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MICROBIAL PROCESSES IN PLANNED RADIOACTIVE WASTE REPOSITORY

Ph.D Thesis

Candidate: Farkas Gyöngyi
Supervisor: Prof. Dr. Kecskés Mihály

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Name of the Ph.D school: Szent István Egyetem Környezettudományi Doktori Iskola

Field of Science: Environmental science

Head of the Ph.D school: Prof. Dr. Menyhért Zoltán

Supervisor: Prof. Dr. Kecskés Mihály

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Signature of Head of the school

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Signature of Supervisor

INTRODUCTION AND AIM OF THE STUDY

Nuclear energy accounts for various proportions of the energy production the industrialized countries, e. g. 78 % in France, 29,35 % in Japan, 29,82 % in Germany, and 18,59-12,82 % in the USA and Russia. The development of the nuclear industry is closely related to the solution of the problem of nuclear waste disposal.

Large national and international research programs are currently under way in Canada, Finland, France, Great Britain, Japan, Spain, Sweden and the USA in which different questions concerning the safety of future underground repositories for nuclear waste are being studied. Depending on their type and quality, radioactive waste materials can be divided into three classes: high- level waste (HLW): $>10^{14}$ Bq/m^3, intermediate-level waste (ILW):10^{10}- 10^{14} Bq/m^3, low-level waste (LLW): $< 10^{10}$ Bq/m^3. HLW consists primarily of spent nuclear fuel. After 1000 years, less than 1% of the radioactivity remains.However , the fuel must be kept isolated for a long period (10 000 years) since the remaining radionuclides can be harmful if they enter the human body. Investigations of LLW and ILW relate, for example, to materials such as ion-exchange resins and laboratory wastes.

The main purpose of a HLW repository is to immobilize radionuclides in such a manner as to hinder their reintroduction to the human environment. The safety principle of a repository is based on multiple barriers. The idea is that, if some of the barriers fail, the remaining ones will be sufficient to ensure the safety of the repository.

At present, all concerned countries have accepted the conception of the multibarrier storage of nuclear wastes, which are first solidified and then placed in a repository located in a stable geological formation.

In Hungary, the amount of HLW produced has been increasing since the four units of the Paks Nuclear Power Plant started operations.

The preferred site is in the area of the Mecsek Hills, in southern Hungary. On the basis of preliminary assessments and technical considerations, the Permian Boda Siltstone Formation in the Mecsek Hills area is being considered as a HLW disposal site. Investigations of the suitability of this formation as a location for a waste repository started in 1994 with the technical assistance of Atomic Energy of Canada Ltd.. The uranium mine is located in the Permian Sandstone Formation close to the Boda Siltstone Formation. The existence of an access tunnel from the Mecsekuran Ltd. uranium mine into the Boda Siltstone Formation at a depth of 1100 m provides an opportunity to characterize some potentially important vertical tectonic structures that could act as groundwater pathways through the siltstone. The Upper Permian Siltstone Formation covers an area of 150 km^2 and ranges in thickness from 700 to 900 m. At present, this formation is being evaulated at one site which is accessible from a uranium mine. The evaluations include geological surveying and hydrogeological, geotechnological and geophysical studies. The temperature of the host rock at a depth of 1100 m is about 49 °C and the groundwater pressure ranges from 40 to 50 bar.

The goal of our microbiology program was to investigate this subterranean environment and particularly to evaluate its potential to cause microbially mediated corrosion of HLW containers.

The aims of the present work were:

- Study of the diversity and distribution of the bacteria in the environment of the planned repository.

- Evaluation of the radiosensitivity of the microorganisms present in that environment.
- Examination of the possible interactions between these subterranean bacteria and a future HLW repository.
- Predictions of possible microbial processes.
- Determination of the importance of the microbial effects as compared with the geological, physical and chemical processes.

MATERIALS AND METHODS

Sampling procedure

In October 1997 and 1998, samples from the preferred site were taken via the access tunnel from the air, groundwater, aleurolite stones and surfaces. The numbers of viable mesophilic aerobic and anaerobic and thermophilic aerobic and anaerobic, bacteria were investigated. After incubation, all well-separated colony types (based on their morphological appearance) were isolated.

Gas production

Reinforced Clostridial Medium and Durham-tubes were used to test for CO_2 and H_2 production and Ruhland Medium with Durham-tubes was used to prove the production of CH_4.

Metal resistances and minimum inhibitory concentrations

The aerobic isolates were cultivated in Minimal Broth (OPCA) or Minimal Agar plates. Metal solutions with different concentrations were added to the agar plates. The minimum inhibitory concentration (MIC) was determined as the lowest concentration of metal that completely inhibited growth.

Investigation of biosorption and bioaccumulation

The aerobic and anaerobic isolates were cultivated at 32 ºC for 24 h and 7 days respectively, in 100 ml Nutrient Broth. The bacterial cells were harvested by centrifugation 6500 rpm for 20 min, washed twice and resuspended in 50 ml model solutions containing 20 ppm Cd^{2+}, Cr^{3+} as cations. The solutions were prepared in bistilled water. After a contact time of 2 h, the cells with the biosorbed metal were collected by centrifugation and the metal ion concentration in the supernatant was measured with a Varian AA-175 atomic absorption spectrophotometer. The concentrations of the metals in the biomass were calculated from the differences in the metal ion concentrations in solution before and after the adsorption process.

Organic acid production

Oxalic, citric and acetic acid production by the isolates was assayed with Boehringer-Mannheim kits.

Radiosensitivity of isolated spore formers

Spores were produced as a surface growth (9 days at 37 °C) on Potato Dextrose Agar (Oxoid CM 139). They were harvested in distilled water, washed three times by centrifugation. The suspension was then heated at 80 °C for 15 min. to inactivate any remaining vegetative cells, cooled, washed three more times and finally resuspended in distilled water to give 10^7 spores/ml.

Reinforced Clostridial Medium (Oxoid CM 149) was utilized for the cultivation of anaerobic spore formers. The aerobic spore suspension were irradiated in air, and the anaerobic ones in N_2 at room temperature, with exposure 1, 2, 3, 4 or 6 kGy irradiation. The aerobic vegetative suspensions were irradiated in air, and the anaerobic vegetative suspensions in N_2 at room temperature, with exposure to 0.2, 0.4, 0.8 or 1.6 kGy.

The irradiation facility was an RH–ϒ–30 ^{60}Co apparatus with a dose rate of 2.0167–2.0475 kGy/h. After irradiation the survivors, were detected on Nutrient Agar Medium (Oxoid CM 67).

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<u>Investigation of biofilm formation</u>

A Nanoscope III (Digital Instruments) AFM apparatus was used. Aleurolite coupons (10 x 10 mm) were polished by using 0.25 μm diamond paste. After washing and sterilizing were inoculated with the isolate K/5. After 0, 2 and 10 weeks, the coupons were removed and there surface were examined by atomic force microscopy.

<u>Media</u>

Nutrient Broth (Oxoid CM 79), used to isolate and cultivate thermophilic aerobic bacteria.

Nutrient Agar (Oxoid CM 67), used to isolate mesophilic aerobic bacteria.

Iron Sulfite Agar (Oxoid CM 79), used to isolate bacteria producing H_2S from sulfite.

API Medium, for sulfate-reducing bacteria wich produce H_2S from sulfate.

Hugh-Leifson Medium, for detection of acid production of isolates.

E.E. Broth (Merck), to detect gas production of anaerobic isolates.

Reinforced Clostridial Medium (Oxoid CM 149), for the cultivation of anaerobic spore-formers.

Chrome-azurol Agar, used for siderophore production.

Ruhland Medium (with Durham tubes), used for CH_4 production

RESULTS

The main purpose of this work was to study the microbial activity in the Hungarian Upper Permian Siltstone (Aleurolite) Formation from the aspect of the safety of future underground repositories for HLW. It was found that:

1.The average mesophilic colony-forming unites of the air samples were 3.5/cm^2 (aerobic) and 0.9/cm^2 (anaerobic). Those of the water samples were 0.39-1.2x10^5/cm^3 (aerobic) and 0.36 - 3.9 x10^3/cm^3 (anaerobic) those of the technical water samples were 10^5 - 10^6/cm^3 (both aerobic and anaerobic). Those of the aleurolite samples were 10^2 - 10^5 /g (aerobic) and 10^1 - 10^3/g (anaerobic).The thermophilic aerobic counts were 0-2.4x 10^2/cm^3 , and the anaerobic counts were 0.43-4.6x 10^4/cm^3.

2. 2.2% of the anaerobic isolates from the groundwater samples proved to produce N_2, 6% of the aerobic isolates from the groundwater samples produced NH_3, and 2.2 % produced H_2.2.4% of the aerobic isolates from the groundwater samples produced N_2, and 3.6% produced NH_3. 1.5% of the anaerobic isolates from the technical water produced N_2 and 2.2% produced CO_2. 2 (aerobic) and 3 (anaerobic) isolates from the surface samples produced CO_2, i.e.1% and 2.2%, respectively. 3 anaerobic isolates

(2.2%) from the aleurolite samples produced H_2. 2 anaerobic isolates (1.5%) from the air samples produced CO_2.

3.The highest proportions of acid producers in the aerobic and anaerobic isolates from the air samples were 63% and 54%. The proportions of acid producers were lowest in the aerobic and anaerobic isolates of the aleurolite: 13 % and 14%, respectively. Organic acids were producted by the isolates as followes: citric acid (0.04-9.2 mg/l), acetic acid (0.11-25.5 mg/l) and oxalic acid (0.7-39.9 mg/l).

4.Almost 20 % of the aerobic isolates exhibited siderophore activity.

5.Altogether 50.6% of the aerobic isolates and 59.4% of the anaerobic isolates were spore-formers.

6. The radiosensitivity of the aerobic and anaerobic isolates was also determined: the D_{10} values of the aerobic spore-formers lay in the range 0.8-2.44 kGy, and those of the anaerobic spore-formers in the range 1.86-4.93 kGy. The D_{10} values of the aerobic and anaerobic vegetative isolates were much lower, in the ranges 0.11-0.57 and 0.22-0.40 kGy, respectively.

7. 5.8% of the aerobic isolates were resistant to 650 mg/l (12.5 mM) Cr^{3+}. 93.7% of the aerobic isolates were resistant to 400 mg/l (1.93 mM) Pb^{2+} 86.1% of the anaerobic isolates were resistant to 200 mg/l (3.8 mM) Cr^{3+}. 81.6% of the anaerobic isolates were resistant to 200 mg/l (0.96 mM) Pb^{2+} and 73.5% of the anaerobic isolates were resistant to 200 mg/l (1.77 mM) Cd^{2+}.

8. The biosorption of Cd^{2+} and Cr^{3+} by the isolates was also studied.The uptakes of Cr^{3+} from the model solutions by the aerobic and anaerobic isolates were 0.55-1.87 and 0.23-2.37 mg metalion/mg biomass. The uptakes of Cd^{2+} from the model solution by

the aerobic and anaerobic isolates were 0.70-1.45 and 0.03-3.62 mg metalion/mg biomass, respectively.

9. Biofilm development on the aleurolite was followed by means of atomic force microscopy.

New scientific results

1. Information was obtained concerning the diversity and distribution of bacteria (aerobic, anaerobic, spore-former, non- spore-former, siderophore-producing and gas-producing bacteria).

2. Microbial processes which affect the safety of a future underground repository were discovered.

3. Bacteria which can immobilize radionuclides were found and isolated.

4. Strains were isolated, which mobilize radionuclides by producing organic acids, together with others which change the environmental conditions by producing gases (CO_2, H_2 or N_2), and also sulfate-reducing bacteria which could be responsible for direct microbial corrosion.

5. The radioresistances of the isolated bacteria were determined. No bacteria with extreme resistance were found, but the radiosensitivities of the isolates varied in a wide range, which indicates the biodiversity in the environment.

6. Biofilm formation by the bacteria isolated from the aleurolite was studied on the surface of the stone. Such biofilms too are responsible for the immobilization of radionuclides.

We do not yet have sufficient information to develop a model which will predict the consequences of these processes: canister and backfill materials have not yet been chosen.

DISCUSSION

The groundwater may play an important role, since the presence of microorganisms can affect the transport of radionuclides from a repository. There is often a very high content of dissolved gases in deep groundwaters. These gases participate in the groundwater chemical equilibria, thereby the influencing bacterial activity.

In our study, N_2, $CH_{4,}O_2$, He, Ar and H_2 were found to be present in the water. There were more mesophilic aerobic and anaerobic bacteria in the technical water (10^5 - 10^6 /cm^3) than in the other samples. Enterobacteria were also found. Sulfate-reducing bacteria are of particular interest in waste disposal because they are important in anaerobic corrosion processes; their activity can change the geochemical environment; and their tolerance to repository conditions has been established. Such bacteria were found in only 4 of 67 samples. It was found only in four samples from 67. The proportion of spore-forming isolates was 50.6 % among the aerobic bacteria and 59.4 % among the anaerobic bacteria.

The D_{10} values of the aerobic spores lay in the range 0.8-2.44 kGy, and those of the anaerobic spores in the range 1.86-4.93 kGy, but extremely resistant bacteria (e.g. *Deinococcus radiophilus*) were not found. The D_{10} values of the vegetative aerobic and anaerobic isolates were much lower: 0.11-0.57 kGy and 0.22-0.40 kGy, respectively. These results are in the same range as the D_{10} values cited in the literature for many species of microorganisms. Among the vegetative bacteria, there were no significant differences in radioresistance between the aerobic and the anaerobic isolates, and the minimum and maximum values of D_{10} did not deviate much from the average. It is also consistent with the results of other microbiological studies that the anaerobic spores are more resistant than the aerobic ones, and that the differences between the D_{10} values in both of the spore-forming groups are higher than those for the vegetative cells.

The most resistant bacteria were isolated from the groundwater. The radiosensitivities displayed by the most resistant microorganisms appear to be of importance in calculations of the time required to inactivate the bacteria in the surroundings of the containers of nuclear fuel waste. At the moment, we do not have any exact information concerning the dose rate at the waste container surface, because our repository is currently under construction. Radioresistant bacteria have a number of other applications which are of interest to the nuclear industry (biosorption, bioprecipitation, and biosensors) and the treatment of radionuclide-contaminated soils.

The gases produced by the gasforming isolates were CO_2 and H_2 (anaerobic isolates). The gas-forming isolates and the organic acid and siderophore producers may contribute to microbial corrosion. The toxic metal resistances of the isolates were high and inducable. The biosorption of Cd^{2+} and Cr^{3+} by the isolates was studied. The biomass concentration was found to have a significant effect on the adsorption. The uptake capacity decreased with increasing cell density. On the other hand, it is possible to achieve the better removal of metals from solution with a higher biomass density.

The Cd^{2+} uptake of aerobic isolates were 0.7-1.5 mg metalion/mg biomass, while the Cr^{3+} uptake were 0.55-1.87 mg metalion/mg biomass. The Cd^{2+} uptakes of anaerobic isolates were 0.03-3.6 mg metalion/mg biomass, and the Cr^{3+} uptake were 0.23-2.37 mg metalion/mg biomass.

The treatments for desorption with Na-carbonate, with Na-citrate and with EDTA were efficent.

Most solid surfaces in aquatic ecosystems harbour biofilms that allow growth to accur. It is usually suggested that the nutrient availability may be larger at a surface than the in surrounding water face, due to physical sorption processes. Once estabilshed at the surface, the bacteria may start to grow, depending on the availability of nutrients and energy. Their original adhesion, thought to be a reversible process, is changed to a irreversible attachement by the production of extracellular polymers. Biofilms tend to retain the radionuclides unless the equilibrium of the biofilm becomes disturbed by changes in the nutrient conditions. Thus, accumulation by biofilms immobilizes radionuclides. Biofilm development on aleurolite surfaces was examined in our laboratory. It is planned to examine the biosorption of metals by biofilms and the radiosensitivity of biofilms are planned to be examined in future work.

The goals of the present work were to investigate this subterranean environment and particularly to evaluate the potential for microbially mediated corrosion of HLW containers and to determine the importance of the microbial effects as compared with the geological, physical and chemical processes. Some of the most likely processes for the release of radionuclides from a repository into the biosphere involve transport via the flowing groundwater. Microorganisms can influence this release in a number of ways: changing the chemical conditions in and around the repository, gas production (CO_2 and H_2), acid production, siderophore production, biosorption and bioaccumulation. Sulfate- reducing bacteria are of particular interest in HLW disposal because they are important in anaerobic corrosion processes. Microbial problems in connection with the disposal of LLW and ILW have not yet been studied in Hungary. In these cases more research is needed on waste and packaging materials (cement, bitumen) and containers (steel).

For an active microbial population to develop, sources of carbon, nitrogen, phosphorus, sulphur and energy are essential, plus certain trace elements. For this reason, the modelling approach adopted focuses upon the availability of the key nutrients (C, N, P and S) and energy within the repository environment in order to determine the composition of the organisms and the maximum biomass production ($C_{160}H_{280}O_{80}N_{30}P_2S$). Although much has been achieved in this period of research, there still remains much work to be done in connection with this complex model.

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