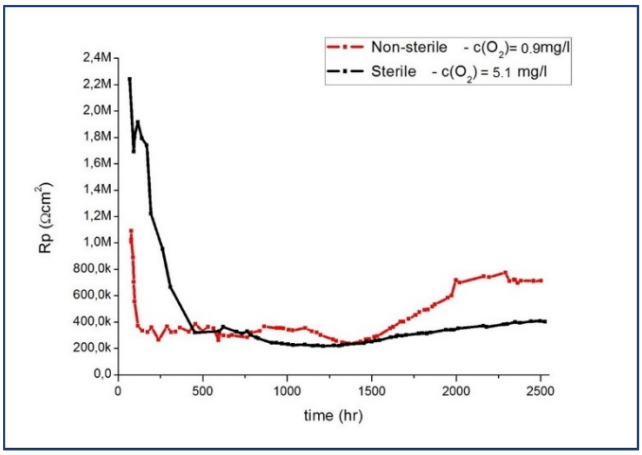

Figure 3: Polarization resistance of the steel samples exposed to sterile and non-sterile environment

An increase of the polarization resistance of the sample in the non-sterilized solution containing SRB showed corrosion inhibition by bacteria during a biofilm formation (1). The full development of a biofilm was not achieved during the experiment. Phase diagrams of the non-sterilized solution containing dissolved oxygen (0.9 $mg \cdot l^{-1}$) show one time constant only, which indicates, that the system was under activation control processes (2). This behavior was attributed to the formation of an unstable conditioning layer based on a mixture of inorganic/organic compounds. Due to a higher concentration of oxygen (5.1 $mg \cdot l^{-1}$) in the sterilized solution at the beginning of the experiment, there is a transition in the spectrum from a two time constant to a one time constant during the first 453 hours. This development is caused by the corrosion potential (E_{corr}) shifts toward active from the passive region (due to decreasing oxygen concentration) during which formed a more defective passive film (Fig. 4).

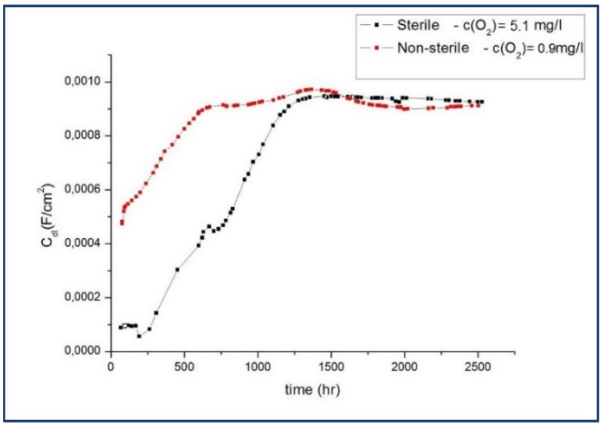

Figure 4: Double layer capacitance of the steel samples exposed to sterile and non-sterile environment

The surfaces of samples after finishing of experiments were very heterogeneous with distinguishable colored areas (corrosion products). Their identification by Raman spectroscopy was unsuccessful due to low thickness of corrosion layers. On the sample exposed to the non-sterile environment, there were also observed dark crystals (Fig. 5). These were determined with certainty as carbonates by Raman analysis. This is the highest probability the calcite ($CaCO_3$) or the siderite ($FeCO_3$) or a mixture of both, but assignment only by Raman analysis isn´t unambiguous.

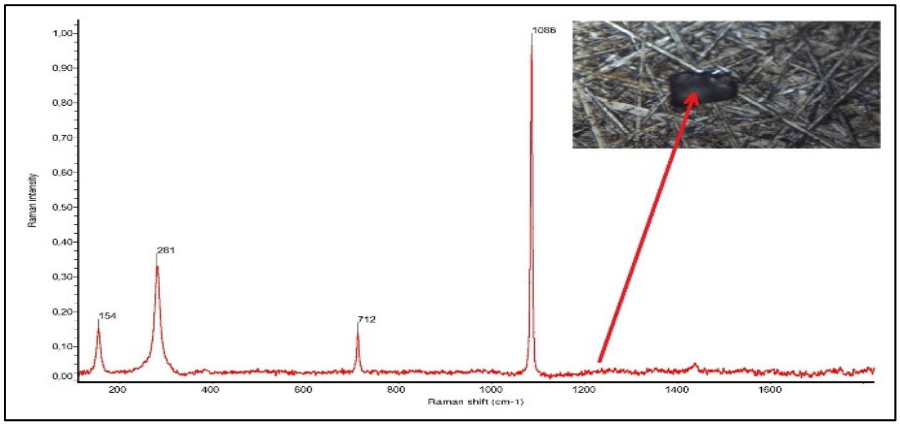

Figure 5 – Raman spectrum of crystal grown on non-sterile steel sample, excitation 532 nm

The metallographic analysis enabled to display corrosion layer and quantify it. In the both cases of samples (sterile and non-sterile environment) were observed thick layer wide in the range from 2 to 3 µm (Fig. 6).

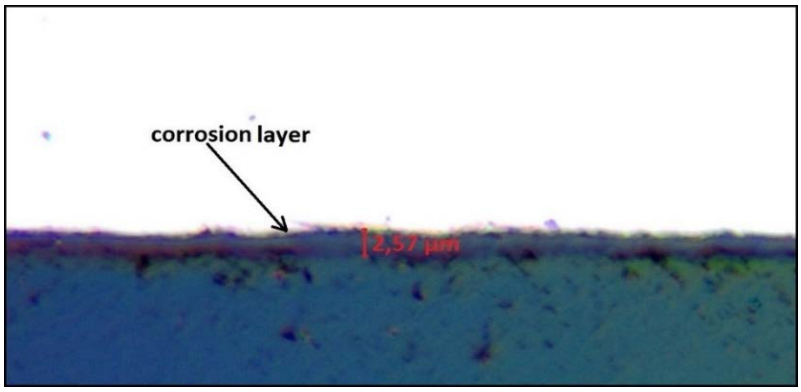

Figure 6: Image of corrosion layer on nonsterile steel sample getting with optical microscope

The abundance of both functional genes (dissimilatory sulphate reductase gene - dsrA and adenosine-5'-phosphosulfate reductase gene - apsA) increased after finishing the experiment in nonsterile sample (Fig. 7). The sterilisation step requires optimisation since sulphate-reducing bacteria were also detected in the nonsterile sample.

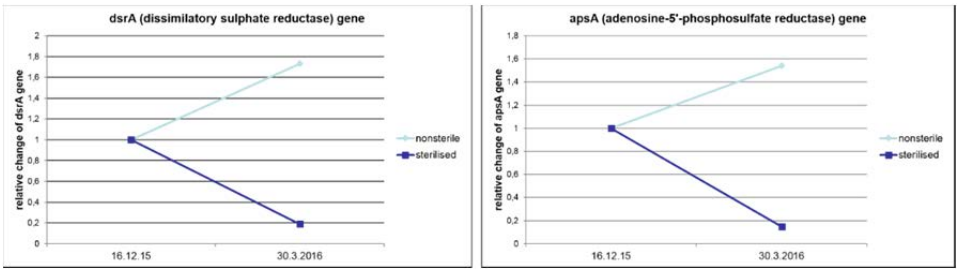

Figure 7: Relative change of functional genes dsrA of sulphate-reducing bacteria

Conclusions

The el-chem experiments were performed with stainless steel 316L in the granitic water from URC Josef containing SRB (parallel with the sterile environment). On the base of EIS measurement and metallographic analysis was found that the corrosion processes have uniform character, and there was observed no local corrosion attack in both experiments. Metallography measurements show that the thickness of corrosion layers after the exposition of 111 days was approximately 2 - 3 µm. The concentration of corrosion products wasn´t sufficient for Raman analysis. On the surface of the sample exposed to the non-sterile solution were observed dark crystals, which were determined by Raman analysis as carbonates. By EIS measurement were recognized that presence of bacteria cause inhibition of corrosion rate. This fact is connected to increase of polarization resistance in the non-sterile solution after ca 1500 hr and to decrease of capacity of the double layer. Measurements were showed, that biofilm wasn´t fully developed. The abundance of sulphate-reducing bacteria increased in the period of the experiment which confirms their role in microbially induced corrosion. The shifts in the microbial community structure after lasting the experiment is planned to be studied using Next Generation Sequencing. This experiment brought an initial insight into the issue of microbially induced corrosion of stainless steel.

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Acknowledgement

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WP2 PROGRESS FROM EPFL:

IC-A AND MA-A1 EXPERIMENTS

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Abstract

Nuclear high-level waste (HLW) disposal is a major engineering challenge and requires consideration of many potential processes that could impact the safety case. Two such concerns are related to the role of microorganisms: One is the corrosion of the steel cylinders that are planned as waste containers by microbially-induced corrosion (MIC, principally by sulfate-reducing bacteria). The second is the build-up of H_2 gas as a result of anoxic steel corrosion and the potentially beneficial role of microorganisms in consuming accumulated H_2. Here, we present planned experiments to investigate these two aspects of microbially mediated impact on HLW.

IC-A experiment

Introduction

This experiment has been ongoing for 3 years and it is planned for 10 years. It is designed to obtain repository-relevant values for the rate of steel corrosion in bentonite (since the steel canisters will be embedded in a bentonite backfill). It consists of individual modules containing steel coupons embedded in bentonite. The bentonite dry density is variable and so are the types of steel coupons used. The bentonite is surrounded by a fritted filter and encased in steel cylinders that are deployed in a borehole (BIC-A) at the Mont Terri Rock Laboratory. These modules are recoverable and the plan is to recover individual modules as a function of time. Additional modules can be deployed once space is available. The presence of bentonite in the modules is intended to limit the potential for microbial growth because of the high density of the clay selected for this experiment.

Materials and methods

We investigated viable counts of aerobic and anaerobic microorganisms in bentonite from three modules retrieved from the borehole in order to evaluate the dry density that results in the least viability of organisms.

Bentonite sampling for microbial analysis took place at the AMEC Foster Wheeler laboratory, Harwell Oxford, United Kingdom. The modules were opened in an anoxic chamber previously cleaned to remove dust with a vacuum cleaner and wiped with a 70% isopropyl alcohol solution. The filters were cut in half along their length in a glove bag to prevent the formation of dust inside the chamber. The chamber was cleaned a second time with the same alcohol solution before sampling the bentonite.

Figure 1: Bentonite cylinder after removal from fritted filter (A) and after cutting to reveal a steel coupon (B).

The filter was cut in half along the long axis and the top half removed. The second part was used as a support to cut the bentonite and was secured in a bench vise. The bentonite was cut and sampled with sterile knifes and spatulas (Figure 1) and packed in sterile sampling bags and then packed in 2 layers of Mylar bags under N_2 atmosphere and kept at 4°C.

The sample sections were cut into smaller pieces under sterile conditions prior to further processing. A suspension was prepared from the small pieces of sample section destined for cell culturing by adding a weighed amount of sample to a known volume of phosphate-buffered saline solution (PBS, i.e., 0.01M NaCl buffered to pH 7.6 with 9 mM Na_2HPO_4 and 1 mM $NaH_2PO_4.H_2O$), which was then stirred for 30 min to 1 hour. Serial dilutions (10^0 to 10^{-3} in PBS) of the suspensions were used in the enumerations.

Aerobic heterotrophs were enumerated on R2A medium (Reasoner and Geldreich 1985). Aerobic plates were poured in a laminar flow hood and incubated at 30°C for 3 to 3.5 days. Anaerobic heterotrophs were also cultured on R2A medium in an anaerobic glove box, and incubated at 30ºC under anaerobic conditions for about 17-28 days. Sulphate-reducing bacteria (SRB) were enumerated by the most-probable number (MPN) method in modified Postgate's B medium (Atlas 1993) and incubated at 30ºC under anaerobic conditions for 35 to 71 days.

Results and discussions

There was a clear correlation between dry density and number of cultivable organisms both under aerobic and anaerobic conditions (Figures 2 and 3). In addition, comparing the colony-forming units (CFU) per gram of the original bentonite used to produce the bentonite cylinders (a maximum of 3.2E+03), it is readily apparent that growth must have occurred, particularly for module 2.

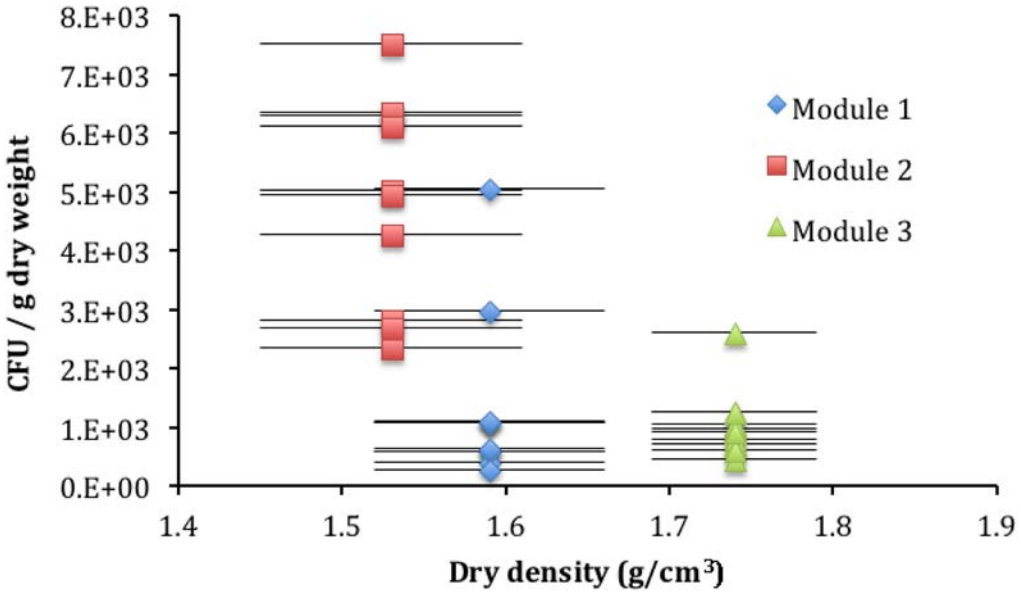

Figure 2: Summary of cultivation results for anaerobic heterotrophic microorganisms plotted as a function of measured dry density

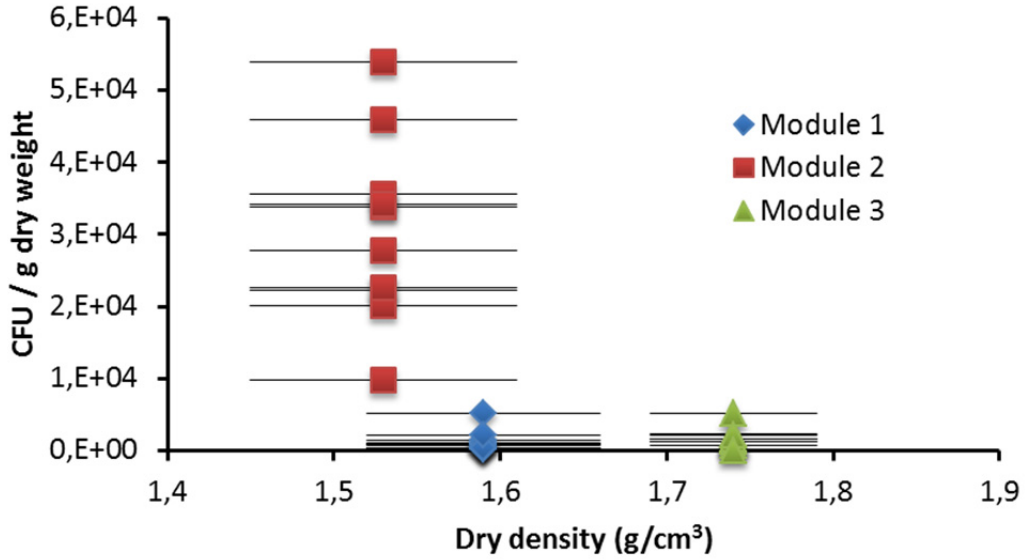

Figure 3: Summary of cultivation results for aerobic heterotrophic microorganisms plotted as a function of measured dry density.

Planned future work

Currently, the experiment does not include a direct control to evaluate the role of microbial processes in steel corrosion. We propose to design modules specifically for this purpose. In order to exclude activity from microorganisms from the bentonite, we will gamma-irradiate the bentonite prior to packing into the module. In order to exclude organisms from the porewater, we will include a 0.2 micron filter between the module cylinder and the bentonite. Additionally, we will combine these two measures in a third module. These modules will provide information about whether microbial activity is extant in the conventional system and whether it impacts the rate of corrosion of steel. We are aware that if sulfate reduction takes place anywhere in the borehole, there is the potential for sulfide to diffuse to the steel coupon surface and cause MIC.

Perspectives

Because the borehole is currently full of modules, we will deploy these three modules in 2018.

MA-A1 experiment

Introduction

An extensive in situ experiment was carried out for 500 days in a borehole at the Mont Terri Underground Rock Laboratory (Bagnoud, 2015). It investigated the consumption of H_2 gas by the indigenous and introduced microbial community by delivering this gas to a borehole. The results showed that (as expected) H_2 consumption was rapid and resulted in the establishment of sulfate-reducing conditions. Thus, it was proposed that the repository could include an engineered gas consumption system, which would rely on the establishment of a sulfate-reducing microbial community that would deplete H_2 and be sufficiently removed from the canister to minimize the impact of sulfide on corrosion. The present experiment intends to provide experimental evidence and consumption rates relevant to repository conditions.

Planned experiments

This new experiment is located at the Mont Terri Underground Rock Laboratory, at the newly drilled borehole BMA-A1, producing about 100 mL/day of water (which is an exceptional yield). The experiment is designed to collect borehole water and deliver to an anoxic chamber located in the gallery while minimizing oxygen contamination. The water will then pass through a series of modules that serve various purposes. The first series of experiments will utilize various sand-bentonite mixtures to evaluate the rate of H_2 consumption (and of sulfate reduction) in a gradient system. The water will be delivered to the outside of a sand-bentonite cylinder, while H_2 is delivered in the center of the cylinder. This electron donor/electron acceptor gradient will result in the establishment of a biofilm at the location where the concentrations of H_2 and sulfate are optimal for growth. The module is designed to be gas tight, thus the influent and effluent H_2 can be measured and the consumption of H_2 calculated. Similarly, the composition of the influent and effluent water will be monitored with respect to sulfate and sulfide (as well as a number of other parameters). The second type of experiment will entail the evaluation of corrosion in bentonite (as a complement to the IC-A experiment). Here, we will be able to evaluate various modules for deployment into the IC-A borehole in 2018. Similarly to the above system, H_2 and borehole water will be introduced into a module containing steel coupons embedded in bentonite. Additionally, a no H_2 system will also be considered. Finally, the third possibility entails obtaining a dry core of Opalinus Clay and interrogating two variants: simply encasing the core in a gas-tight system and measuring gases coming out (we expect H_2, CH_4 and higher hydrocarbons). Because the core will be dry, we don't expect significant microbial activity. The second variant is to deliver borehole water and H_2 to the core and to characterize the microbial community that establishes as a biofilm in this system.

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MICROBIAL INFLUENCE ON BENTONITE-TRANSFORMATION

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Abstract

Bentonites are clay silicates consisting mostly of montmorillonite. Due to their mineral composition, bentonites are characterized by a high swelling capacity and low hydraulic conductivity. Therefore, Bentonite is a promising raw material for serving as a natural clay barrier for the disposal of highly radioactive waste. To address the question, whether and to which extend microbial activity affects the respective parameters, selected bentonites can be supplied with a pore water solution and afterwards incubated for short- and long-term analysis at different temperatures in order to simulate the evolution of microbial activity and the resulting impact on Bentonite-transformation. For characterization the respective Bentonite samples will be analyzed considering their geochemistry, molecular biology and mineralogy – three divisions that influence each other directly. We expect that the obtained results could reveal variations in microbial diversity and a correlation with changes of geochemical parameters, which could affect the composition and solubility of minerals, and furthermore, the beneficial properties of bentonite. The gained information can be further used to indicate a trend in bentonite transformation, which is a prerequisite for evaluating the influence of prokaryotes on safety-relevant processes and properties in radioactive waste repositories.

Introduction

Prokaryotes are an essential component of the earth's biota, catalyzing unique and essential transformations within the biogeochemical cycles of the biosphere (1). Soil and subsurface represent an important habitat for prokaryotes which use for their energy supply chemical sources like fluids and gases that migrate as well as components of the minerals itself (2). The same is true for Bentonite, which is because of its properties like a high swelling capacity and low hydraulic conductivity a very attractive clay mineral for the disposal of radioactive waste ((3), Figure 1).

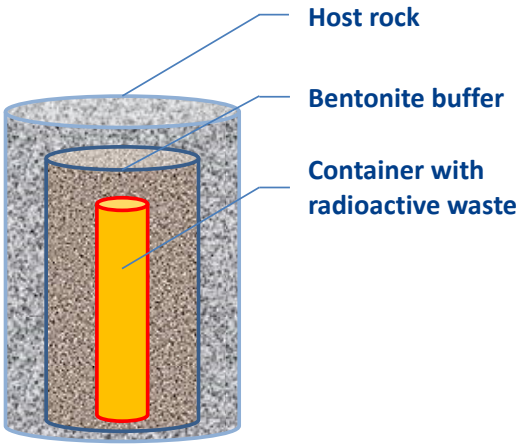

Figure 1: Illustration of a possible disposal of radioactive waste using Bentonite as natural barrier.

Over the last years a couple of studies were performed where microorganisms were detected in the Opalinus Clay from the Mont Terri URL, Switzerland (4, 5, 6, 7). Multidisciplinary approaches were

used to study the microbial diversity in Boom clay formation, a deep-subsurface clay deposit in Mol, Belgium (8) and Spanish bentonites (9). Depending on the choice of Bentonite and the host rock (clay stone, granite or salt rock), a variation of electron donors (e.g. Fe^{2+}, Mn^{2+}, H_2, CO, CH_4, H_2S) and electron acceptors (e.g. O_2, Fe^{3+}, Mn^{3+}, CO_2, SO_4^{2-}) could serve as a potential energy source for prokaryotes. Just after closing the repository, oxygen will be the favored electron acceptor, since the free energy yield will be highest. Since oxygen will be consumed over the time, anaerobic processes will dominate in a closed repository, namely anaerobic respiration and fermentation. Characteristic, anaerobic metabolites could be CO_2, H_2, Fe^{2+}, CH_4 and organic acids.

Within WP2 task T2.4 Microbial degradation of bentonite buffer we want to address the question whether the indigenous microbial metabolic activity do influence the geochemistry and mineralogy of the Bentonite by changing the composition and solubility of minerals and ions. This could influence the mentioned swelling capacity and the hydraulic conductivity and, thus, the potential of mobilization and immobilization of compounds and ions – a crucial point when talking about the final disposal of highly radioactive waste. Within the framework of the MIND-project the influence of microorganisms on Bentonite-transformation, is addressed. In order to simulate an anaerobic environment, which will be adjusted after closing the repository, Bentonites are supplied with an anaerobic, sterilized pore water solution (10). The Batch experiments, including controls, will be analyzed and characterized regarding their geochemistry, molecular biology and their mineralogy.

Description and Discussion

We are planning to use two Bavarian Bentonites from southern Germany. A natural Bentonite (from a mineral deposit near Landshut in Bavaria, Germany) and the corresponding industrial Bentonite (Bentonite B25, Federal Institute for Geosciences and Natural Resources (BGR) Hannover; Steffen Kaufhold) will be compared. For this purpose a set of different batch experiments containing Bentonite and oxygen-free pore water-solution are planned to be made. Furthermore, organic carbon sources like acetate lactate and/or methanol could be added in order to stimulate the growth and activity of certain species (Figure 2).

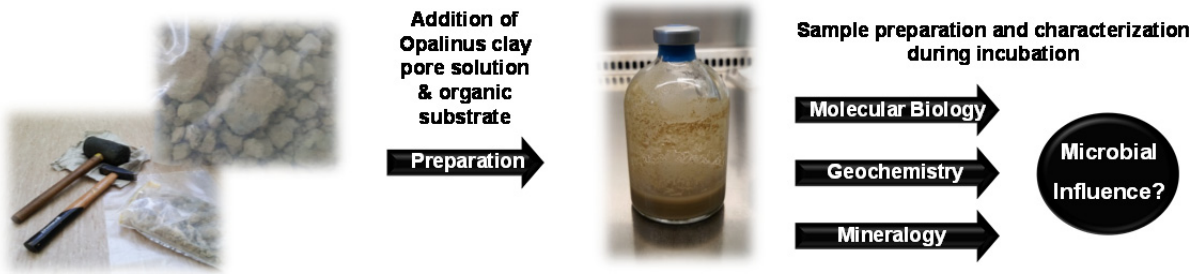

Figure 2: Sample preparation of natural Bentonite. The Bentonite is in a first step crushed under sterile conditions. Afterwards, the Bentonite is added to a sterilized, anaerobic pore water solution (with or without substrate) within a serum bottle. The batches are incubated at different temperatures without shaking in the dark. During the course of time, samples are taken in order to analyze geochemical parameters, microbial diversity and the mineralogy.

While incubating, samples at different times are analyzed. The samples are opened within a glove-box with an oxygen-free atmosphere. Different geochemical parameters like O_2, pH, and E_h are analyzed *via* sensors. The iron concentration (Fe^{2+}, Fe^{3+}) is analyzed with the Ferrozine-assay (11) and the SO_4^{2-} concentration can be determined by ion chromatography. The consumption of eventually added organic compounds (e.g. acetate or lactate) can be monitored by HPLC-analysis. The formation of gaseous compounds can be quantified *via* gas chromatography.

Depending on the geochemical results, batches can be selected for analyzing their microbial diversity. In a first step, the DNA has to be extracted and purified. Therefore, we already successfully

used a protocol adapted from Selenska-Pobell (12). To get a first overview about the microbial community and its changes over time, we will use the approach of RISA-analysis (Ribosomal-Intergenic-Spacer-Analysis). An intergenic region located between 16S rDNA and 23S rDNA, which is unique for each species, is here amplified (9). The more diverse the present community is, the more complex is the RISA pattern consisting of multiple DNA fragments of different lengths (Figure 3, A).

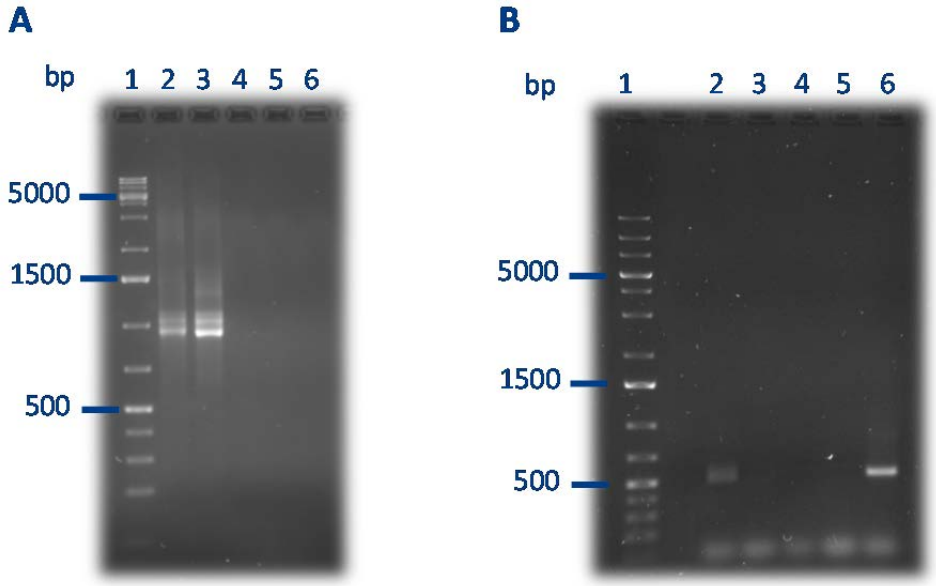

Figure 3: Electrophoretic separation of DNA-fragments. A: RISA (Ribosomal Intergenic Spacer Analysis)-profile using oligonucleotides 130r and 969f (9). 1: DNA Ladder 2: Bentonite1 3: Bentonite2 4: Control (buffers used for DNA-extraction) 5: TE-buffer (Tris-EDTA) 6: water. B: DNA-fragment as a result of a PCR (Polymerase Chain Reaction) using oligonucleotides 519r and 28f (13). 1: DNA-Ladder 2: Bentonite1 3: Control (buffers used for DNA-extraction) 4: TE-buffer (Tris-EDTA) 5: water 6: E.coli (positive control).

On the basis of the geochemical parameters and the RISA-profile of the respective batches, we will choose samples for Next Gen sequencing. For this purpose we chose the approach of a PCR (Polymerase Chain Reaction) in order to amplify a variable, for each species characteristic, region within the 16S rDNA (~500 bp) using specific oligonucleotides (28F, 519R (13)). The method of sequencing will be Next Gen sequencing.

Changes in the microbial diversity (from aerobic conditions to anaerobic conditions) could be reflected in the geochemical parameters, meaning that an increase of e.g. Fe^{2+} should correlate with an increase of Fe-reducing organisms and an increase of SO_4^{2-} -ions should correlate with an increase of sulphate-reducing organisms (14, 15). The formation of both metabolites (Fe^{2+} and SO_4^{2-}) could in turn correlate with a decreasing oxygen concentration as well as with a decreasing redox-potential (E_h).

In a last step, we want to do the most challenging part, namely the characterization of the molecular structure of the respective Bentonites. Here we would like to use Scanning Electron Microscopy, X-Ray Diffraction and/or Transmission Electron Microscopy. An increase of Fe^{2+} could here have a big influence on the molecular structure, since it is known that a high Fe^{2+}-content within the Bentonite could have the consequence of a transformation from Montmorillonite to Illite, called Illitization. As a consequence of this structural change, the swelling capacity is going to decrease and the hydraulic conductivity will increase. So the beneficial properties of Bentonites would not be present anymore. To see if this really happens, as a consequence of the microbial activity, these structural analyses are necessary for the evaluation of Bentonite as a barrier material.

In order to evaluate the results in a proper way, it is essential to have controls and a “kind of” statistics. In Table 1 we show a possible batch experiment designed for the analysis of one Bentonite sample incubating at one temperature.

Table 1: Possible set up of one batch experiment with one Bentonite at one temperature.

Sample No.	Bentonite (à 20 g)	Opalinus clay pore solution (à 40 ml)	Substrates (e.g. Lactate, Acetate, Methanol…)
1-12	Yes	Yes	Yes
13-24	Yes	Yes	-
25-30	Yes (sterilized)	Yes	Yes
31-36	Yes (sterilized)	Yes	-
37-42	-	Yes	Yes
43-48	-	Yes	-

Conclusions

The ubiquitous distribution of Prokaryotes and their influence on geochemical processes demonstrates the importance to analyze their effect on Bentonite, a material thought to be a very promising barrier material for the disposal of radioactive waste. In order to see if microbes do influence the beneficial properties of Bentonites, it is necessary to analyze the microbial metabolic activity within the Bentonites. Just then we can draw a conclusion, whether Bentonites are suited for the respective application.

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15 YEARS EXPERIMENT OF BENTONITE-WATER-COPPER INTERACTIONS IN AEROBIC AND ANAEROBIC CONDITIONS

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Abstract

Bentonite clay is applied to protect the copper canister in KBS-3 method, and therefore its proper functioning over the time period of about 100 000 years must be studied carefully. One of the most interesting system is composed of bentonite and copper, and their chemical and microbial interaction and reactions. While both dissolution of copper and microbial activity is assumed to be very slow, there are not many long term experimental systems available for this type of study. Our experimental systems were allowed to react 15 years before starting to analyse the materials. The main observations were clearly seen microbial both in aerobic and anaerobic conditions, and formation of copper mineral in the bentonite near the copper.

Introduction

In the KBS-3 method bentonite is compacted into confined space at high dry density of about 1,600 kg/m3, which after total wetting has absorbed about 400 kg of water. However, even though the total porosity is comparable high (over 40%) the mass transport is limited to happen by molecular diffusion only and no water flow is taking place. In order to maintain the target density, the bentonite must be in confined volume and therefore producing swelling pressure of typically few MPas, and the experimental system have to withstand the swelling forces. Therefore, many experiments carried out with highly compacted bentonite are batch type in that respect that only post mortem analysis are available and depending on the system size the experimental time is typically months or longer (diffusion type transport couples the equilibration time to square of the system size).

In 1997 the long-term experimental set up was initiated as an aim to study long-term effect of compacted bentonite and the changes in chemistry and microstructure in aerobic and anaerobic conditions. Bacterial and fungal diversity studies were not originally planned but were analysed at the end of the experiment by molecular methods.

Description

The experimental arrangement consisted of the compacted Na-bentonite (MX-80) enclosed in a copper cylinder which was allowed to react through a steel sinter with a solution outside. One of the test parcels was kept during the experiments under the nitrogen atmosphere and one under normal atmospheric conditions. After 15 years the experiments were dismantled and the chemical composition of the bentonite and external water, microstructure of the bentonite, external solution and copper surfaces and minerology mineralogy of the bentonite were studied. The anaerobic experiments were bubbled with nitrogen and the oxygen was sucked away from bentonite under vacuum. All the work phases were handled inside the anaerobic glove box and the plastic bottle was inside hermetic box during the experiments. The experiment was performed in the anaerobic chamber during the years. In order to study microorganisms in bentonite DNA and RNA were extracted by two kits PowerWater DNA Isolation kit (water, biofilm on the copper surface) (MoBio Laboratories, Inc., CA, USA) and ZR Soil Microbe DNA MidiPrep (bentonite) in accordance with the

manufacturer's protocol. As a proxy for bacterial biomass, quantitative PCR (qPCR) was used to determine the amount of 16S rRNA gene copies or transcripts in each sample. The amount of sulfate reducing microbes was determined on base of copies or transcripts of β-subunit of dissimilatory sulfite reductase (dsrB) gene. The bacterial and fungal diversity were investigated by PCR-DGGE analysis of 16S rRNA gene fragment and ITS region for fungi (1,2, 3).

Discussions

The main chemical changes during the experiment were copper diffusion and precipitation as cuprite and malachite in bentonite and replacement of cations as exchangeable cations in aerobic experimental set up. The amount of carbonates increased in external water as well as calcite and siderite. Microbiological studies revealed abundant diversity of bacteria and fungi. SRBs were detected as well iron oxidizing bacteria. Sulphate reducing bacteria were detected in both anaerobic and aerobic experiment. The functional role of these microorganisms in transformation of minerals by oxidizing or reducing is still to be explored.

Conclusions

The experiment was not originally planned for microbiological studies and therefore no samples in the beginning of the experiment for microbiology and therefore no information of the changes in microbial communities during time was achieved. However, this study show that wide diversity of active bacteria and fungi are naturally inhabiting bentonite and can stay viable under compacted conditions.

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MIND WP2: OVERVIEW OF PLANNED BGS EXPERIMENTS ON MICROBIAL INTERACTIONS WITH STEEL AND BENTONITE

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Abstract

The British Geological Survey's (NERC-BGS) contribution to Work Package 2 of the MIND project investigates the microbiological influence on steel corrosion and bentonite degradation in pressurized flow experiments. Progress on the workplan has involved the design and manufacture of the apparatus required to run these experiments. Ongoing work involves the commissioning and testing of the equipment which is not due to be due to be completed until month 18 (December 2016). Plans for sample preparation, experimental set up and operation of a constant volume flow cell, along with the associated collection of microbial, mineralogical and physical data, are presented here.

Introduction

As part of the studies undertaken as part of Work Package 2 in the Microbiology In Nuclear Disposal (MIND) project, the British Geological Survey (NERC-BGS) will be conducting a series of laboratory experiments to understand the extent to which microorganisms can accelerate the corrosion of steel, that will be used for canisters storing radioactive waste, and the degradation of bentonite clays used as a buffer material. Previous BGS research on bentonite and iron interactions in non—microbiological studies of corrosion [1][2] have characterised the mineralogical changes associated with corrosion of steel embedded in bentonite. Although this work did not specifically consider microbiological influence, there were some interesting findings that could have relevance to the microbiological activity in bentonites. Those previous studies found that alteration of the bentonite was associated with the liberation of iron from corrosion of steel or cast iron. This resulted in the enrichment of iron in smectite. The formation of aragonite adjacent to the corroding metal indicated that exchangeable calcium ions were migrating towards the iron. Water in the bentonite was consumed by iron corrosion, which led to shrinkage of the bentonite and the development of halite-saturated porewater. This leads to intriguing possibilities for the survival and activity of microorganisms in such an environment; lack of physical space is recognised as a constraint on microbial activity in clays, but localised shrinkage around corroding steel suggests that habitable space may be created adjacent to corroding metal. Alternatively, if porewater in these shrinkage cracks is highly saline, this could decease microbial activity [3]. Experiments to be conducted as part of this work package will investigate the extent to which microbial activity can alter the amount and nature of steel corrosion and bentonite clay alteration, and assess the potential impact these could have on the engineered barrier system.

Description and Discussion

To investigate the influence of microbial activity on both the corrosion of steel and the degradation of clay, laboratory flow experiments will be set up in a constant volume cell (Fig. 1). Samples for this experiment will be prepared using FE Bentonite obtained from Nationale Genossenschaft für die Lagerung radioaktiver Abfälle (Nagra) and unalloyed steel (DH-SE21-14 LGC standards). These materials will be used to prepare samples as shown in Fig. 1 with steel chips embedded in a section

of the sample near to the inlet end of the sample. A relatively larger down steam section lacking steel chips will allow the propagation of the reaction front to be monitored.

Prior to sample preparation, the bentonite will be sterilised by gamma irradiation. To establish the influence of microorganisms on these processes, “biotic” samples will be inoculated with a mixed microbial culture derived from unsterilized bentonite. This will allow abiotic reactions that cause steel corrosion and clay degradation to be compared to microbially influenced reactions. The inoculum will be prepared by using the unsterilized bentonite to grow enrichments cultures for sulphate reducing bacteria, iron reducing bacteria, methanogens and iron oxidizing bacteria. Following enrichment, the number of cells will be determined by most probable number counts and epifluorescence, and a known number of cells from each of the enrichment cultures will be added to bentonite used to prepare the sample. The bentonite will then be allowed to dry out in an anaerobic cabinet.

Samples will be compressed to dry densities between 1400 kg/m^3 and 1700 kg/m^3 to establish the limits of microbial activity. Hydration and hydraulic behaviour will be monitored using a synthetic groundwater (likely to be supplemented with organic carbon source) supplied to the sample via a high precision syringe pump. The final composition of this groundwater is still to be finalised.

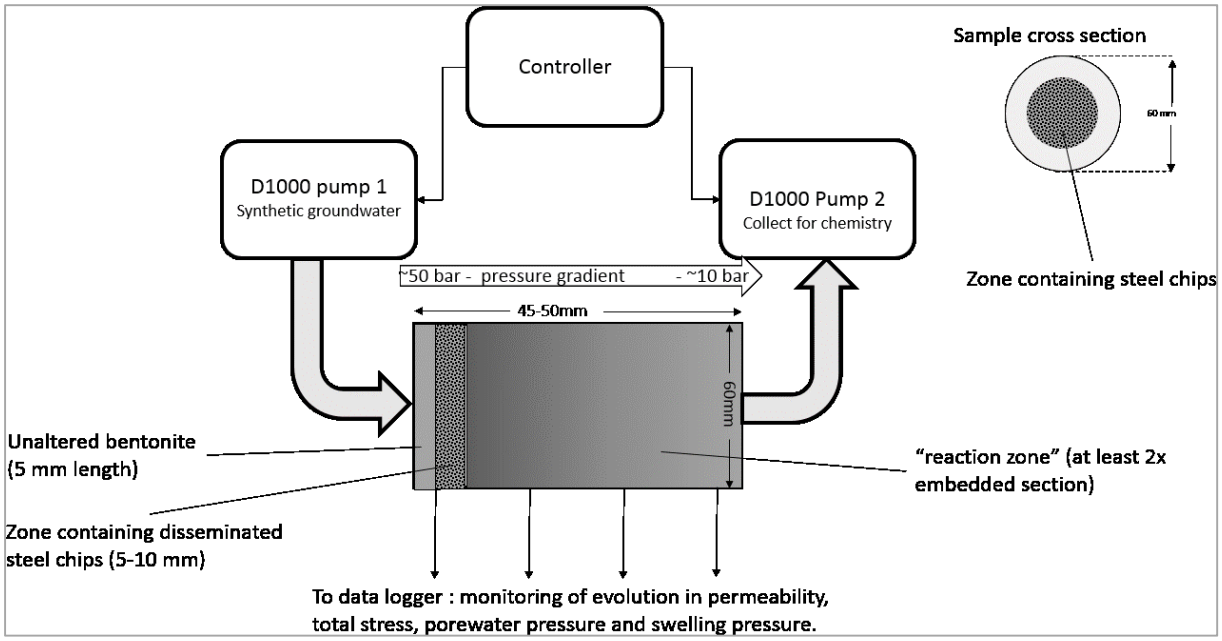

Figure 1: Schematic diagram showing the experimental setup of the flow experiments

Once a sample has been prepared it will be placed into a constant pressure vessel (Fig. 2). The pressure vessels are made from grade two titanium, each fitted with 5 load cells (2 axial and 3 radial) to continuously monitor changes in stress. Filters at either end of the sample allow water to be injection and gas/water to be collected. The load cells will allow initial hydration to be monitored to determine the start swelling pressure. The application of a hydraulic gradient across the sample (maintained using the syringe pumps to control inflow and outflow) will allow permeability to be monitored throughout the experiment (Fig. 3). During this time in- and out-flow will be monitored along with total stress to establish if microbially influenced corrosion of the steel has a direct effect on swelling behaviour of the samples.

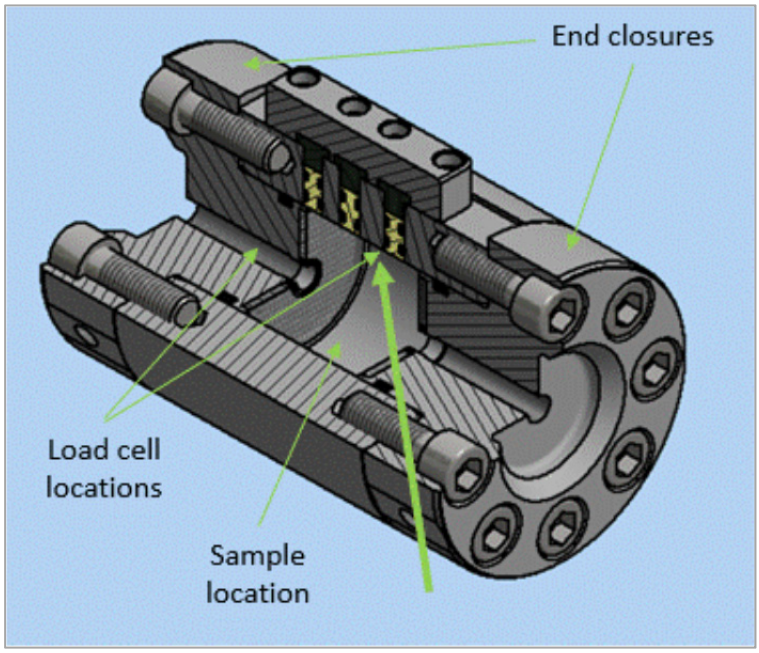

Figure 2: Diagram showing the constant pressure cell

At the end of the experiment a range of standard microbiological techniques (e.g. most probable number counts, epifluorescence microscopy) and molecular techniques (e.g. quantitative polymerase chain reaction, denaturing gradient gel electrophoresis, Illumina sequencing) will be applied as appropriate to identify spatial and temporal changes to the microbial community. Detailed mineralogical evaluation by high-resolution scanning- and transmission-electron microscopy petrographic thin sections and detailed x-ray diffraction analysis will be used to evaluate alterations to the bentonite, determine corrosion products and compare products and extent of corrosion in the biotic and abiotic experiments. The effect of alteration on cation exchange capacity (CEC) and exchangeable cations will be quantified. The chemical composition and volumes of fluids and gases evolved will also be determined. The results of the physical, chemical and biological analysis will be combined to provide a detailed understanding of the impact of microbial activity on corrosion of steel and on the mineralogy and microstructure and therefore the swelling capacity of the bentonite. This will allow us to identify the key attributes in determining the long term performance of the engineered barrier system. The use of a range of starting dry densities in these experiments will help to determine the upper limits of microbial activity and identify whether reduced bentonite density, for example close to the canister and rock wall, are likely to be important areas for microbial activity, and thereby assess their impact on buffer integrity.

Progress to date has largely been in the design and manufacturing of the pressure equipment used in this experiment. Currently, work is ongoing to commission and test the apparatus which is anticipated to last until month 18 (December 2016). The experimental phase will begin in January 2017 and run until the end of this project.

Conclusions

This detailed laboratory study, performed under representative *in situ* conditions will enable the fundamental processes governing microbial activity and its impact on canister corrosion and bentonite engineered barrier system performance to be examined. This task complements other *in situ* tests proposed within the work package, thereby providing important information under a range of relevant repository conditions.

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MICROBES AND CEMENT: FOR BETTER OR WORSE?

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Abstract

Within a geological repository for radioactive waste, the concrete plugs and seals of the repository drifts are generally designed to have a relatively low alkaline pH (pH 11), in order not to disturb their direct environment (e.g. bentonite backfill, host rock) to a great extent. The long term behaviour of seals and plug systems is an urgent priority issue in the strategic research agenda of the IGD-TP (key topic 3, subtopic 10) [1].

Within the MIND project, the interaction between micro-organisms and cementitious materials used in plugs and seals is investigated at different interfaces. The impact of microbial activity on cement integrity can be either detrimental or advantageous, depending on the dominant reaction and the niche where it occurs.

This extended abstract provides a brief introduction to the topic, followed by the experimental set-up as planned within the MIND project.

Introduction

Within construction and engineering, cement has been used for centuries. Even in the Roman ages, various kinds of cement were used, resulting into constructions of which some are still standing more than 2000 years later. Indeed, cementitious materials are preferred in construction because of their long time durability.

Within subsurface radioactive waste disposal however, the evolution of cementitious materials in interaction with other repository materials, the host rock and its ground water, needs to be assessed. The chemistry of cementitious materials and the speciation behaviour of (some) radionuclides are strongly correlated. These correlations therefore need to be considered in performance assessment.

In current disposal development projects, cement compositions are being optimized, in order to reduce negative interactions of the latter with the waste matrix, backfill materials and the geological layer, all the while maintaining the necessary properties for durability and sealing.

Various cementitious materials are planned to be used in radioactive waste disposal:

(i) as part of the engineered barrier,

(ii) as plugs and seals of the disposal drift

(iii) in construction or lining of the disposal drift.

Within this study, the focus is on plugs and seals, which are generally designed to have a relatively low alkaline pH (pH 11), in order not to disturb their direct environment (e.g. bentonite backfill) to a great extent. The long term behaviour of seals and plug systems is an urgent priority issue in the strategic research agenda of the IGD-TP (key topic 3, subtopic 10).

Cement and microbes: concept

In conditions where cementitious materials are in contact with water, they become exposed to micro-organisms. Microbes have been described to survive and even thrive at high pH. Such micro-organisms are named alkaliphiles and have been observed in both natural and man-made high pH environments [2]. Especially in the case of plugs and seals with relatively low alkaline pH, and at interfaces with the host rock or backfill, microbial activity is expected to affect cement integrity.

Microbial activity is known to affect the mineralogy, chemistry and structure of cementitious materials, which can be either deleterious or beneficial for the cementitious construction and its durability. Quite some microbes are known to produce metabolites like organic acids and sulphur compounds, which are considered aggressive for cementitious materials. The dissolution of $CaCO_3$, the main component of cement, by a variety of fairly weak organic acids like acetate has been reported [3], following equation 1:

$$CaCO_3 + H^+ \rightarrow Ca^{2+} + HCO_3^- \qquad \text{Equation 1}$$

This reaction leads to leaching of Ca^{2+} and lowering of pH, the latter opening niches for less alkaliphilic micro-organisms and deterioration the cement as such. The dissolved Ca^{2+} is able to reprecipitate, either as $CaSO_4$ (gypsum) or back as $CaCO_3$, with or without (direct or indirect) microbial interaction. If such reprecipitation occurs at the cement interface, it might result into a protective film, thereby enhancing cement strength. On the other hand, gypsum might be transformed into the voluminous ettringite, which would cause cracking of the cement.

The impact of microbial activity on cement integrity can therefore be either detrimental or advantageous, depending on the dominant reaction and the niche where it occurs. The question remains to which extent the sum of all microbial processes, in the given *in situ* environmental conditions, will influence the long-term performance of seals and plug systems.

Description of laboratory experiments

In order to assess the dominant metabolic processes of microbial communities indigenous to the bentonite backfill or infiltration host rock water, with respect to their impact on cementitious materials, both SCK•CEN and MICANS will conduct a series of laboratory-based experiments which are complementary in their approach.

In various batch set-ups (Fig. 1), SCK•CEN will subject cementitious reference materials to long term incubation in the presence or absence of backfill materials, host rock waters and microbial inocula, under a range of parameters to estimate the boundary conditions of the processes (e.g. neutral and high pH, different bicarbonate concentrations in the water, addition of relevant carbon sources like plasticizers and grinding agents). Microbial activity, biofilm development and overall shifts in microbial communities will be monitored by state of the art enzymatic, microscopy and molecular techniques, while cement integrity will primarily be monitored by electron microscopy and chemical analysis of the liquid phase.

Fig. 1 Experimental conditions for laboratory incubation of cementitious materials with microbial inocula.

MICANS will perform laboratory experiments with compacted bentonites (backfill reference) in contact with concrete in titanium test cells with instruments for registration of the load (Fig. 2). After incubation at various times, the microbial effect on pH and Eh, profiles perpendicular to the concrete will be analysed using microsensors. The stability of the concrete will be analysed and sterile controls will be compared with systems with residing micro-organisms.

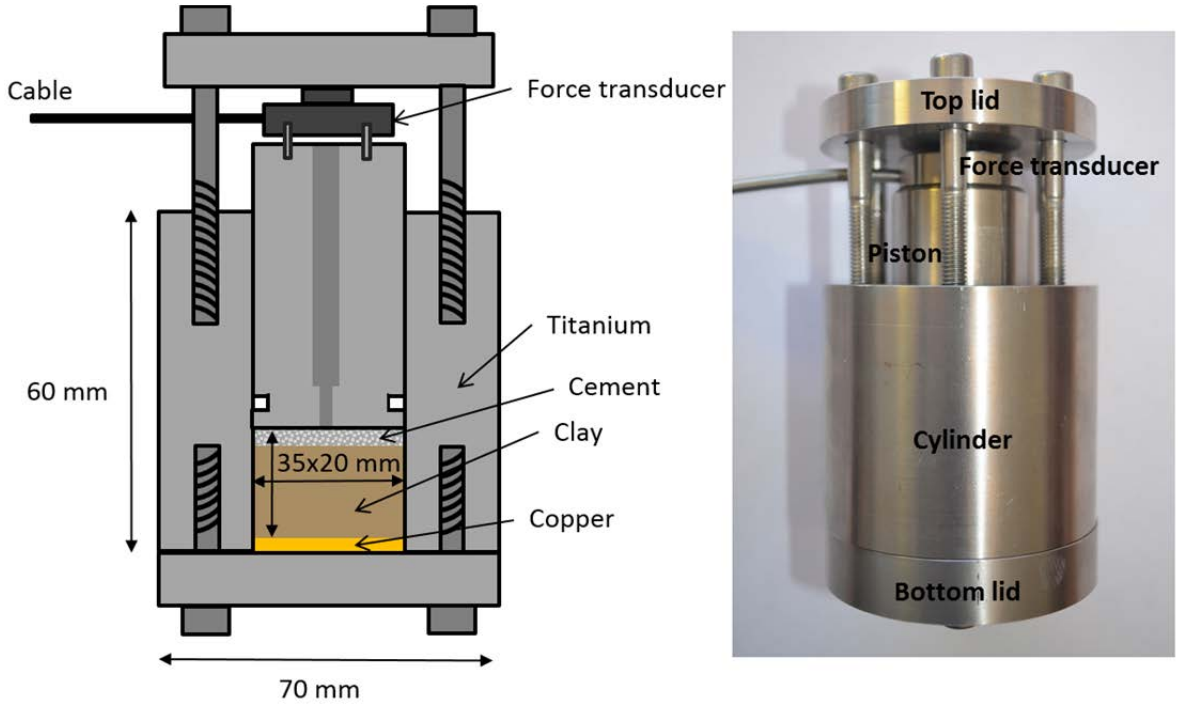

Fig. 2 Titanium test cell for laboratory experiments with compacted bentonite in contact with cementitious materials.

Expected outcome

During and/or after incubation of the experimental set-ups, microbial, molecular, chemical and microscopy analysis will provide an insight in the mechanisms affecting either deterioration or enhancement of cement integrity. Complex metabolic pathways are expected, of which intermediate and end-products will be elucidated, as well as reaction kinetics and boundary conditions. These results will be correlated to microbial community patterns and shifts, dominant microbial species and

surface colonization. Ultimately, these results will be evaluated towards their use for the assessment of the long term performance of seals and plug systems.

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MICROBIAL SULPHIDE-PRODUCING ACTIVITY IN BENTONITE CLAYS AT DENSITIES FROM 750 – 1600 KG M^{-3}

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Abstract

This work sought to identify possible threshold bentonite densities with a corresponding swelling pressures for MX-80, Asha2012 and Calcigel above which microbial sulphide-producing activity is practically inhibited (all others conditions being favourable for the microbes). This work also sought to evaluate how viability of SRB decreases as density and swelling pressure increase.

Introduction

In the Swedish model for geodisposal of spent nuclear fuel (SNF) the bentonite barrier has an important function in maintaining the integrity of the copper canisters isolating the spent fuel (SKB 2010). In the repository a highly compacted bentonite with a bulk wet density between 1950 and 2050 kg m^{-3} and a corresponding swelling pressure of ~5 MPa at full water saturation is projected. The bentonite is intended to hinder outward transport of radionuclides and inward transport of corrosive groundwater components, and to act as a buffer against rock movements. Corrosion may consequently threaten the integrity of the canisters and is thus of special concern. In a future SNF repository, the dominant copper corrosive elements will be O_2 and S^{2-}. Oxygen introduced during repository construction is expected to be reduced, mainly by microorganisms, soon after repository closure (Kotelnikova 2002). Once the oxygen is reduced, anaerobic microbial processes will prevail in the repository. The anaerobic microbial process of concern is the dissimilatory reduction of SO_4^{2-} to hydrogen sulphide by sulphate-reducing bacteria (SRB). The presence and activity of SRB have been detected in groundwater at repository depth (Hallbeck and Pedersen 2012) as well as in various types of commercially available bentonites including Asha, Calcigel and Wyoming MX-80 (Svensson et al. 2011). Sulphate-reducing bacteria have also been found in a full scale demonstration repository (Arlinger et al. 2013) and in various pilot and full scale tests of bentonite performance (Karnland et al. 2009; Lydmark and Pedersen 2011).

Until now, laboratory research on survival and activity of SRB in bentonite as functions of density and swelling pressure has only been performed with Wyoming MX-80 bentonite at wet densities of 1750 and 2000 kg m^{-3} (Bengtsson et al. 2015). While the sulphide-producing activity was significant at 1750 kg m^{-3} it was close to nil at 2000 kg m^{-3}. A precise threshold could not be identified. Further, it is not known if different bentonites have diverse influence on SRB activity and survival. This work seeks to identify possible threshold bentonite densities with a corresponding swelling pressures for MX-80, Asha2012 and Calcigel above which microbial sulphide-producing activity is practically inhibited (all others conditions being favourable for the microbes). This work also seeks to evaluate how viability of SRB decreases as density and swelling pressure increase.

Methods and materials

The experimental design in this work consisted of test cells made of a titanium cylinder with a piston in which bentonite cores were installed together with SRB, copper discs and additions needed for SRB sulphide-producing activity, and then incubated under relevant conditions (Figure 1). The cells

were filled with Wyoming MX-80, Asha2012 or Calcigel bentonite powder with addition of a bacterial cocktail consisting of three different species of SRB (except controls). By adjustment of the amount of bentonite and corresponding space, the swelling pressure generated by the bentonite could be regulated and also monitored by a force transducer connected to a data collection system. Test cells were adjusted to dry densities from 750 to 1600 kg m^{-3} during simultaneous water saturation with a salt solution. When the bentonites had reached planned swelling pressures and were fully water saturated, the piston and bottom lid were removed. A start of the experiments, a 40 µm pore size titanium filter that was attached to the bottom lid and kept the bentonite from swelling out into the inlet hole during water saturation, was replaced with a copper disc that simulated a copper canister. On the opposite bentonite core side to the copper disc, $^{35}SO_4^{2-}$ together with lactate was added. The test cells were then closed again and the force transducer, piston and top lid were refitted. The test cells were repeatedly sampled after up to 3 months from the addition of $^{35}SO_4^{2-}$ and lactate. The radioactivity of the $Cu_x{}^{35}S$ that had formed on the copper discs was localized and quantified using electronic autoradiography. Samples were taken from different layers of the bentonite core and analysed for distribution of ^{35}S, of SO_4^{2-} and most probable number of SRB. A finite difference method was used to derive the SO_4^{2-} to S^{2-} reduction rate in the bentonite from data of the amount of Cu_xS on the copper discs.

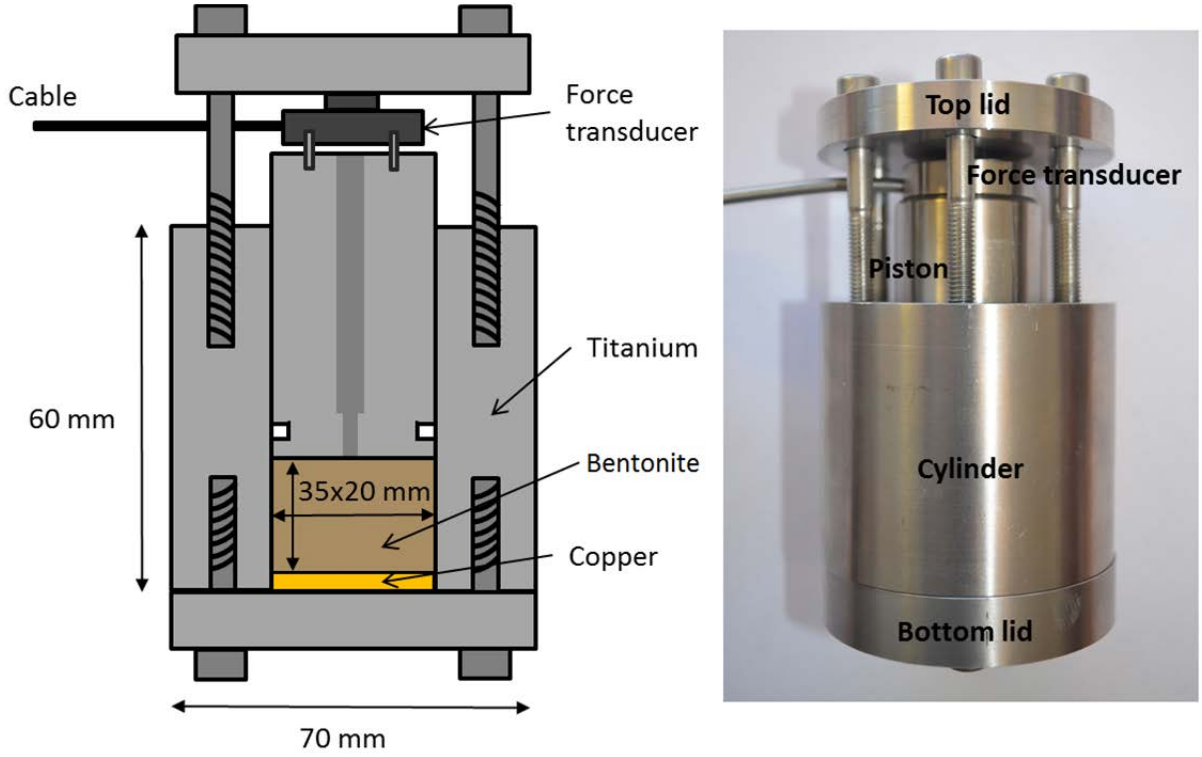

Figure 1 Left: A schematic cross section of a test cell. Right: An assembled test cell, spacers are not mounted.

Results and Discussion

The present experimental set-up was reproducible, enabling detailed and controlled experiments on the activity of sulphide-producing bacteria in compacted bentonites as function of a large array of independent variables such as electron donor and acceptor, temperature, carbon source, species of microorganisms, type of bentonite, groundwater composition, density and swelling pressure. With the present experimental configuration, it will be possible to test additional bentonites with the goal to understand what factors, in addition to swelling pressure, control microbial sulphide producing

activity. Bentonite cores from various full-scale experiments can be trimmed to fit the test cells and be compacted to different densities and swelling pressures. Experiments similar to those presented in this report can subsequently be performed to investigate the viability of SRB and the potential for microbial sulphide-producing activity in collected bentonite samples that have been exposed to varying experimental conditions in the field.

All three tested bentonites below the respective threshold swelling pressure without added SRB had a sulphide-producing activity that was approximately equal to that of bentonites with added SRB. Consequently, there were inherent species of SRB in all tested, commercially available bentonites. Prolonged heat exposure for 7 days at 110 °C did not kill these SRB which corroborates previously published observations on the exceptional heat resistance of dehydrated microorganisms in bentonites. It is very likely that the bentonites in a future SNF repository will be infested with a large diversity of SRB and many other types of microorganisms. Safety evaluation must include parameters that confirm inactivity of SRB in buffers. That need should be possible to achieve based on this and future research. However, back-fill materials must also be compacted above the threshold for each particular bentonite. Else, microbial activity will be on-going as was found for the prototype repository (Arlinger et al. 2013).

The viability of added and inherent SRB was linked to density and swelling pressure of the respective bentonites. SRB could be cultivated in all test cells except for some cells without added SRB and with high swelling pressures. Because the method of detection was based on cultivation, it cannot be excluded that there were viable but not cultivable microorganisms in the bentonites. New methods in nucleic acid analysis are being developed and will hopefully assist this issue in a near future. The effect on microbial activity from a drop in swelling pressure due to bentonite erosion, or any other density lowering process, is unknown but should be possible to investigate with the developed experimental methodology. Evolution has shaped microorganisms to always respond quickly when favourable growth conditions appear. If there is a drop in in swelling pressure below the respective threshold level, microbial growth can occur that rapidly increase the numbers and activity of favoured microorganisms, if energy for growth is available. One important source of energy can be H_2 from corroding metals and CH_4 and H_2 from geological sources. Organic residues in the bentonites can also foster microbial growth and activity.

Sulphide-producing activity was clearly linked to density and swelling pressure. There were range windows for each bentonite in which virtually all sulphide-producing activity stopped. Comparing the windows for density and swelling pressure showed that windows for swelling pressure were much narrower than were the windows for density. Consequently, it is swelling pressure, not density *per se*, that control microbial sulphide-producing activity in compacted bentonites. However, the cut-off swelling pressure was different for different bentonites. Therefore, there must be at least one more factor controlling sulphide-producing activity. At present, the type of factor, or factors, are unknown and can only be speculated on, but such speculation is out of scope of this report. For now, research focus has been on viability and activity of SRB. It is not self-evident that other groups of microorganisms behave as SRB do. There may exist microorganisms that can be active at higher swelling pressures than SRB. Analysis of a variety of different microorganism groups could provide information if the cut-off observed for SRB is “universal” or only valid for SRB.

The observation of a total consumption of lactate and reduction of a corresponding amount of SO_4^{2-} to S^{2-} at swelling pressures below the thresholds was obtained in all experiments. The modelling of SO_4^{2-} reduction rates based on radioactive SO_4^{2-} on the copper discs will then only be correct for the first sampling occasion of these test cells. Modelling of test cells above the threshold with low or no activity will still be valid because there has been excess SO_4^{2-} and lactate in these test cell for the full experimental time. There was a large discrepancy between modelling results based on consumption of SO_4^{2-} and amount of S^{2-} on the copper discs. The sulphide-producing activity must have been much larger in the bentonites than indicated by the modelling based on copper disc radioactivity.

Either are the assumed and used diffusion coefficients not correct, or, is there an unknown S^{2-} retardation factor in compacted bentonites that appears to increase with increasing density.

Acknowledgements

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EFFECT OF IRRADIATION ON POLYSYRENE AND MICROBIAL COMMUNITY: EXPERIMENTAL DESIGN AND FIRST RESULTS

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Abstract

Radioactive ILW and LLW contain different types of organic compounds. Selected material in the MIND project is: bitumen, organic ion exchange resins and halogenated polymers (PVC), which are present in significant amounts in the national inventories. This work study the effect of radiation on polystyrene resins under neutral and alkaline pH in simulated repository conditions in batch experiments. Microbial degradation of radioactive waste will be carried out in batches inoculated with granite pore water from the Josef Underground (JU) facility, depth to ~100 m that is used for a wide range of research activities and/or from the Bukov Underground Research Facility (URF) - depth ~700 m, that operated by SURAO.

Introduction

Representative mixture of acidic and base ion exchangers have the same ratio as wastes from NPP. Irradiation of waste will carried out in irradiation chamber. Microbial degradation will be carried out in batches inoculated with natural granite pore water. The experiments consider the physical and chemical conditions that the organic materials will be subjected to during storage and geological disposal.

Material and methods

Spent resins are identical as from VVER nuclear power plants.

Selected samples:

- tested material: ion exchangers, mixture of Catex and Anex
- **Catex** – strongly acidic resin – with sulfonyl functional group R – SO_3^- , mechanicaly very resitant

- **Anex** – strongly base anion resin – with quarternary amonium functional group R – N^+ $(CH_3)_3$, mechanicaly resitant
- ratio (catex/anex) 2 : 1
- exchange capacity (spent) ca 90%
- spent mixture contains a low amount mechanically damaged balls (ca 10%)
- main sorbed ions: Na^+ , K^+ , BO_3^{3-} , NO_3^-
- dimmension of bead, diameter 0.3 – 1.2 mm

Figure 1 – Surface of mixture ion exchangers, dimension 50 mm

Irradiation facility

Irradiation probe ROZA with source using ^{60}Co ϒ (cobalt 60) radiation

Nominal activity 500 TBq

Dose rate range 0,25 – 7 kGy/h

Irradiation probe is suitable for medium size samples and vessels. The rod cobalt source is released from the shielding container to the working position to the central hole. Samples are fixed on the holders near central probe or are situated around - on stainless steel drum. Ion exchangers are situated in vessel with parallel front and back walls for homogennious irradiation and are rotated at regular intervals.

Figure 2 – Irradiation facility ROZA, open position (without source)

Irradiation chambre PANOZA with source using ^{60}Co ϒ radiation

Nominal activity 100 TBq

Dose rate range 3 – 100 kGy/h

Irradiation chamber PANOZA is suitable for larger size samples. Chamber contains the rod cobalt source which is hiden in the shielding container. To the working position is pull out from covering using mechanism on steel rope. Samples can be situated on the holders close to ϒ source. This arrangement is suitable for larger irradiated doses and more rapid processes. But samples can be placed conversely at a greater distance from the source (ca 2 m) close to wall. Dose rate achieve lower values which more simulates degradation processes at longer duration. There is also irradiation Thermobox for exposure at elevated temperatures up to 90 °C. Total dose is about 1 MGy. It has been considered in previous irradiation studies of organic materials.

Dosimetry

The irradiation time is calculated based on actual radioactivity of gamma source and the distance of the sample from the source. Dosimetric pellets are used for precise measurement. They are situated directly on the surface or inside the sample. Adsorbed dose is determined by Alanine / EPR system (electron paramagnetic resonance). The alanine free radical yields an EPR signal that is dose dependant, yet is independent of the dose rate and energy and is relatively insensitive to temperature and humidity. The evaluation of irradiated dosimeters is performed by EPR spectrometry. Dose determination duration is short term – in minutes.

Figure 3 – Equipment for evaluation of total dose and alanine pellete dosimeters

Post irradiation processes and analysis:

- Water - Granitic and/or cementitious water
- Range of pH 7 – 10 (up to 12)
- After irradiation is required inoculation by various cultures
- Natural or anthropogenic alkaline water will be obtained for batch experiment inoculation in order to obtain microbial consortium adapted to higher pH
- The incubation will be carried out under strictly anaerobic conditions in argon atmosphere
- Analyses of degradation products will be performed using HPLC and GC-MS

Conclusions

The first irradiation experiments will be to ensure that sufficient soluble organic material is produced. Changes of wastes composition after irradiation and after exposition of inoculated underground water from relevant environment will be determined.

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DEEP GROUND WATER SOURCES IN THE CZECH REPUBLIC: CHARACTERIZATION OF MICROBIAL DIVERSITY

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Abstract

The microbial diversity of deep ground water and biofilm samples from first, Bukov Underground Research facility (URF) and second, Josef Underground research Centre (URC) was studied using amplicon sequencing of 16S rRNA gene (targeting variable V4 region). The species composition differed between the two localities but also among different water sources within the same locality. Only two water sources (VITA at Josef UCR and BK18 at Josef URC) contained bacteria representing typical anaerobic environment including sulphur and iron reducing bacteria.

Introduction

The water present beneath the Earth's surface between saturated soil pore spaces or the fractures of rock formation is known as a deep ground water. Porous rock such as granite, limestone and gravel present deep down the ground possess innumerable small spaces which are able to hold water. This pore water has strong potential to host microbial consortia. In order to remain alive and propagate, it is crucial for microbes to turn over several ground water components between different reduced and oxidized state [1]. Part of this microbial community is expected to colonize and influence the safety performance of radioactive waste repository [2]. In this study, two deep ground water sources 1) Bukov Underground research facility (Bukov URF) and 2) Josef Underground research centre (Josef URC) in the Czech Republic (Fig. 1) were analysed to describe microbial diversity.

Material and methods

We studied water and biofilm samples from Bukov Underground Research Facility (URF) located in Vysočina Region, Moravia (operated by the Czech Radioactive waste Repository Authority - SURAO). The site is located 600 m below the surface in the crystalline rock. Josef Underground Research Centre (Josef URC; Central Bohemia) represented second site studied (Fig.1). The height of overlying strata (granitic rock) is 90 -110 m.

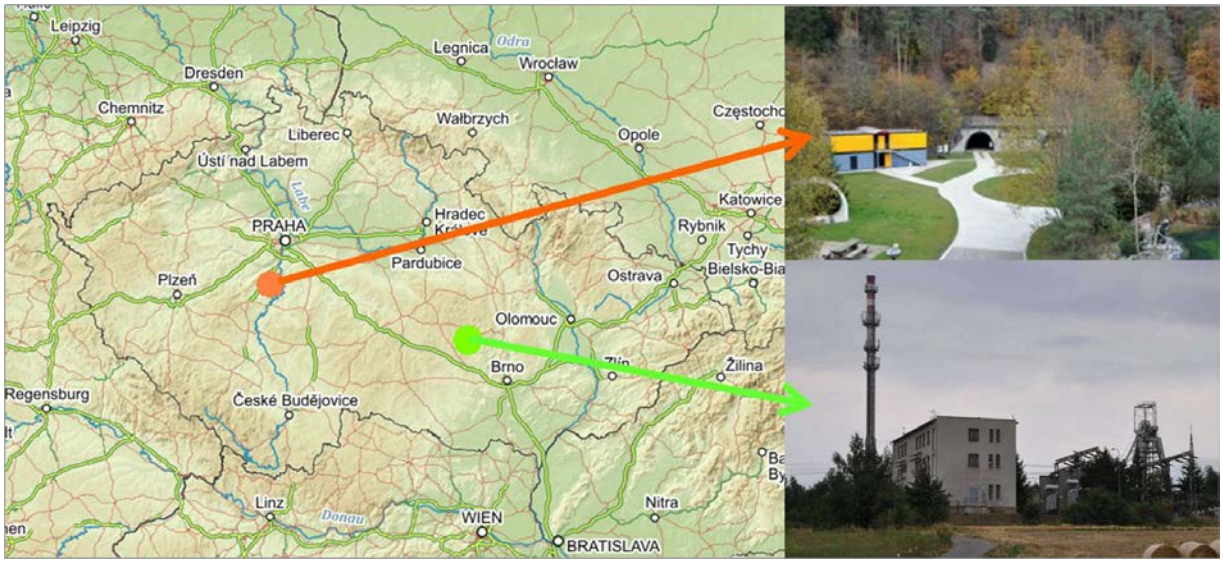

Figure 1: Location of deep ground wated sources in the Czech Republic for MIND project. Orange: Josef URC; green: Bukov URF.

Sample collection

Deep ground water and biofilm samples were collected into sterile bottles (Fig.2) and stored at 4°C. Before analysis, water samples were concentrated by filtration through a 0.22 µm membrane filter.

Figure 2: A) Josef URC, B) biofilm at Bukov URF near BK23, C) collection of ceiling water sample from Bukov URF, and D) water source from BK06, Bukov URF.

DNA extraction

Fast DNA Spin kit for soil (MO BIO, USA) was used for DNA extraction from both water and biofilm samples.

16S rRNA gene amplification and amplicon library preparation

First, 16S rRNA gene was amplified using primers 530F [3] and 802R [4] targeting the variable V4 region. Same primers carrying Ion Torrent adaptor sequences and unique Tag barcodes were used for the amplicon preparation.

Emulsion PCR and sequencing

We used Ion Torrent platform for amplicon sequencing. The process consists of following steps: i) emulsion PCR, ii) enrichment, iii) sequencing carried out on a 314 chip using the Ion Torrent personal genome machine system.

Data analysis

Sequence data were analysed by the pipeline SEED v. 1.2.3 [5]. Sequences with insufficient quality or mismatches in tags were removed from the dataset. All sequences with minimal read length of 275 bp were clustered into operational taxonomic units (OTUs) and chimeric sequences were removed using UPARSE implementation in USEARCH 7.0.1090 [6] with a 97% similarity threshold. The consensus from each OTU was constructed from an MAFFT alignment [7] based on the most abundant nucleotide at each position. The OTUs were identified and their environmental requirements were assessed by megaBLAST and BLASTn algorithms against GenBank nt/nr database.

Results and discussion

Josef URC water was sampled at two sites: VITA and HV1 located relatively close to each other. 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 has the 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 (e. g. *Desulfobolaceae* OTU004, *Desulfomicrobium* sp. OTU020, *Desulfovibrio* sp. OTU047, *Desulfovibrio* sp. OTU054, etc.) and fermenting anaerobic bacteria (*Spirochaeta* sp. OTU093). Sulphate reduction and oxidation of various organic compounds were the main metabolic processes detected in VITA. The microbial community in HV1 was species poor and limited mainly by scarcity of electron acceptors. Markers of the redox S cycle points at autotrophy and subsequent heterotrophy.

Bukov URF water was collected from seven different sources together with two biofilm samples (Fig. 3). The most important factor determining microbial diversity was the opportunity to oxidize reduced sulphur and iron containing compounds. BK23 was influenced by a higher concentration of iron. This is in agreement with the biofilm composition at the BK23 source (Fig. 2). The biofilm contained *Gallionella* sp. (OTU146) and more iron oxidizing autotrophs (e. g. *Ferriphaselus* sp. OTU066). Heterotrophic

bacteria detected in BK23 were different from those detected in all other sources, for example *Sphingopyxis* sp. (OTU111) 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, BK7, BK15 and the ceiling, is more or less homogenous, probably due to the anthropogenic impact.

In BK18, anaerobic bacteria dominated, e.g. *Desulfobulbus sp.* and Fe(III) reducing *Ferribacterium* sp. (OTU021). Some members of oxidizing bacteria and other autotrophs were present as a probable sign of anthropogenic activity. Overall, low abundance of microorganisms is most probably caused by the scarce energy sources. The relatively high diversity in this nutrient-limited environment was unexpected. We are not able to explain this phenomenon, although it was observed previously.

Table below (Tab. 1) represents the results of the NGS amplicon analysis: only selected (most common) OTUs with marked abundancies are shown.

locality	Bukov URF									Josef		determination
sample type	water	water	water	water	biofilm	water	water	water	biofilm	water	water	
sample name / OTU	BK06	BK6B	BK07	BK15	BK15 - biofilm	STROP	BK18	BK23	BK23 - biofilm	VITA	HV1	
1	16	15	1387	2378	390	1753	23	55	48	3	0	*Thiobacillus* sp.
2	312	297	2404	867	261	677	102	46	1	1	0	*Sulfuritalea* sp.
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* sp.
6	327	450	60	1384	612	763	7	161	3	0	0	*Sulfiricella denitrificans*
7	635	690	106	126	419	205	5	156	48	1	0	*Gallionella* sp.
8	0	0	0	0	0	0	1	0	1	0	3405	unclass. alpha proteobacterium
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* sp.
16	138	98	42	23	1	287	1	414	3	1	0	unclass. gamma proteobacterium
17	15	92	0	5	39	1171	0	0	8	0	0	unclass. delta proteobacterium
19	0	5	0	0	0	4	212	1	0	0	0	*Acinetobacter* sp.
20	0	0	0	0	0	0	0	0	0	1282	0	*Desulfomicrobium* sp.
21	0	0	0	0	0	0	1166	0	0	0	0	*Ferribacterium* sp.
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* sp.
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* sp.
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* sp.
35	12	15	4	14	8	12	311	0	1	6	4	*Ralstonia* sp.
38	0	3	4	0	0	0	71	3	0	0	0	*Novosphingobium* sp.
47	1	0	0	2	0	0	105	10	0	450	23	*Desulfovibrio* sp.
51	31	2	19	20	64	0	27	104	90	0	0	*Hyphomicrobium* sp.
52	8	1	97	3	35	0	16	46	0	15	74	*Brevundimonas* sp.
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* sp.
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* sp.
66	0	4	0	1	7	0	0	216	149	0	0	*Ferriphaselus* sp.

Table 1 Results of the NGS amplicon analysis from water and biofilm samples. Only selected (most abundant) OTUs are shown. Numerical values indicate the number of sequences detected in the respective sample.

Conclusions

The microbial community structures of the samples collected at Bukov URF and Josef URC were very different. Only two water sources (VITA at Josef UCR and BK18 at Josef URC) contained bacteria representing typical anaerobic environment including sulphur and iron reducing bacteria.

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MOLECULAR ANALYSIS OF MICROORGANISMS IN THE CZECH BENTONITE

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Abstract

Two Mg-Ca bentonite samples from the Czech Republic were analysed using molecular-biological approach. The diversity of microbial communities present in, first, homogenized, and second, raw bentonite samples from Černý vrch (NW Czech Republic) was studied. IonTorrent platform was used for the amplicon sequencing of 16S rRNA gene targeting the variable V4 region. The microbial communities in the bentonite samples were relatively similar in both samples which suggests that the process of homogenization does not affect the composition of the bacterial community to a large extent. Beta- and Alphaproteobacteria dominated in both bentonite samples. Typical soil bacteria as well as chemolitotrophs that could utilize NH_3, Mn^{2+}, Fe^{2+}, S^{2-} as electron donors were present.

Introduction

The globally accepted strategy for the management and treatment of high level and long lived radioactive waste is to dispose of the waste in a deep and stable geological formation [1]. The concept of the containment is based on a multi barrier system (Fig.1) with different materials such as a metal container, bentonite and the host rock. The microbial community of the host rock or buffer material for the deep geological repository may compromise the effective performance and the safety of the radioactive waste disposal system [2]. Therefore, emphasis has been given to understanding the activity and diversity of microorganisms existing in repository.

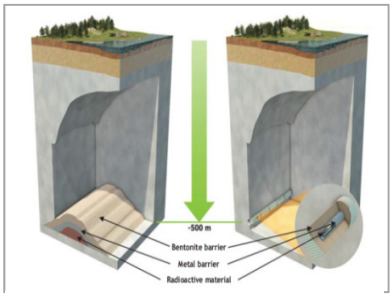

Figure 1: Czech concept for deep geological repository.

In the Czech Republic, bentonite from Černý vrch is planned to be used as a buffer material in the waste repository, however nothing is known about its microbial diversity. Therefore, our study is the first attempt made to investigate the structure of microbial community of the Czech bentonite samples.

Material and methods

Bentonite samples

We studied two Czech Mg-Ca bentonite samples. These bentonites are being used in other Czech projects related to planned underground repository. Both samples were collected at the same locality (Černý vrch, NW Czech Republic) in 2014. First, homogenized bentonite (Fig.2A) was commercially obtained from Keramost a.s. (the product is called “Bentonite a montmorillonite”; “BaM”). Second, raw unhomogenized bentonite (Fig. 2B) collected at the same locality was studied.

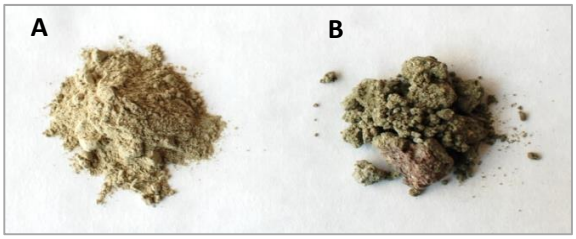

Figure 2: Bentonite samples from Černý Vrch: A) Homogenized "BaM" bentonite, B) raw bentonite.

DNA extraction and quantification

Genomic DNA was extracted from 10 g of 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 [2-3]. Qubit 2.0 or Agilent 2200 Tape Station was used for the quantification of genomic DNA.

16S rRNA gene amplification and amplicon library preparation

Firstly, 16S rRNA gene was amplified using primers 530F [4] and 802R [5] targeting the variable V4 region. Same primers carrying Ion Torrent adaptor sequences and unique Tag barcodes were used for the amplicon preparation.

Emulsion PCR and sequencing

We used Ion Torrent platform for amplicon sequencing. The process consists of following steps: i) emulsion PCR, ii) enrichment, iii) sequencing carried out on a 314 chip using the Ion Torrent personal genome machine system.

Data analysis

Sequence data were analysed by the pipeline SEED v. 1.2.3 [6]. Sequences with insufficient quality or mismatches in tags were removed from the dataset. All sequences with minimal read length of 275 bp were clustered into operational taxonomic units (OTUs) and chimeric sequences were removed using UPARSE implementation in USEARCH 7.0.1090 [7] with a 97% similarity threshold. The consensus from each OTU was constructed from a MAFFT alignment [8] based on the most abundant nucleotide at each position. The OTUs were identified and their environmental requirements were assessed by megaBLAST and BLASTn algorithms against GenBank nt/nr database.

Results and dicsussion

The homogenized bentonite (BaM) and the raw bentonite samples from Černý vrch were more similar than we expected in terms of the microbial community structure based on the results from the operational taxonomical unit (OTU) analysis. 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. Chemolithotrophic bacteria with a possible corrosion capability were present as well, though in lower abundances (Table 1). Typical soil bacteria, including OTU023 *Massilia* sp., OTU040 *Bradyrhizobium* sp., OTU045, OTU065, OTU068 *Lysobacter* sp., OTU067 *Methylocapsa* sp., OTU081 *Microbacteriacea* sp., OTU095 *Acidobacteria* sp., and probably OTU105, OTU114, OTU122 were also present. These bacterial taxa are also known to inhabit oligotrophic environments.

Interestingly, chemolithotrophs that could utilize NH_3, Mn^{2+}, Fe^{2+}, S^{2-} as electron donors were present in relatively high abundances in both bentonite samples (e. g. OTU001 *Thiobacillus* sp., OTU007 *Gallionella* sp., OTU026 *Rhodobacteraceae* and OTU063 *Nitrosomonas* sp.). 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.

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

Table 1: Results of the amplicon sequencing showing only selected (most abundant) OTUs. Numerical values indicate the number of respective OTUs detected.

Conclusions

The microbial communities in the bentonite samples were relatively similar in both samples, although the first one was homogenized commercial material and the second one was raw bentonite sampled directly in the mine; in other words homogenization caused only small differences in the bacterial community structure. Beta- and Alphaproteobacteria dominated in both bentonite samples. *Thiobacillus* sp., *Gallionella* sp., *Rhodobacteraceae*, and *Nitrosomonas* sp. capable of oxidizing NH_3, Mn^{2+}, Fe^{2+}, S^{2-} were present in both samples.

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Acknowledgement

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DEVELOPMENT OF METHODS FOR SEPARATION OF MICROORGANISMS FROM BENTONITE CLAYS

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Abstract

This work tried to establish an indirect DNA extraction method by cell separation, since direct DNA extraction from bentonite clays with commercial DNA extraction kits resulted in no measurable DNA yield at Micans. The efficiency of the methods was estimated from spiked bentonite clays as well on FEBEX clay.

Introduction

In the concept for a Swedish high-level radioactive waste (HLW) repository in a deep igneous rock formation, bentonite clay should act as a buffer material. The bentonite clay will be placed around the copper canisters containing the waste. The environmental conditions in the repository are anaerobic therefore oxygenic corrosion of the canisters will not constitute a long-term problem. The analysis of the microbiome of the buffer material is important because various bacteria can perform processes that affect the condition of the buffer material and eventually the repository. Those processes could include reduction of sulphate to hydrogen sulphide by sulphate-reducing-bacteria (SRB) (Pedersen *et al.* 2000) and radionuclide migration or transformation of clay minerals (Lopez-Fernandez *et al.* 2015). Previous experiments at Micans trying to directly extract DNA from bentonite resulted in no measurable yield of DNA. An indirect DNA extraction method seems to be more favourable to analyse the microbial community of bentonite clay. The general idea in this study is to replace the polyvalent cations with monovalent cations. This should lead to an increasing electrostatic repulsion between the clay particles and the negatively charged soil bacteria. Additionally, detergents dissolve extracellular polymeric substances that are involved in adhesion of bacterial cells to soil particles. At last a physical force disperses the clay and enhances desorption of bacterial cells (van Elsas *et al.* 2006).

The aim of this study was to establish a cell separating method. This method would enable subsequent DNA extraction and downstream analyses of the microbial community of bentonite clay. In the following experiments it will be estimated if the chosen method is efficient enough to separate bacterial cells from spiked bentonite clay in a high number, for following DNA extraction. In these experiments it is desirable not to lyse the cells to be able to calculate cell number with total number count (TNC).

Methods and materials

For the experiments the bacteria *Bacillus subtilis* (Culture Collection of the University of Göteborg (CCUG), Göteborg, Sweden, no. 48815A) and *Pseudomonas fluorescens* (CCUG no. 32456A) were cultivated in nutrient broth (Scharlau, Barcelona, Spain,) for 16 h at 30 °C and 140 rpm. Those bacteria were used to spike the bentonite powders MX-80, Asha and Calcigel (SKB, Stockholm, Sweden) The total number count (TNC) was estimated to be able to calculate the amount of bacteria per gram of bentonite clay. Afterwards the spiked bentonite was either dehydrated by air drying or by freeze drying. The spiked bentonite clay powders were stored at 4 °C until cell extraction.

Three different buffers were tested to estimate which is the most suitable for cell extraction of bacteria from spiked bentonite clay. Each buffer contains a detergent which dissolved extracellular polymeric substances and dispersed the clay particles. A chelating agent in the buffers replaced the polyvalent cations with monovalent cations and increased electrostatic repulsion between the clay particles and the negatively charged soil bacteria (van Elsas *et al.* 2006). 50 mL of each buffer was stirred on a magnetic stirrer and $NaCl_2$ was added to a final concentration of 0.5 M. 5 g of each spiked bentonite clay powder (MX-80, Asha and Calcigel) was slowly added to each buffer. The homogenized slurries were transferred to a 50 mL Falcon tube and centrifuged at 1000 × g for 15 minutes at 10 °C, to collect coarse particles. Afterwards the supernatant was centrifuged at 4565 × g for 1 hour at 10 °C, to collect the microbial cells and remaining clay particles. The pellet was resuspended in 3 mL of sterile 1 × phosphate-buffered saline (PBS). Carefully 1 mL Nycodenz 80% (w/v) (SIGMA-ALDRICH Chemie GmbH, Schnelldorf, Germany) was placed beneath the sample and centrifuged at 4565 × g for 30 minutes at 10 °C. The remaining clay particles formed a pellet on the bottom while the detached bacteria remain in the supernatant and interphase. 1 mL of this supernatant was filtered and stained with DAPI with Vector shield mounting medium (Vector Laboratories, Burlingame, CA, USA). The stained polycarbonate filters were mounted to slides and the bacterial cells were counted with a Zeiss Axio Scope.A1 microscope (Carl Zeiss AB, Stockholm, Sweden) under UV-light.

After the estimation of the TNC after cell extraction ($TNC_{a.c.}$) the recovery rate was calculated. First the $TNC_{a.e.}$ was multiplied by the total volume of the sample and then dived by the amount of spiked bentonite powder, used for the cell extraction, to be able to compare it to the TNC before cell extraction ($TNC_{b.c.}$). The recovery rate was calculated with the Formula 1.

Formula 1

$$Recovery\ rate = \frac{TNC_{a.c.} \times 100\%}{TNC_{b.c.}}$$

Results and Discussion

In this study bacteria were detached from clay particles by replacing the polyvalent cations with monovalent cations and increasing the electrostatic repulsion between the clay particles and the negatively charged bacteria with the buffer described in Gabor *et al.* 2003 (Figure 1) This buffer was chosen to estimate the recovery rate from spiked bentonite clays.

This procedure made it also possible to detached and extracted bacterial cells from hydrated non-spiked FEBEX clay (Figure 2) FEBEX clay of the core B-C-60-18 was hydrated in 0.9% sterile NaCl-solution and the extraction procedure was performed twice. 147 000 amol ATP mL^{-1} (SD ±15 700 amol ATP mL^{-1}) were measured for this extraction.

The TNCs before extraction for Calcigel/MX-80 were 7.4×10^8 cells g^{-1} and for Asha $3{,}3 \times 10^8$ cells g^{-1}. The amount of extracted bacterial cells for Calcigel was 6.2×10^4 cells g^{-1} and for MX-80 5.4×10^4 cell g^{-1}. The calculated recovery rate for Calcigel bentonite powder was 0.25% and for MX-80 0.22% (Table 1). The cell extraction for spiked Asha resulted in no visible *B. subtilis* cells. Even if it was possible to detach and extract bacterial cells the recovery rate for spiked bentonite clay was very low. One possibility is that a lot of bacterial cells remained attached to the clay particles and were centrifuged down in the low centrifugation step together with the clay. Another possible reason for the low recovery rate could be that a vast amount of bacterial cells died through the spiking and the dehydration through freeze drying. Vegetative cells of it are sensitive to drying (Morgan *et al.* 2006). In this study no protective agents were used for the dehydration by freeze drying, which endangered the survival of the bacterial cells. Furthermore, it could be that the bacterial cells need to be hydrated before cell extraction, because it is a critical step for the revival of cells after drying (Morgan *et al.* 2006). Dehydrated bacterial cell walls are less permeable.

Hence it is possible that DAPI cannot stain all extracted bacterial cells. It is a rod-shaped bacterium however, in Figure 1 some of the bacterial cells appear more coccoid then rod-shaped. This indicates that dehydrated bacterial cells are more difficult or not possible to stain and need to be hydrated before cell extraction.

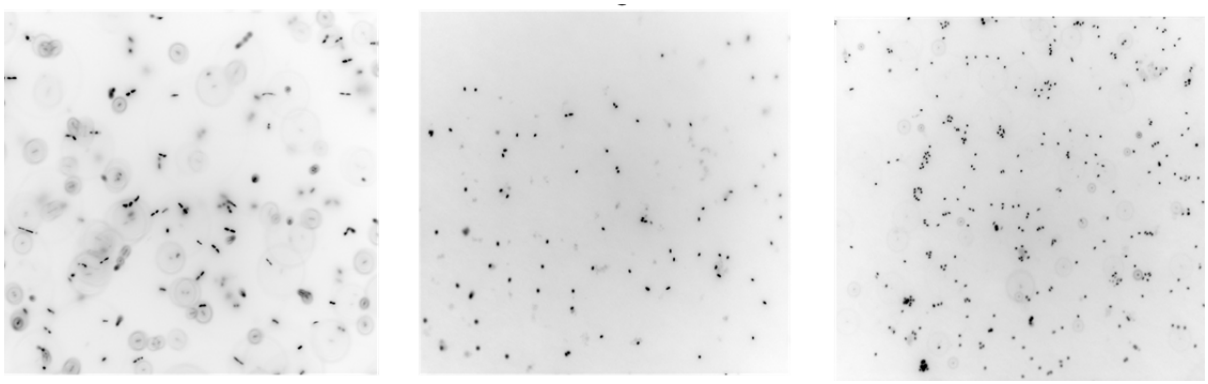

Figure 1 Images of P. fluorescens extracted from spiked bentonite clay powder. The left image shows extracted bacterial cells from spiked Asha bentonite clay powder, the middle image from spiked Calcigel bentonite clay powder and the right image from spiked MX-80 bentonite clay powder. All samples were stained with DAPI Vector shield mounting medium.

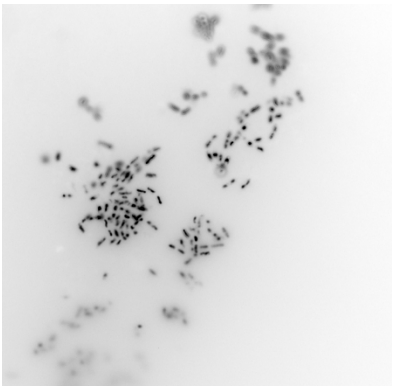

Figure 2 Images of extracted bacterial cells from FEBEX clay after hydration. The image shows extracted bacterial cells from non-spiked FEBEX clay. The clay was hydrated by incubation in 0.9% NaCl solution before cell extraction. The sample was stained with DAPI Vector shield mounting medium.

Table 1 TNC after cell extraction

Bentonit clay powder	**Amount of bentonit clay powder (g)**	**$TNC_{a.e.}$ (cells mL^{-1})**	**Standard deviation**	**cells g^{-1}**	**Recovery rate (%)**
Calcigel	5	3.1×10^6	1.6×10^5	1.86×10^6	0.25
MX-80	5	2.7×10^6	2.8×10^5	1.62×10^6	0.22

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BIOINFORMATIC ANALYSIS OF NUCLEIC ACID DATA WITH FOCUS ON SAFETY FUNCTIONS

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Abstract

New methods for sampling attached microorganisms have been developed for determination of amount of biomass per surface area. The methodologies were applicable for determination of microbial diversity. The first part of this project addressed how biofilms could be investigated using DNA extraction and sequencing. Different methods for sampling of microorganisms attached to rock surfaces were developed and tested. Extraction and analysis of DNA was tested as well. One part of the project was on the adaptation of a flow cell method for *in situ* development, sampling and analysis of microbial biofilms on solid materials introduced in groundwater flowing from deep aquifers. The project also utilized swab materials for sampling of biofilm materials from newly drilled fracture surfaces. Finally, this project used a pressure filtration method to collect large quantities of DNA from planktonic microorganisms for diversity analysis of sequence libraries.

Introduction

Investigations of potential sites for a spent nuclear fuel (SNF) repository in Forsmark, Sweden have revealed diverse cultivable populations in all analysed groundwater samples from depths of a few meters down to approximately 1000 m. The presence of active microbial populations in repository environments must consequently be addressed to facilitate safe implementation of geological disposal of SNF. It has repeatedly been show that subterranean microorganisms rapidly attach to mineral surfaces and that attached microorganisms are at least as metabolically active as are planktonic microorganisms. While there were well developed methods for the analysis of numbers and diversity of planktonic microorganisms in deep groundwater there were no methods for assaying numbers and diversity of attached microorganisms on aquifer fracture surfaces during the site investigations in Forsmark.

Three methods were used to collect DNA from microorganisms. The first method utilises swabs for detachment of microorganisms from fracture surfaces in conjunction with drilling operations. The second method collects planktonic microorganisms with a filtration equipment. The third method involves flow cells with garnet grains that are overflown with groundwater for a period of time and thereafter sampled and analysed for ATP and DNA. All three developed methods can be used in a series. Swabbing and analysis of ATP and DNA during drilling will produce a data archive with information on original diversity on aquifer surfaces. The analysis can await data from flow logging and found water conducting aquifer samples can be further processed. If such aquifers are packed off and pumped, the filtration method will provide information on planktonic diversity while the use of FCs will provide data on microbial attachment, growth diversity.

Methods and materials

DNA extraction

The PowerWater® DNA Isolation Kit, (order no. 14900-100-NF, MO BIO Laboratories, Immuno diagnostics, Hämeenlinna, Finland) was used for DNA extraction. The extracted DNA was quantified and subsequently stored at −20 °C until 454 pyro-sequencing.

Quantification of extracted double stranded DNA

Extracted nucleotide eluates were first quantified using a ND-1000 UV-vis spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA), for quality control of the extraction efficiency and purity. Double stranded (ds) DNA concentrations were quantified fluorometrically using the Stratagene MX3005p fluorometer with MXPro software (Agilent Technologies Inc., Santa Clara, CA, USA) and the Quant-it™ Picogreen reagent kit from Molecular Probes (cat. no. P7589; Invitrogen, San Diego, CA, USA) according to the manufacturer's specifications.

454 FLX Titanium 16S rDNA v6v4 pyrosequencing

A Bacterial 16S rDNA v4v6 amplicon library for sequencing was generated by using the degenerated forward (518F, CCAGCAGCYGCGGTAAN) [1] and reverse primer (1064R, CGACRRCCATGCANCACCT) [2]. Conditions for the PCR reaction was; 1X Platinum HiFi Taq polymerase buffer, 1.6 units Platinum HiFi polymerase, 3.7 mM $MgSO_4$, 200 µM dNTPs (PurePeak polymerization mix, ThermoFisher), and 400 nM primers. Between 5 and 25 ng of sample DNA was added to a master mix to a final volume of 100 µL and this was divided into three replicate 33 µL reactions. A no-template negative control for each sample series was included. Cycling conditions included an initial denaturation at 94 °C for 3 minutes; 30 cycles of 94 °C for 30 seconds, 57–60 °C for 45 seconds, and 72 °C for 1 minute; and a final extension at 72°C for 2 minutes using an Bio-Rad mycycler. The quality and concentration of the amplicon library was evaluated by using the Agilent Tapestation 2000 instrument from Agilent according to manufacturer's protocol. The reactions were cleaned and products under 300 base pairs were removed using Ampere beads at 0.75 × volume (Beckman Coulter, Brea CA). The final products were re-suspended in 100 µL of 10mM Tris-EDTA + 0.05% Tween-20, quantified using PicoGreen Quant-IT assay (Life Technologies), and assayed once again on the Tapestation 2000 instrument. Amplicons were further titrated in equimolar concentration before emulsion-PCR based on their dsDNA concentrations. A GS-FLX Sequencer was used to generate pyrotag sequence reads with the Roche Titanium reagents.

After sequencing, data was run through a quality control process. Each read was trimmed for primer bases from the beginning and the end of each read, barcode key was identified and removed and sequences likely to be of low-quality based on assessment of pyrosequencing error rates was removed [3]. A Bioinformatic Trimming anchor site (565F-a) TGGGCGTAAAG was used to trim sequences to the same biological length. Further sequences were screened for chimeras by using the UCHIME algorithm [4]. The 454 pyrotag sequence clustering into operational taxonomic units (OTUs) was done by an open reference OTU picking methodology using the USEARCH algorithm which uses both a de novo and reference based approach [5]. After OTU-picking a representative sequence from each OTU was selected and used for processing to assign a taxonomic classification based on the Greengenes gg_13_8 database (http://greengenes.lbl.gov/cgi-bin/nph-index.cgi).

After classification of data the representativeness of sequences was tested by rarefaction analysis and Chao as well as abundance-based coverage (ACE) indexes were used to estimate richness. Samples were not normalized or subsampled when alpha- or beta diversity was analysed [6]. To statistically estimate abundance and evenness for each sample, Shannon and Simpson indices were calculated. Distance calculations for phylogenetic tree construction were done by Unifrac distance

measure and Unweighted Pair Group Method with Arithmetic Mean (UPGMA). The generated tree was visualized in FigTree v 1.4.0 software. To compare groups and visualize relationships of samples based on their composition of OTUs a Principal Coordinate Analysis (PCoA) was done in the Emperor software allowing for three-dimensional visualization of clustering based on metadata for samples [7].

Bioinformatic processing, statistical analyses and data visualization

The amplicon 16S rDNA sequencing data was analysed and evaluated using the Quantitative Insights into Microbial Ecology software (QIIME) version 1.9.1. (www. http://qiime.org). Data graphics design and statistical analyses were performed in Statistica 13 (Statsoft Inc., Tulsa, OK, USA).

Sampled sites

The multidisciplinary Swedish Deep Drilling Program project 'Collisional Orogeny in the Scandinavian Caledonides' (COSC, (http://www.ssdp.se/projects/cosc/) drilled a 2500 m deep hole close to the mountain Åreskutan in Jämtland, Sweden. During drilling fresh fracture surfaces from rock-groundwater and rock-rock interfaces were sampled for DNA. Before the drilling was started, laboratory trials were conducted to find the best way to sample with the different new types of sampling swabs and storage techniques. A pressure filtration method was used to collect large quantities of DNA from planktonic microorganisms for diversity analysis of sequence libraries. The DNA from pressure filtrated groundwater from boreholes in the Äspö HRL tunnel, Sweden and DNA from flow cells attached to these tunnel boreholes and surface boreholes in Olkiluoto, Finland, were sequenced. The flow cells were loaded with glass beads and garnet grains as solid supports for biofilm development. The amounts of biomasses and the DNA library diversities from these two materials were compared and evaluated.

Results and Discussion

High-throughput sequencing by means of 454 pyrosequencing is based on emulsion PCR and does not require the preparation of clone libraries before sequencing. DNA extracted from the biomass can be directly used for the analysis of microbial communities based on the 16S rDNA gene. Using sequencing platforms, such as GS FLX Titanium sequencing on the 454 sequencing platform (Roche, Basel, Switzerland), it is possible to obtain thousands of sequences both cost and labour efficiently compared with previously used sequencing techniques. The method produces a huge number of sequences covering most microorganisms in the sampled populations, providing conclusive information about genus/species diversity. The massive number of sequence library data then have to be processed using bioinformatics tools. Recently, there has been a change from use of the 454 pyrosequencing platform to use of the Illumina sequencing platform. This is because the 454 pyrosequencing platform was recently bought by Hoffman LaRoche who soon after that announced the discontinuation of the 454 sequencing platform in 2013 in favour for their own Illumina platform. From 2015 and onwards sequencing will, therefore, be performed using the Illumina platform. With the high-throughput sequencing methods the composition of the microbial communities can be thoroughly characterized. The differences in community composition between samples can be accurately detected and, due to the high number of sequences obtained, rare microorganisms present at only low levels, i.e., below 0.1% of the community, can be detected[8].

The usefulness of high-throughput sequencing is briefly illustrated by the information in Figure 1. The proportion of important genera, such as the sulphate-reducing bacteria (SRB) can be revealed. The data is limited to 20 observations from three locations but do still show clear differences in the representation of SRB between the sampled groundwater locations, possibly related to differences in groundwater origin and composition. There is a large array of additional analysis methods than can be applied on DNA from groundwater and biofilms. For instance, 16S sequence libraries were used to infer flow paths in Äspö HRL [9] and a subglacial lake on Iceland [1]. Information of metabolic strategies

of microbial populations in ecosystems can also be obtained [10]. Genes for specific metabolic processes can be amplified and sequenced which can give insights in potential dominating processes in the deep biosphere [11,12]. Quantitative DNA analysis methods can reveal on-going microbial activity [13]. If full genomes are sequenced, detailed information about possible metabolic pathways is obtained as has recently been published for Äspö HRL groundwater samples [14]. However, is should be noticed that all investigations referred to above have been performed on planktonic microbial populations, often with limited amount of extracted DNA which increases the risk for reagent contamination [15]. The methods developed, tested and described in this report overcome the risk for reagent contamination because they collect enough large amounts of DNA to overcome reagent contamination issues. They also include the major part of the deep biosphere biota, the attached microorganisms, and that aspect and approach is novel. It remains to collect and analyse more samples to avoid errors in conclusions due to a small dataset. Such work is in progress for the Olkiluoto site in Finland.

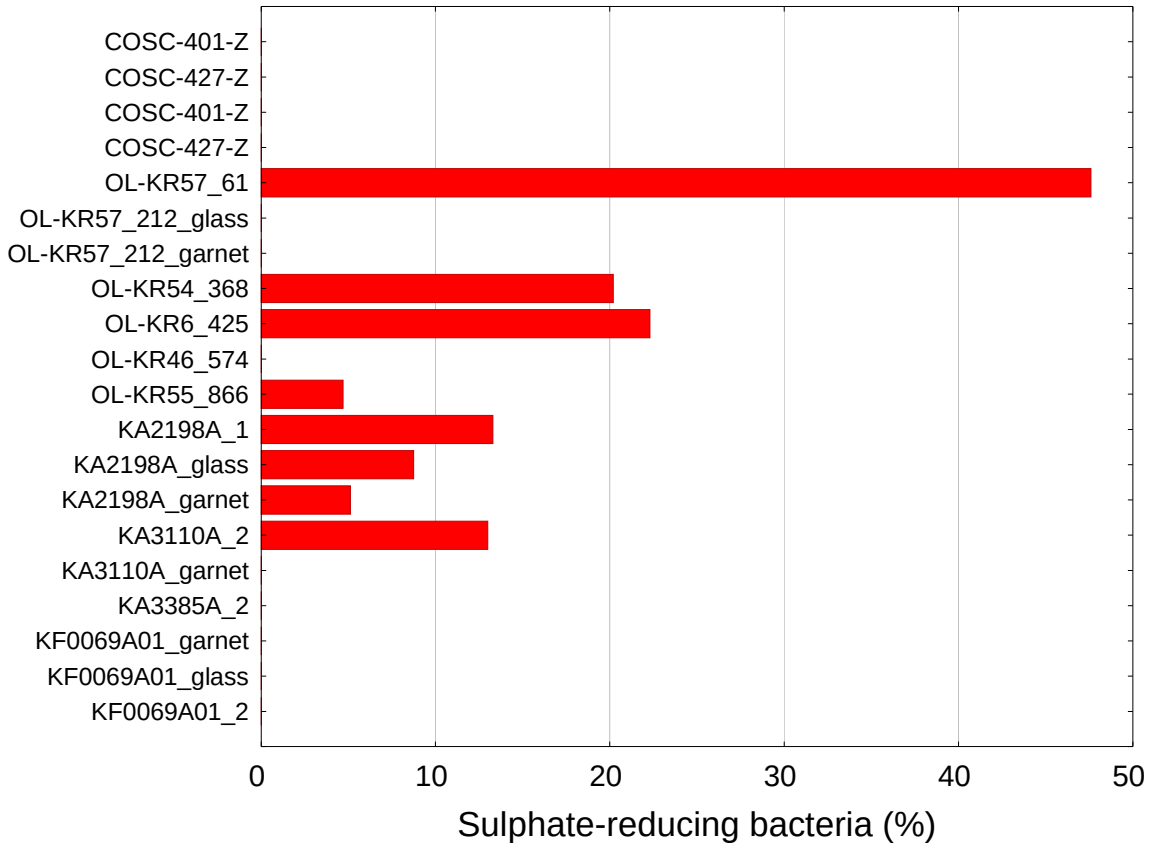

Figure 1. The proportion of 454-pyrosequences belonging to sulphate-reducing taxa.

Acknowledgements

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MICROBIAL PROCESSES WITHIN BENTONITE BARRIER MATERIALS

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Abstract

The United Kingdom has been generating nuclear waste since the 1940's. Radioactive Waste Management is responsible for implementing geological disposal of the UK's radioactive waste. Wastes are to be disposed of in a future Geological Disposal Facility (GDF), and will include higher activity wastes, and potentially, other nuclear materials, as defined in the White Paper on implementing geological disposal (DECC, 2014). The UK's radioactive waste inventory is described in figure 1. High heat generating wastes (HHGW) consist mostly of spent fuel (SF) from current/future power stations, and high level waste from SF reprocessing. Bentonite is a clay-based barrier material considered for use in many HHGW disposal concepts. Bentonite delivers several safety functions including long-term low permeability, chemical environment favouring disposal canister integrity, attenuates the migration of radionuclides released from the waste, limits the transfer of soluble corroding agents to the container, and minimizes microbial activity. This project aims to extend the UK's current knowledge base concerning microbial processes within bentonite, by exploring the following objectives:

1. Demonstrate the effect of bentonite density/swelling pressure on microbial activity, in relation to general microbial colonization, as well as on microbial Fe(III) reduction.
2. Understand the impact of gamma irradiation on aforementioned processes, including the impact on *in situ* processes, and the mineralogical structure of bentonite.
3. Consider the influence of temperature and evolving resaturation on the microbial population within the bentonite buffer and their interaction with bentonite, using samples from the FEBEX *in-situ* experiment (ENRESA, 2000) at the Grimsel Test Site (Figure 2).

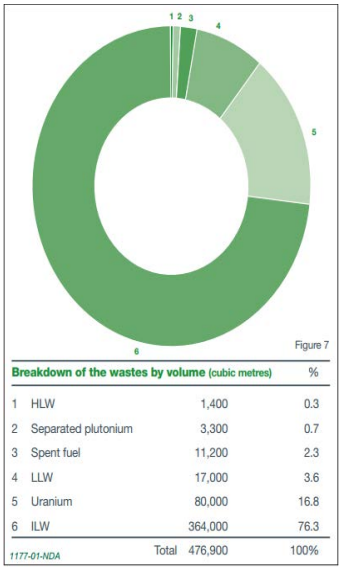

Figure 7

Breakdown of the wastes by volume (cubic metres)			%
1	HLW	1,400	0.3
2	Separated plutonium	3,300	0.7
3	Spent fuel	11,200	2.3
4	LLW	17,000	3.6
5	Uranium	80,000	16.8
6	ILW	364,000	76.3
1177-01-NDA	Total	476,900	100%

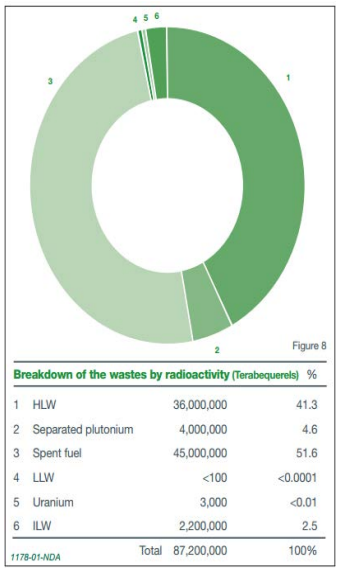

Figure 8

Breakdown of the wastes by radioactivity (Terabequerels)			%
1	HLW	36,000,000	41.3
2	Separated plutonium	4,000,000	4.6
3	Spent fuel	45,000,000	51.6
4	LLW	<100	<0.0001
5	Uranium	3,000	<0.01
6	ILW	2,200,000	2.5
1178-01-NDA	Total	87,200,000	100%

Figure 1: Overview of the UK's radioactive waste inventory as of 2011 (NDA 2010)

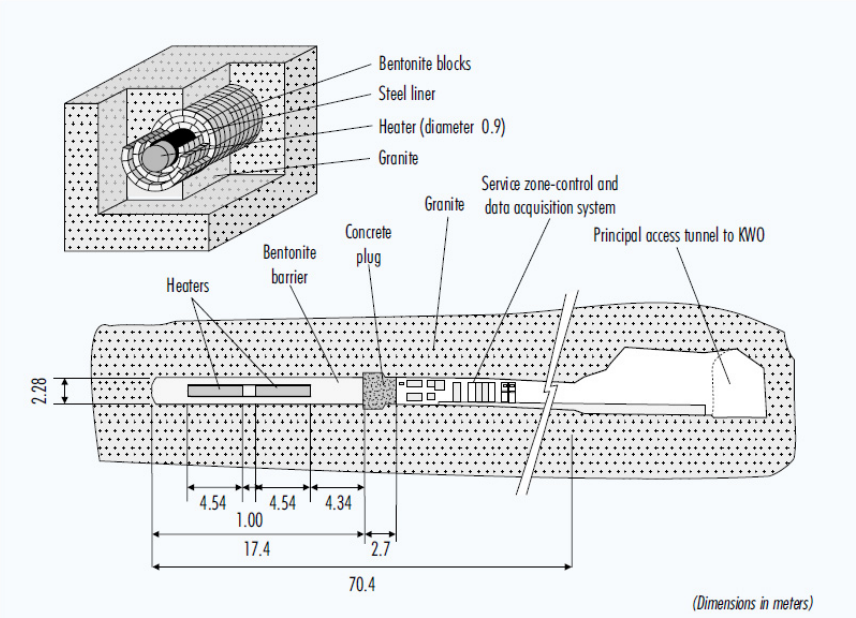

Figure 2: Layout of the FEBEX in-situ test (ENRESA 2000)

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