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Microbial Analytics Sweden AB

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Svensk Kärnbränslehantering AB
Swedish Nuclear Fuel
and Waste Management Co

Box 250, SE-101 24 Stockholm
Phone +46 8 459 84 00

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Abstract

The Prototype repository is an international project to build and study a full-scale model of the planned Swedish final repository for spent nuclear fuel. The Prototype consists of two sections with four and two full-scale copper canisters, respectively. In 2011, the outer section with two canisters (nos. 5 and 6) was excavated.

Groundwater surrounding the Prototype has been demonstrated to include microorganisms such as iron-reducing bacteria (IRB) and sulphate-reducing bacteria (SRB) with the ability to affect the repository through reduction of structural Fe(III) in the buffer or by the production of sulphide, respectively. During excavation, samples were taken for microbiolgical and molecular biological analysis from backfill, buffer, and canister surfaces and analysed with an emphasis on microbial presence and number.

The underground environment is anaerobic, but the construction of a repository will raise the oxygen levels. Oxygen is not favourable for the longevity of the copper canister, but oxygen levels will decrease over time, partly due to microbial activity that consumes oxygen. Therefore, evaluating the presence and numbers of the heterotrophic aerobic bacteria that consume oxygen as well as monitoring the oxygen levels are important.

The oxygen content of the bentonite itself is also a primary concern, and a method for measuring how the oxygen diffuses through the clay has long been needed. In the work reported here, we performed two pilot studies to address this need. One of these studies tested a method for differentiating between oxygen saturation in aerobic versus anaerobic bentonite; this method has potential for further development.

The tunnel above the Prototype canisters was backfilled with a mixture of bentonite and crushed rock. Sixty-three randomly chosen samples from a cross-section through the backfill were analysed for culturable heterotrophic aerobic bacteria. All but one exhibited growth, with four samples exhibiting numbers over 10^6 colony-forming units per gram wet weight (CFU gww^{-1}). These four samples were all taken near the tunnel ceiling, supporting the possibility of an influx of nutrients from surrounding groundwater. This milieu seems to favour SRB over IRB, according to the most probable number (MPN) results for these samples.

Microbial numbers in buffer and on canister surfaces seem to be low judging from the present results. However, from samples from buffer areas with high water saturation and low density, i.e. three samples from buffer ring 5 in deposition hole 6, we were able to grow up to 1.3×10^4 cells gww^{-1} in IRB medium. DNA from these samples was extracted and then sequenced to obtain information about the species cultivated. Alignment indicated that species of anaerobic thermophilic bacteria, such as *Thermacetogenium phaeum*, and other aerobic bacteria with the potential to form spores, such as *Thermaerobacter subterraneus*, could be found in the buffer. These bacteria must have been dormant since the formation of the bentonite blocks, but are obviously still viable when given appropriate growing conditions.

Molecular biology methods were also able to find traces of SRB on canister surfaces, though these bacteria were not viable and could not be cultivated.

Contents

1 Introduction

The Prototype repository is an international project to build and study a full-scale model of the planned deep repository for Swedish spent nuclear fuel. The current model, KBS-3, is based on encapsulation of the nuclear waste in copper canisters, which are then deposited deep underground embedded in bentonite clay. The bentonite is intended to function as a barrier against inward and outward transport of water and radioactive material, and as a buffer against rock movements, to maintain the integrity of the copper canisters. The Prototype repository differs from a real storage facility in that it is drained. This makes the swelling pressure lower in the Prototype repository than in a real storage facility. The project is being conducted at the Äspö Hard Rock Laboratory (HRL) in crystalline rock at a depth of approximately 450 m.

Since the construction of the Prototype, extracted pore water has been analysed for gas composition, microorganisms, chemistry, and redox potential to evaluate biogeochemical processes taking place at the site (Eriksson 2007, Lydmark 2010, 2011).

The underground environment is anaerobic, but the construction of a repository will raise the oxygen levels. Oxygen is not favourable for the longevity of the copper canisters; however, the oxygen level will decrease with time, partly due to microbial activity that consumes oxygen. Therefore, evaluating the presence and numbers of oxygen respiring microbes as well as monitoring the oxygen levels are important.

Another compound that could shorten the lifespan of the copper canisters is sulphide. Sulphide has not been found in high enough concentrations in this near-anaerobic environment to pose a direct threat to the canisters. However, higher concentrations of sulphide may be produced by the reduction of sulphate by sulphate-reducing bacteria (SRB). This bacterial group has been found in groundwater from environments surrounding the Prototype (Eydal et al. 2009) as well as in the bentonite clay (Masurat et al. 2010a). The proposed repository model uses high bentonite swelling pressure to restrict bacterial activity, a mechanism tested and confirmed in recent studies (Masurat et al. 2010a, Persson et al. 2011). However, the boundary between the bentonite and the copper canister may not exhibit the same harsh environment for SRB, and could perhaps even be a suitable place for biofilm formation (Persson et al. 2011). However, due to the barrier function of bentonite, SRB have to stay viable in the bentonite and/or migrate through it from the groundwater to reach high enough numbers to produce sulphide concentrations that could threaten the canisters.

The excavation of Prototype section 2 (Figure 1-1) enabled studies of bacterial growth and migration in buffer and backfill as well as of bacterial abundance near the copper canisters. This report evaluates the presence and numbers of the microbes found at the Prototype repository. In addition to microbial assays, the oxygen levels within bentonite drill cores were analysed.

1.1 Design of the Prototype repository

The Prototype has six full-scale deposition holes distributed in two sections, as shown in Figure 1-1. The inner section farthest from the main tunnel, section 1, contains four deposition holes, while the outer section nearest the main tunnel, section 2, contains two. A full-size, electrically heated canister was placed in each deposition hole. The heaters were installed to mimic the heat emitted by the nuclear waste during actual storage; however, due to technical problems with the power supply cables, the power was reduced on canister 6 from approximately 1,600 to 1,100 W.

Around, below, and above each canister were ten rings and four blocks of compacted bentonite, filling the space between the canister and rock; this bentonite is referred to as buffer. The Prototype tunnel was filled with a mixture of sodium-exchanged bentonite and crushed rock (30% and 70%, respectively), hereafter denoted backfill (Gunnarsson 2002).

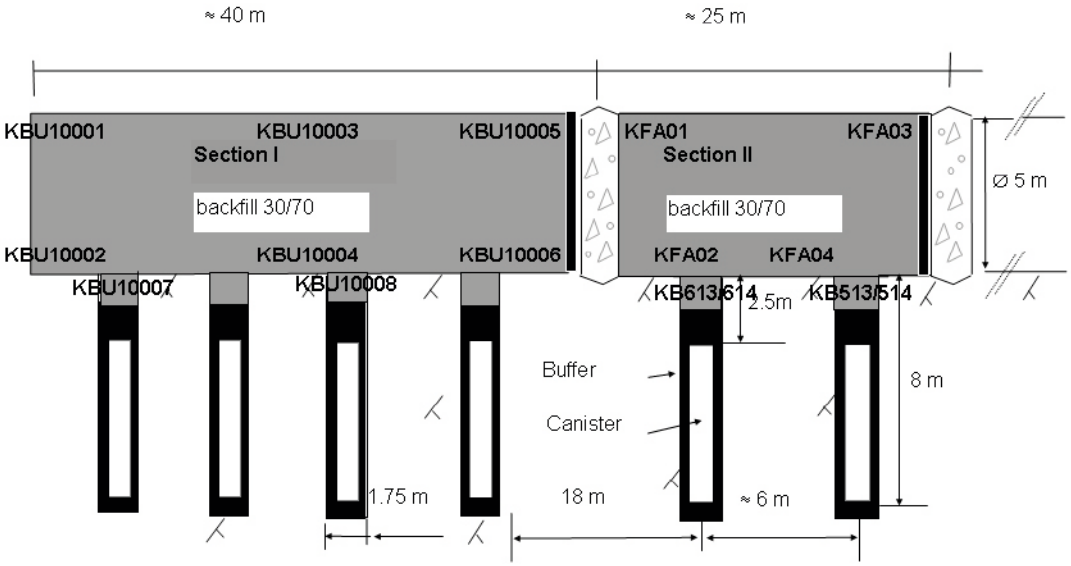

Figure 1-1. *Schematic of the Prototype repository (adapted from IPR 99-34).*

1.2 Microorganisms

The Prototype repository enabled studies of various microbial processes taking place at a facility resembling an actual storage facility of type KBS-3. Though microbial activity may reduce the oxygen level in the Prototype repository, various bacterial life strategies could nevertheless compromise the stability of the copper canister through sulphate reduction ending in sulphide production or iron reduction potentially reducing Fe(III) in montmorillonite, hence potentially affecting its properties and long-term stability (Stucki et al. 2002). As stated before, as microorganisms present at the Prototype repository may affect the repository environment in various ways, this research focused on three kinds of bacteria: SRB, culturable heterotrophic aerobic bacteria (CHAB) and iron-reducing bacteria (IRB).

Bacteria able to grow in oxygenated environments are called aerobic bacteria and can be detected by cultivation on Petri dishes with agar and media containing organic carbon. This kind of bacteria can respire oxygen and thus reduce the oxygen content of the buffer much faster than can abiotic processes. This could be beneficial for the repository, since oxygen itself is corrosive to copper. However, many bacteria able to respire oxygen also have the ability to excrete compounds not favourable for a repository for high level radioactive waste. Examples of such compounds are organic acids and bioligands that, for example, can mobilize several radionuclides (Johnsson et al. 2006, Kalinowski et al. 2006).

Other bacteria of interest are the SRB and IRB, which can be detected by MPN analysis and using molecular biology methods, such as polymerase chain reaction (PCR) and sequencing. During the oxidation of either inorganic (i.e. hydrogen gas) or organic compounds, SRB reduce sulphate to sulphide, a compound also highly corrosive to copper canisters. SRB able to oxidize inorganic compounds are called autotrophic SRB, while those able to oxidize organic compounds are called heterotrophic SRB. Both autotrophic SRB (*Desulfotomaculum spp*.) and heterotrophic SRB (*Desulfovibrio africanus*) have previously been found in bentonite (Masurat et al. 2010b, Svensson et al. 2011). The presence and survival of SRB will therefore have to be evaluated at the repository site. Concerning IRB, studies have demonstrated that members of the iron-reducing genus *Shewanella* can perform relatively rapid iron reduction at atmospheric pressure and room temperature by using australian nontronite (NAu-1; iron rich smectite) as an iron source (Kim et al. 2004). This is another smectite mineral compared to what normally dominates bentonite clay (montmorillonite).

As the reduction of structural iron in smectite is an undesired process that may reduce the swelling capacity of the buffer (Stucki et al. 2002), it is important to evaluate the presence and numbers of IRB in the buffer materials. The proposed theory is that microorganisms will be inactive within the bentonite buffer as long as the density remains high and the water content low.

A minor part of this project also involved methane oxidizing bacteria (MOB) which also are a bacterial group of importance for a final repository. MOB in fact belongs to the domain archaea and can be both aerobic and anaerobic, but it is the anaerobic oxidizing of methane (AOM) that is the relevant process for the sustainability of a respository. The AOM-process is still under debate, but the hypothesis is that SRB are able to use a metabolic intermediate of the AOM-process which accelerates the reduction of sulphate to sulphide.

1.3 Sampling

Section 1, the inner section farthest from the main tunnel, has four deposition holes labelled 1–4; section 2, the outer section nearest the main tunnel, has two deposition holes labelled 5 and 6 (Figure 1-2).

The samples discussed in this report were taken from the canister surfaces and the bentonite buffer surrounding the canisters in deposition holes 5 and 6, from the backfill of section 2, and from titanium sample collectors embedded in the buffer and backfill. In addition, experiments evaluating the composition of gas in bentonite were carried out.

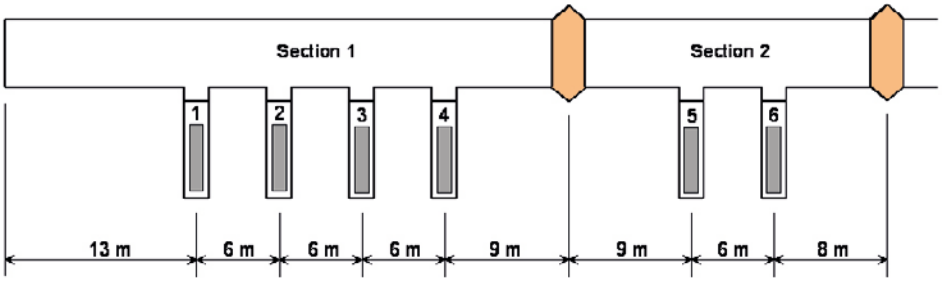

Figure 1-2. *The deposition holes in the Prototype repository (from AP TD F63.1-04-018).*

2 Materials and methods

2.1 Culturable heterotrophic aerobic bacteria

Petri dishes containing agar with nutrients were prepared as described by Hallbeck and Pedersen (2008) for determining the number of CHAB. Bentonite samples, dispersed overnight as described in Sections 2.9 and 2.10, were diluted in ten-times dilution series in sterile analytical grade water containing 1.0 g L^{-1} of NaCl and 0.1 g L^{-1} of K_2HPO_4. From each dilution, 0.1-mL portions were spread using a sterile plastic rod on the plates in triplicate. The plates were incubated in the dark as described in Table 2-1, after which the number of colony-forming units (CFUs) was counted; plates with 20–200 colonies were counted.

2.2 Medium preparation, inoculations, and analysis of most probable number of culturable iron- and sulphate-reducing bacteria and methane-oxidizing bacteria

The procedures described by Widdel and Bak (1992) for preparing anoxic media were modified and used to determine the MPN of microorganisms, as described by Hallbeck and Pedersen (2008). Five tubes were used for each ten-times dilution, resulting in an approximate 95% confidence interval lower limit of 1/3 of the obtained value and an upper limit of three times the value (Greenberg et al. 1992). Media were prepared for IRB and SRB. Aerobic medium was also prepared for MOB, as described by Pedersen et al. (2008). The cultivation time was about eight weeks to ensure that slow-growing microorganisms would be included in the results.

2.3 Microelectrodes for measuring sulphide, oxygen, pH, and E_h

Four types of electrodes were used for microscale measurement of sulphide, oxygen, pH, and E_h (all from Unisense A/S, Aarhus, Denmark); the electrodes were calibrated according to the manufacturer's protocol. The signal, or electric current, generated from the electrodes upon analysis was measured using a manual picoammeter (model PA2000; Unisense A/S) and then transferred to an A/D converter connected to a PC. Changes of saturation, concentration, and potential were recorded using SensorTrace BASIC software (Unisense A/S). The data were analysed and plotted using STATISTICA 64 (StatSoft Inc., Tulsa, OK, USA).

Table 2-1. Incubation temperatures and times for CHAB.

CHAB samples from	Incubation temperature	Incubation time
Backfill	20°C	7 days
Buffer	20°C	7 days
	40°C	3 days
Bentonite from copper corrosion experiment	18°C	7 days
	40°C	7 days
	55°C	7 days

2.4 DNA extraction and analysis

Total genomic DNA was extracted from swabs using the PowerSoil DNA isolation kit (cat. no. 12888) and from bentonite clay using the PowerMax Soil DNA isolation kit (cat. no 12988), both from MO BIO Laboratories, Carlsbad, CA, USA, according to the manufacturer's protocol. From IRB MPN cultures, 9 mL of culture were filtered onto 0.22-µm Water filters using vacuum pressure (cat. no. 14880-100-WF; MO BIO Laboratories) and total genomic DNA was extracted using the PowerWater DNA isolation kit (cat. no. 14800-100-22; MO BIO Laboratories) according to the manufacturer's protocol. Nucleotide concentrations were measured using the Nanodrop ND-1000 UV-vis spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA) and dsDNA concentrations were measured 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. The extracted DNA was stored at −20°C and subsequently used for PCR and cloning.

2.4.1 PCR screening

For all DNA extracts, PCR screening was conducted using the universal 16S rRNA primer set 27f (5´– AGAGTTTGATCCTGGCTCAG-3´) and 1492r (5´– GGTTACCTTGTTACGACTT-3´) (Lane 1991), numbers corresponding to positions in *Escherichia coli* Brosius (Brosius et al. 1978). Furthermore, the APS7f (5´– GGGYCTKTCCGCYATCAAYAC-3´) and APS8r (5´– GCACATGTCGAGGAAGTCTTC-3´) primer set specific to the adenosine-5´-phosphosulphate reductase alpha subunit gene (*apsA*) was used for screening SRB in swab DNA extracts (Friedrich 2002).

In the reactions, 1 µL of either apsA primer and 0.5 µL of either 16S rRNA primer were used for final primer concentrations of 400 nM for aps and 200 nM for 16S rRNA; the total reaction volume was 25 µL. Amplification of the *16S rRNA* gene was initiated by denaturation at 98°C (30 s) followed by a second denaturation at 98°C (30 s) and then a total of 30 cycles at 60°C (30 s) and 72°C (40 s) followed by a final terminal extension at 72°C for 5 min. Amplification of the *aps* gene was initiated by denaturation at 98°C (5 min) followed by a second denaturation at 98°C (30 s) and then a total of 31 cycles at 58.8°C (60 s) and 72°C (90 s) followed by a final terminal extension at 72°C for 7 min. Amplifications were performed using a Bio-Rad MyCycler PCR machine and iProof High-fidelity Mastermix proofreading polymerase (cat. no. 172-5310; Bio-Rad Laboratories, Hercules, CA, USA). PCR products were visualized on a 1% agarose gel containing ethidium bromide for amplicons of the appropriate size.

2.4.2 Cloning and sequencing

The diversity of IRB MPN culture microorganisms was examined by their *16S rRNA* gene sequence and swab-collected SRB microorganisms were identified by their *aps* gene. The PCR was conducted as described in Section 2.4.1. The amplification products were purified using a QIAquick gel extraction kit (cat. no. 28704; QIAGEN, Solna, Sweden) following the manufacturer's protocol. To produce 3´A overhangs of the blunt-ended iProof polymerase product, 1 µL of Taq polymerase (cat. no. 18038-042; Invitrogen) and additional dATP were added to the reactions, which were then incubated for 30 min at 72°C.

The purified samples were cloned into the linearized PCR 2.1-TOPO vector and transformed into chemically competent TOP10´F *E. coli* cells using the TOPO TA cloning kit (cat. no. K4550-01; Invitrogen) following the manufacturer's instructions. White clones containing the insert were randomly selected and each colony was inoculated in 1 mL of yeast-tryptone medium containing kanamycin (40 mg mL^{-1}) and incubated overnight at 37°C and 220 rpm. The recombinant plasmids were extracted from the bacteria using the Miniprep kit (cat. no. 27106; QIAGEN) following the manufacturer's protocol.

The sequencing was performed using the Value Read Tube service (Eurofins MWG Operon, Ebersberg, Germany) with the M13r sequencing primer (5´– CAGGAAACAGCTATGAC-3´) provided by Eurofins MWG for aps clones and the Value Read Plate service using the 907r internal 16S rRNA gene primer (5´ – CCGTCAATTCCTTTRAGTTT-3´) (Lane 1991) for the 16S rRNA clones.

Raw data sequences were screened for vector contamination using VecScreen, a specialised Basic Local Alignment Search Tool (BLAST), and screened for chimeric sequences using the Bellerophon program (Huber et al. 2004). Sequence data were analysed and aligned using the Lasergene 9 software package, the sequence alignment and assembly editor MegAlign, and SeqMan (DNASTAR, Madison, WI, USA). The *apsA* reference gene *Desulfovibrio aespoeensis* with accession number EF442013 was used as a sequence mask for the alignment of aps gene clones and the *16S rRNA* reference gene *E. coli* Brosius with accession number J01695 (Brosius et al. 1978) was used for the 16S rRNA gene clones. Furthermore, the clones were compared to sequences available in the BLAST nucleotide database on 2012-09-21. Sequence homology was analysed using either the nucleotide-nucleotide algorithm or the 16S rRNA microbial algorithm. Sequences not exhibiting a 100% match to database records were assigned individual accession numbers in GenBank (http://www.ncbi.nlm.nih.gov/genbank/).

2.4.3 Cell extraction from bentonite in an attempt to enable molecular analysis of material with low cell number

Survival of laboratory-grown bacteria in bentonite has been investigated, and naturally occurring SRB have been detected in bentonite (Masurat et al. 2010a). However, previous studies have concluded that SRB activity inside compacted bentonite should be very low or absent (Motamedi et al. 1996, Stroes-Gascoyne et al. 1997, Pedersen et al. 2000, Masurat et al. 2010a, Pedersen 2010). So far, no DNA extraction method has enabled molecular analysis of bacteria in bentonite exhibiting very low cell numbers, due to limitations attributable to the excessive volume needed to compensate for the low cell number. Furthermore, extractions are hampered by bacteria and DNA adhering to clay surfaces, and also, clay material may promote incomplete cell lysis or inhibiting enzymatic reactions (Kallmeyer et al. 2008).

In an attempt using molecular analysis to determine whether bacteria grow and survive in bentonite from buffer or backfill or in bentonite near the copper canister surface in the Prototype repository, we developed a DNA extraction method, using Nycodenz (cat. no. 1002424, GE Healthcare Bio-Sciences AB), involving pre-density gradient cell separation from bentonite before DNA extraction (Kallmeyer et al. 2008). This separation method could allow for TNC and ATP quantification of cells extracted from bentonite.

Briefly described, a primary slurry of bentonite from canister 5 ring 7 buffer and a known amount of SRB was prepared, after which the extracellular polymers, which bind the cells to the clay compartments, were hydrolyzed promoting cell detachment. After ultrasonic treatment and density gradient centrifugation through a Nycodenz solution, the cells were supposed to separate from bentonite and thus enrich the numbers of cells for DNA extraction by reducing the starting material of clay compartments. The SYBR Gold nucleotide stain (cat. no. S11494, Invitrogen) was used in detecting extracted cells by means of microscopy.

Cell extraction efficiency was low at about 1% and there were problems with sediment background fluorescence, which led to undercounting due to bentonite material shielding cells from view. In conclusion, to determine, using molecular methods, whether bacteria survive within bentonite exhibiting low cell numbers, we need to develop a more sensitive method for DNA extraction.

Bentonite samples, intended for DNA extraction, collected from backfill (see Section 2.9) and buffer (see Section 2.10) were excluded, since no protocol has so far yielded suffcient DNA concentrations from bentonite for downstream molecular analysis from bentonite, such as bacterial species identification. Further DNA extractions from bentonite located near canister 6 yielded no detectable DNA, indicating either very low cell numbers or an extraction protocol not efficient enough for such low cell numbers. In swab samples from canister 5, we could detect the *aps* gene present in SRB using both PCR methods and sequencing; it would therefore be interesting to study whether SRB were present inside the bentonite at that site as well.

2.5 Backfill samples

During excavation of the Prototype repository, 143 backfill samples were taken along a profile starting from the floor level of tunnel section 3,551.154 m and ending at the roof level at 3,556.241 m (the green line shown in Figure 2-1). Every sample was given a unique identifier and the sample locations are described using three coordinates, where *x* is the horizontal distance from the centre of the tunnel, *y* is the vertical distance from the centre of the tunnel, and *z* is the tunnel length, all measured in meters. For a full list of samples and sampling dates, see Appendix A1. All samples were individually vacuum-packaged and sent, all on the same occasion, to Microbial Analytics AB for analysis.

2.6 Buffer samples

Five bentonite profiles were sampled from rings 9, 7, 5, 3, and 1 in each of deposition holes 5 and 6 (Figure 2-1). Whenever possible, these profiles were taken in positions subject to different moisture and temperature conditions, with an emphasis on bentonite with relatively high moisture and low temperature during operation. Each profile consisted of two bentonite blocks, one in contact with the canister surface and another in contact with the rock boundary layer. For a full list of samples and location angles in the depositions holes, see Appendix A2. Immediately after sampling, the bentonite blocks were laminated and flushed with nitrogen, and then sent to Microbial Analytics AB for analysis.

In addition to the above samples, five 47-mm buffer drill core samples for gas analysis were also taken from ring 7 (70–110°) in deposition hole 5. Immediately after sampling, the cores were put in custom-made nitrogen-flushed gas-tight brass holders with an inner diameter of 50 mm. After insertion of the core, the holder was re-flushed with nitrogen to a total pressure of 2 bar.

2.7 Canister samples

Samples were collected from the canister surface on two occasions, 2010-05-30 and 2011-11-01, in deposition holes 6 and 5, respectively.

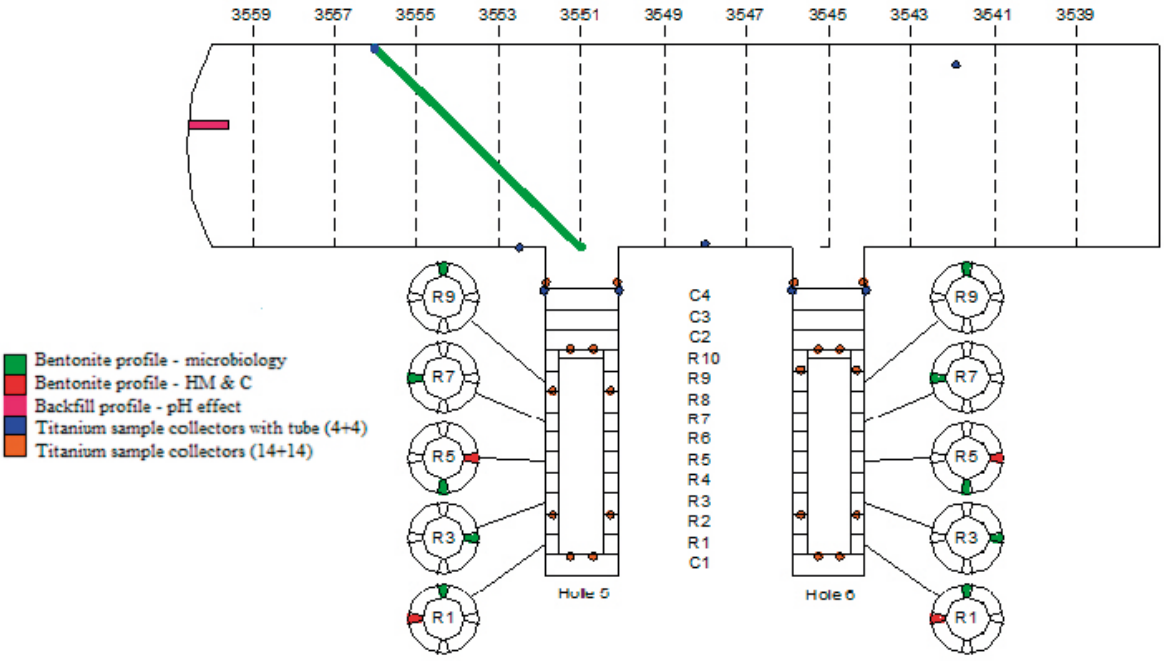

Figure 2-1. *Sampling points in the outer section of the Prototype repository.*

2.7.1 Deposition hole 6

Samples for microbiological and molecular biological analysis were taken from the bentonite and canister surface corresponding to ring 8. Microbiological samples were taken at 65° and 245° and molecular biological samples only at 245°.

One gram of bentonite per sample that had been in contact with the canister surface was scraped off with a sterile scalpel and put in an appropriate sampling vessel for DNA extraction according to Section 2.4 or for SRB culture (i.e. a tube containing 9 mL of SRB medium) according to Section 2.2 (Figure 2-2). Samples and controls were taken in batches of 4–10 samples according to Appendix A3.1. The batches were used for: DNA extraction with the PowerSoil maxi and PowerSoil kits (see Section 2.4), clay DNA extraction (see Section 2.4), and SRB cultures at 20°C and 40°C.

Swab samples were taken from the canister surface in the following manner. DNA/RNA-free cotton swabs were soaked in sterile phosphate buffered saline (PBS) and then used to thoroughly swab an area on the canister surface (approximately 10 cm^2). The swabs were then transferred to appropriate sampling vessels for DNA extraction or culture (see Appendix A3.1).

Negative controls containing only a sterile scalpel or swab, spiked controls with the addition of 0.1 mL *D. aespoeensis*, and positive controls with the addition of water from borehole KJ0052F01 were also prepared according to Appendix A3.1. The atmosphere in the tubes for SRB culture was changed to nitrogen immediately after sampling. SRB culture tubes were analysed for sulphide production using the $CuSO_4$ method, as in MPN analysis (see Section 2.2), but with single samples with either positive or negative results.

2.7.2 Deposition hole 5

Samples for microbiological and molecular biological analysis were taken from the bentonite and the canister surface corresponding to ring 7. Microbiological samples were taken at 25–70° and 70–90°, and molecular biological samples only at 25–70°.

Sampling was performed as described in Section 2.7.1 but with the following alterations: Molecular samples for PowerSoil maxi and clay DNA extraction were excluded. Samples spiked with *D. aespoeensis* culture were also included for the molecular biological samples. The groundwater added for positive controls was changed from KJ0052F01 to KA3110A groundwater.

Furthermore, bentonite residues and coating remaining on the canister after the bentonite ring had been removed were scraped off with a sterile scalpel and put in tubes containing SRB medium (Figure 2-3). Two batches of five samples each were made, one grown at 20°C and another at 40°C. Negative, positive, and spiked controls were also included, two of each (see Appendix A3.2).

Figure 2-2. *Left: The method whereby, using a sterile scalpel, bentonite samples are scraped off the buffer surface that had been in close contact with the copper canister. Right: The sampling site in deposition hole 6.*

In addition to the above sampling, a method test was also performed as follows: 5 mL of water from KA3110A and 5 mL of a *D. aespoeensis* culture were applied to the canister surface after sampling was completed. The liquids were given time to dry (~20 min). The treated surface was then scraped and swabbed for SRB cultures at 20°C and 40°C and for DNA extraction using the PowerSoil kit, resulting in a total of 20 method test samples (see Appendix A3.2).

2.8 Microbial-induced anaerobic copper corrosion

In deposition hole 5, a copper ring was installed within ring 10 as an additional method to assess anaerobic copper corrosion. Upon excavation, a sample from the bentonite surrounding the copper ring was sent to Microbial Analytics for evaluation of the presence of SRB, IRB, CHAB, and MOB.

Sampling was carried out as in Section 2.10, but the incubation was conducted at three temperatures, i.e. 18, 40, and 55°C (Table 2-1). In addition, samples for the evaluation of MOB presence were also taken and transferred to tubes containing 4.5 mL of MOB medium (see Section 2.2).

2.9 Sub-sampling of backfill samples

A total of 63 samples were selected to provide an overview of the backfill material. To avoid contaminating sample surfaces during excavation, each backfill sample was cut in half with a sterile scalpel. From each sample, one piece weighing about 1 g was cut out with a sterile scalpel and placed into a test tube containing saline solution, as described in Section 2.1. The test tubes were then held at 4°C overnight, to allow the sample to disperse in solution.

Five of these 63 buffer samples were selected for additional analysis. From each of these, three pieces weighing 1 g each were cut: one piece was treated as described above, one was placed in SRB medium, and one was placed in IRB medium (2.2). The test tubes were held at 4°C overnight, to allow the samples to disperse in solution.

The next day, the test tubes containing the samples in solution were placed on a shaker for further dispersion. Samples in growth media were then cultured according to Section 2.2, while the samples in saline solution were cultured on agar plates according to Section 2.1.

Figure 2-3. *Left: The method whereby samples are scraped off the canister surface with a sterile scalpel. Right: The sampled area on the canister surface.*

2.10 Sampling of buffer

Upon arrival at the laboratory, the samples were immediately placed in an anaerobic environment inside an anaerobic box (COY Laboratory Products, Grass Lake, MI, USA). To avoid contaminating the buffer, the first millimetre of bentonite was scraped off each sampling area with a sterile tool; 1-g samples were then taken at three different positions in the ring in order to create a microbial profile. The sampling positions were: bentonite in close contact with the boundary layer, bentonite in close contact with the canister, and bentonite from the middle of the ring (here referred to as the "bulk").

Ten samples were taken from each ring: four from the bentonite in close contact with the boundary layer, four from bentonite in close contact with the canister, and two from the bulk. Samples were cut with a sterile scalpel and then put in saline solution for CHAB analysis (see Section 2.1) and in SRB or IRB medium for MPN analysis (see Section 2.2). Samples were held at 4°C overnight to disperse before inoculation. For full list of samples, see Appendix A2.

2.11 Sampling and analysis of titanium cup content

Twelve hydrochemical sample collectors were placed in the bentonite in each deposition hole (Figure 2-1). A sample collector consists of a titanium cup with a titanium filter placed on top. Pore water from the bentonite was assumed, after saturation of the material, to flow through the filter and into the cup.

The following eight additional sample collectors were installed: four in the backfill, two in the rock–bentonite interface at the top of deposition hole 5, and two in the rock–bentonite interface at the top of deposition hole 6 (Figure 2-1). The same sample collectors were used as described above, except a tube made of polyether ether ketone (PEEK) was connected to the bottom of the cup. This enabled the retrieval of in situ samples during the test period. For a full list of sample collectors, see Appendix A4.

When the test was over and the bentonite excavation had started, the cups were located, vacuum-packaged, and sent to Microbial Analytics for analysis. Upon arrival, a 3-mm hole was drilled in each cup and the water inside was analysed using microelectrodes according to Section 2.3.

2.12 Sampling and analysis of drill core gas content

Upon arrival at the laboratory, the composition of gas in four of the five drill cores sampled from ring 7 in deposition hole 5 (see Section 2.6) was analysed. For each drill core, two gas vials (part no. 392611857; Agilent Technologies) with butyl rubber stoppers were flushed with N_2 and evacuated down to 2–4 × 10^{-2} bar. A short PEEK tube with a needle (cat. no 613-3924; BD Bioscience) was mounted on the drill core holder. The needle was inserted through the stopper and one of the taps on the holder was opened so that gas from inside the holder could flow into the gas vial (Figure 2-4).

The O_2 content was then analysed using a Bruker 450-GC gas chromatograph (Bruker Daltonics Scandinavia AB, Solna, Sweden) equipped with a pulsed discharge helium ionization detector. Samples of 100 µL were withdrawn from the gas sample vials using a helium-flushed 250-µL syringe (model 1825RN; Hamilton, Reno, NV, USA) and immediately injected into the gas chromatograph sample loop. The O_2 was separated using a Molecular Sieve 5A PLOT column (25 m × 0.32 mm) (Agilent Technologies Inc., Santa Clara, CA, USA) column with helium as the carrier gas. The integrated chromatograms obtained were referenced to calibration curves and reported as the fraction of the total amount of gas.

The fifth drill core was taken out of the holder and put in an aerobic plastic container. This drill core served as a positive control with respect to oxygen throughout the experiment. All drill cores were stored in room temperature for 41 days to let the gases equilibrate.

After 41 days, the drill core that served as the positive control was replaced in its holder, which was flushed with N_2. The gas compositions of all other drill cores were analysed again, as above, and the results from day 0 were compared with the new results.

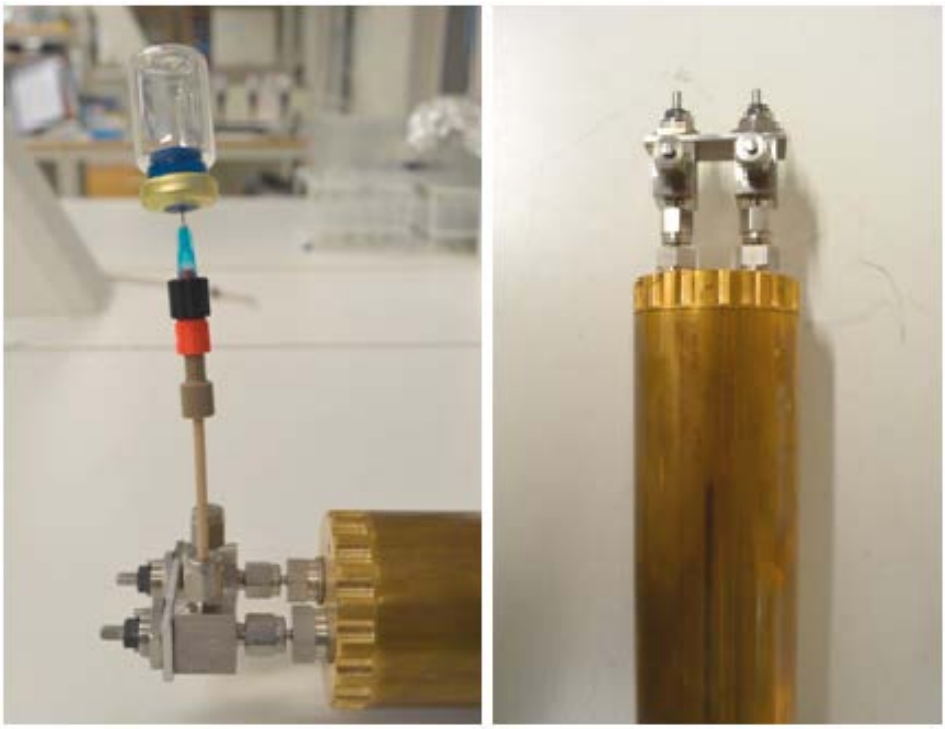

Figure 2-4. *Left: PEEK tube with needle and gas vial in position to collect gas sample. Right: design of drill core holder with two gas taps at the top.*

The whole procedure was repeated once more after 41 additional days, together with analysis of the positive control, creating three datasets of gas composition data for 0, 41, and 82 days post sampling for four samples and one positive control. If the bentonite contained oxygen, it would diffuse out of the core and mix with the N_2 atmosphere in the gap between the core and the holder.

2.13 Analysis of oxygen profiles in buffer with microelectrodes

To evaluate the possibility of using microelectrodes to measure the oxygen saturation of the bentonite, an important part of the final repository discussion, two experiments were carried out. The first experiment used bentonite from the buffer of deposition hole 6 of the Prototype repository, which had been in contact with air for at least four months and was therefore considered aerobic. A hole 30 mm deep and 5 mm wide was drilled into the bentonite and then filled with anaerobic water (Figure 2-6). The bentonite was afterwards immediately placed in a portable nitrogen-flushed anaerobic box (Figure 2-5) and real-time measurements of the oxygen saturation in the anaerobic water were then made for approximately one hour using an oxygen microelectrode according to Section 2.3.

The second experiment was carried out as above except using an anaerobic piece of bentonite from deposition hole 5. Before each experiment, the oxygen saturation of the atmosphere inside the box was analysed.

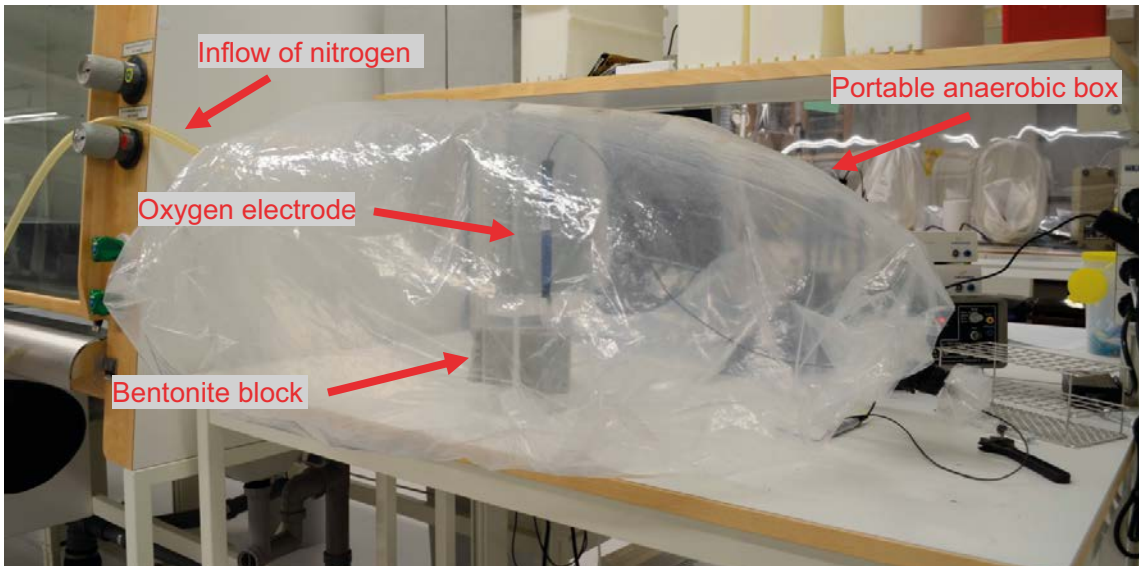

Figure 2-5. *Oxygen saturation in bentonite measured using a microelectrode inside a portable anaerobic box.*

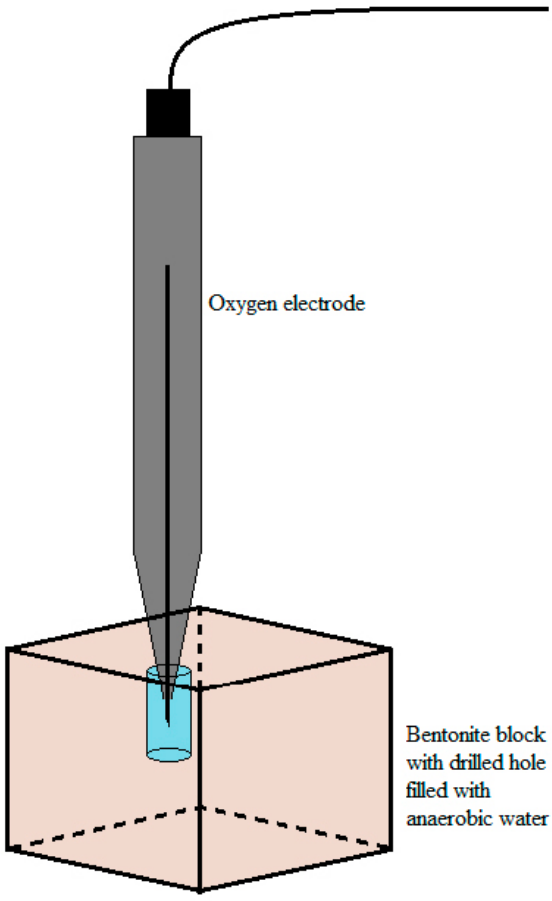

Figure 2-6. *Schematic figure of oxygen saturation measurement with microelectrode.*

3 Results

3.1 Backfill

3.1.1 Culturable heterotrophic aerobic bacteria

All but one of the 63 randomly chosen backfill samples exhibited bacterial growth to various extents. The samples that exhibited the most bacterial growth were found in the upper half and around the edges of the backfill material bordering on the tunnel wall, as shown in Figure 3-1.

3.1.2 Most probable number

From the 63 backfill samples analysed for CHAB, five were randomly chosen for MPN analysis. Sample PXP000953, which gave the highest CHAB number, also gave the highest total MPN cell number of the five samples. However, sample PXP00970, which was in the range of 10^6 to 10^7 cells per gram wet weight (cells gww^{-1}) for CHAB, was found to produce the lowest total MPN cell number. Samples PXP000926, PXP000953, and PXP000970, which were taken in the upper half, near the tunnel ceiling, all had more SRB than IRB; the reverse was the case for samples PXP001000 and PXP001028, which were taken in the middle of the tunnel.

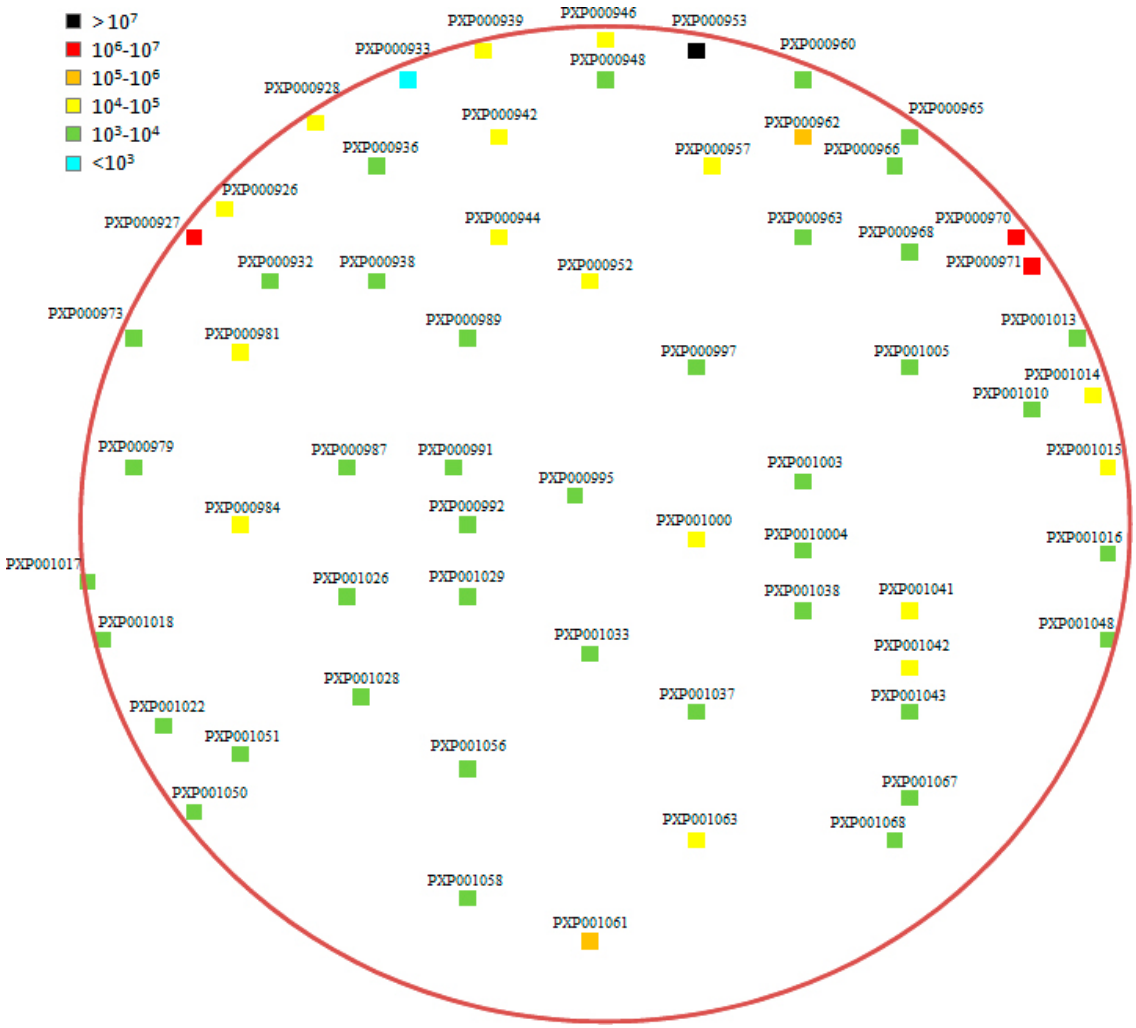

Figure 3-1. *Plot showing the CHAB results for 63 backfill samples in a profile starting from the floor level of tunnel section 3,551.154 m and ending at the roof level at 3,556.241 m. Bacterial numbers are according to the intervals shown in the legend in colony-forming units per gram wet weight (CFU gww^{-1}). Numbers $<10^3$ CFU gww^{-1} are considered below detection.*

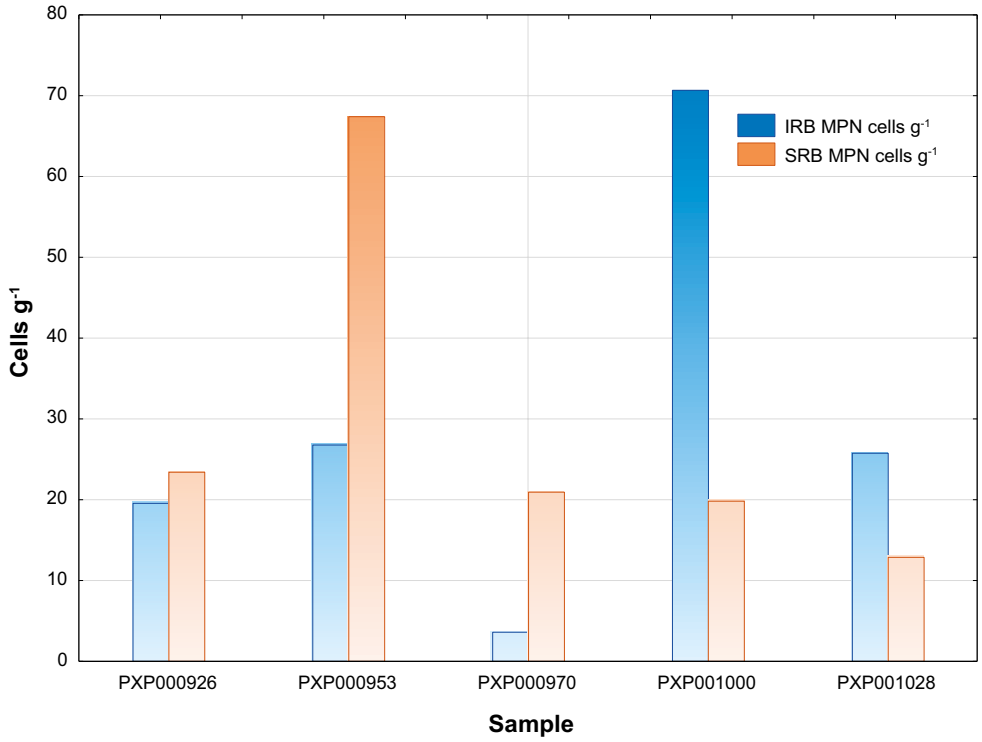

Figure 3-2. *Bar graph illustrating MPN results for IRB and SRB from five randomly chosen backfill samples. Samples PXP000926, PXP000953, and PXP000970 were taken in the upper half, near the tunnel ceiling, while PXP001000 and PXP001028 were taken in the middle of the tunnel.*

3.2 Deposition hole 6

3.2.1 Buffer

Culturable heterotrophic aerobic bacteria

All CHAB samples from deposition hole 6 were < 1.8×10^4 CFU gww^{-1}; this indicates very low numbers of aerobic bacteria, since no growth could be observed (see "Discussion").

Most probable number

Of the 20 samples analysed, three samples cultured in IRB media could be cultivated to a level above 18 cells gww^{-1}. These were two samples from ring 5 near the rock boundary layer and one from within the bulk, all cultured at 40°C (Table 3-1).

Culture media inoculated for SRB exhibited no growth. For the full list of samples, see Appendix A2.1.

Table 3-1. Positive IRB samples from buffer in deposition hole 6.

Sample ID	Weight IRB bentonite (g)	IRB MPN cells mL^{-1}	IRB MPN lower limit	IRB MPN upper limit	IRB MPN cells gww^{-1}	IRB MPN lower limit	IRB MPN upper limit
K6 Ring 5 Buffert 40 Berg 3	0.14	≥ 160	–	–	10,286	–	–
K6 Ring 5 Buffert 40 Berg 4	0.11	160	60	530	13,091	4,909	43,364
K6 Ring 5 Buffert 40 Bulk 2	0.11	3	1	12	245	82	982

Microbial diversity in IRB MPN cultures

To obtain information on the species that had been cultivated in the IRB MPN cultures from deposition hole 6 ring 5, these species were cloned as described in Section 2.4.2.

Cloning and sequencing revealed a total of six clones from within the bulk and 11 from the rock boundary layer. The species found represent the phylum Firmicutes and classes Clostridia and Bacilli. Each genus was 87–100% similar to database records in GenBank (as of 2012-09-21). Sequences not exhibiting a 100% match to database records were submitted to GenBank and assigned individual accession numbers (Table 3-2).

3.2.2 Canister

SRB growth indicated by metabolic product of sulphide

Measurement of sulphide in samples from deposition hole 6 revealed no SRB activity, since all samples were below detection (< 25 mg L^{-1}).

DNA analysis: PCR screen for SRB

Specimens sampled according to Section 2.7.1 for molecular biological analysis were not confirmed positive by PCR screening with 16S rRNA and aps primers for the presence of universal *16S rRNA* and *apsA* genes, respectively, regardless of specimen type and DNA extraction method. These results did not indicate high numbers of bacteria present at the canister surface, within the bulk, or at the rock boundary layer.

Table 3-2. 16S rDNA clones detected in IRB MPN cultures from deposition hole 6, ring 5. Samples originated from within the bulk and from the rock boundary layer. Previously undescribed clones were assigned individual accession numbers in GenBank. Expected (E) value is a weighted average of all possible values.

Sample ID	Assigned Accession no.	Sample origin	Most similar record in database	Identity %	E-value	Accession no.	*n* observations
K6 Ring 5 Bulk clone 111056:3	JX950120	Bulk	*Thermaerobacter subterraneus*	87.0	0.00	NR_028814.1	1
K6 Ring 5 Bulk clone 111056:7	JX950122	Bulk	*Thermacetogenium phaeum*	99.9	0.00	NR_024688.1	1
K6 Ring 5 Bulk clone 111056:2	JX950119	Bulk	*Bacilli bacterium JAM-FM0401*	99.6	0.00	AB362269.1	2
K6 Ring 5 Bulk clone 111056:6	JX950121	Bulk	*Syntrophothermus lipocalidus*	95.0	0.00	NR_040796.1	1
K6 Ring 5 Bulk clone 111056:1	JX950118	Bulk	*Desulfitibacter alkalitolerans*	93.0	0.00	NR_042962.1	1
K6 Ring 5 Rock clone 111063:1	JX950123	Rock	*Thermacetogenium phaeum*	95	0.00	NR_024688.1	1
K6 Ring 5 Rock clone 111063:2	JX950124	Rock	*Thermaerobacter composti*	96	0.00	AB454087.1	1
K6 Ring 5 Rock clone 111063:3	JX950125	Rock	*Thermaerobacter litoralis*	88	0.00	NR_043281.1	5
K6 Ring 5 Rock clone 111063:6	JX950126	Rock	*Pelotomaculum thermopropionicum*	92	0.00	NR_040840.1	1
K6 Ring 5 Rock clone 111063:7	JX950127	Rock	*Desulfotomaculum thermobenzoicum strain DSM 619*	89.3	0.00	AJ294430.1	1
K6 Ring 5 Rock clone 111064:2	JX950128	Rock	*Bacilli bacterium JAM-FM0401*	99.6	0.00	AB362269.1	2

3.3 Deposition hole 5

3.3.1 Buffer

Culturable heterotrophic aerobic bacteria

The number of CHAB present in samples from buffer in deposition hole 5 were all below 1.8×10^3 CFU gww^{-1} (see "Discussion").

Most probable number

All but one MPN analysis for IRB produced values below 1.8 cells gww^{-1}. The culturable sample from deposition hole 5 sampled from ring 9 located near the copper canister cultured at 40°C had 6.36 cells gww^{-1} (Table 3-3).

Table 3-3. Positive IRB sample from buffer in deposition hole 5.

Sample ID	Weight IRB bentonite (gww)	IRB MPN cells mL^{-1}	IRB MPN lower limit	IRB MPN upper limit	IRB MPN cells gww^{-1}	IRB MPN lower limit	IRB MPN upper limit
K5 Ring 9 Buffert 40 Kapsel 3	0.99	0.7	0.2	2.10	6.36	1.82	19.1

The remaining 19 IRB samples and all 20 samples cultured in SRB media were below detection for all buffer samples from deposition hole 5 (see Appendix A2.2).

3.3.2 Canister

SRB growth indicated by metabolic product of sulphide

Samples for the microbial analysis of SRB growth, indicated by the presence of the metabolic product sulphide were taken from the canister surface as in Section 2.7.2. Two cultures inoculated with bentonite that had been in contact with the canister surface cultivated in SRB media at 40°C had a positive value of sulphide, 26 mg L^{-1}, indicating the presence of viable SRB.

All other SRB cultures from bentonite or canister surface material were below the detection limit (< 25 mg L^{-1}); however, one of the swab samples for which the canister surface was spiked with KA3110A groundwater to test the sampling method contained 33 mg L^{-1} of sulphide.

DNA analysis: PCR screen for universal bacteria and cloning of the aps *gene*

To evaluate the presence of bacteria, SRB in particular, at the boundary between the copper canister and the bentonite layer, samples for molecular biological analysis were taken as in Section 2.7.2 and analysed as in Section 2.4. The universal *16S rRNA* gene could be detected in eight of ten samples taken at ring 7, indicating the presence of bacteria at the site. Furthermore, two of the eight samples positive for the *16S rRNA* gene also exhibited a positive result for the *apsA* gene, indicating the presence of SRB. These double positive samples were cloned as in Section 2.4.2 to identify the SRB species present. The closest relative found when aligned to known *aps* sequences in GenBank on 2012-03-13 was *Desulfovibrio aespoeensis strain Aspo 2* with accession number EF442013.1. Isolated clones exhibited > 99% similarity to database records. The method control sample spiked as in Section 2.7.2 confirmed that the methods were working properly, by indicating detection of the *16S rDNA* and *apsA* genes as expected.

3.3.3 Bentonite from copper corrosion experiment

Bentonite samples taken from the vicinity of the copper ring installed at the top of the canister in deposition hole 5 were all below detection except for IRB cultured at 40°C, which gave a slightly positive MPN result at 2.9 cells gww^{-1}.

3.4 Titanium cups

A total of 27 titanium cups were sent to Microbial Analytics AB for analysis of sulphide, oxygen, pH, and E_h using microelectrodes. However, since no water could be found in any of the cups, further analysis was precluded.

3.5 Gas in buffer

Two experiments were conducted to evaluate the gas content of the bentonite, one using drill cores from deposition hole 5 and another using aerobic and anaerobic buffer material. Both experiments were based on gas diffusion to equilibrate concentration differences.

3.5.1 Drill cores

No obvious difference in O_2 content could be measured between the four drill cores and the positive control (Figure 3-3), which indicates that this method is too blunt for this application. In addition, all drill cores, except no. 1, had high starting values on day 0, possibly due to technical sampling problems (see "Discussion").

3.5.2 Electrodes

The oxygen saturation of the atmosphere inside the box was approximately 3.5% and 1.5% in experiments 1 and 2, respectively.

Experiment 1 – aerobic bentonite: In Figure 3-4, the oxygen saturation rises in the oxygen-free water from 1% to about 6%; it then begins to level out before drastically decreasing to about 3%, which corresponds to the saturation of oxygen in the box at the time of sampling.

Experiment 2 – anaerobic bentonite: The path of the red line in Figure 3-4 contrasts markedly with that of the blue line. The oxygen saturation flattens out quickly above 0.5% before drastically increasing to about 2%, which corresponds to the approximate oxygen saturation in the box at the time of sampling.

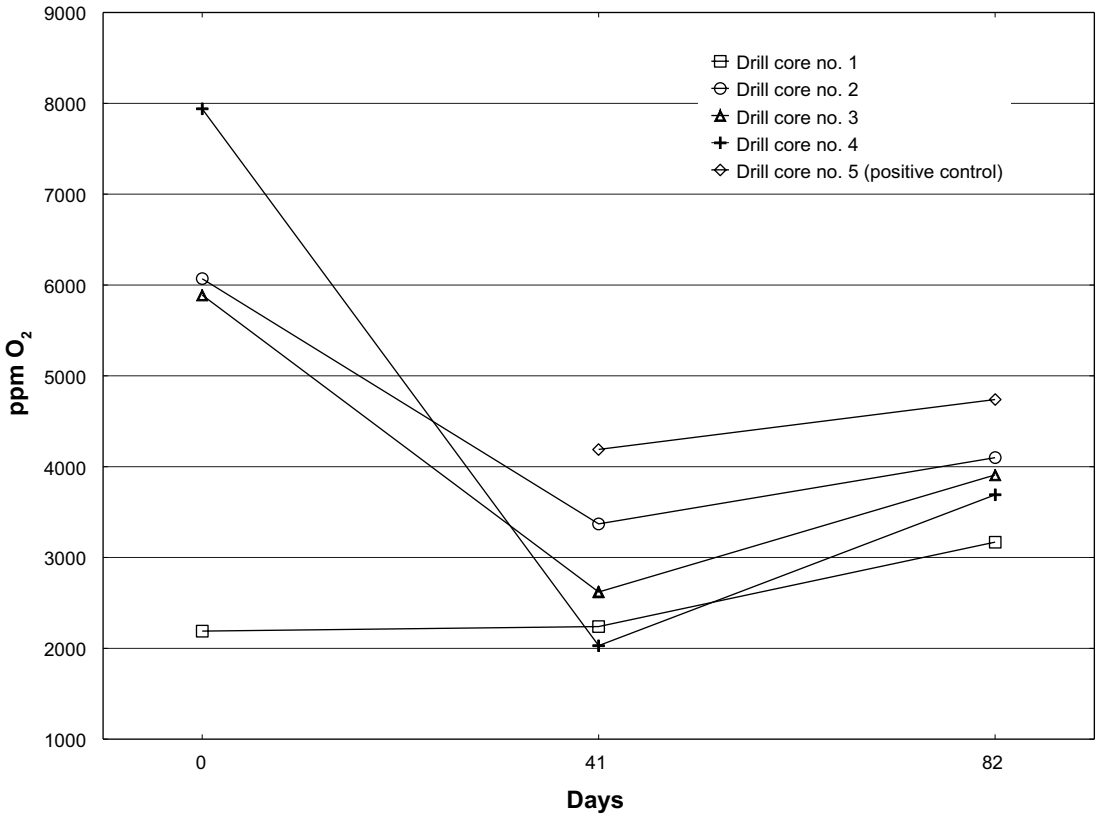

Figure 3-3. *Gas content of drill cores nos. 1–5, measured on days 0, 41, and 82.*

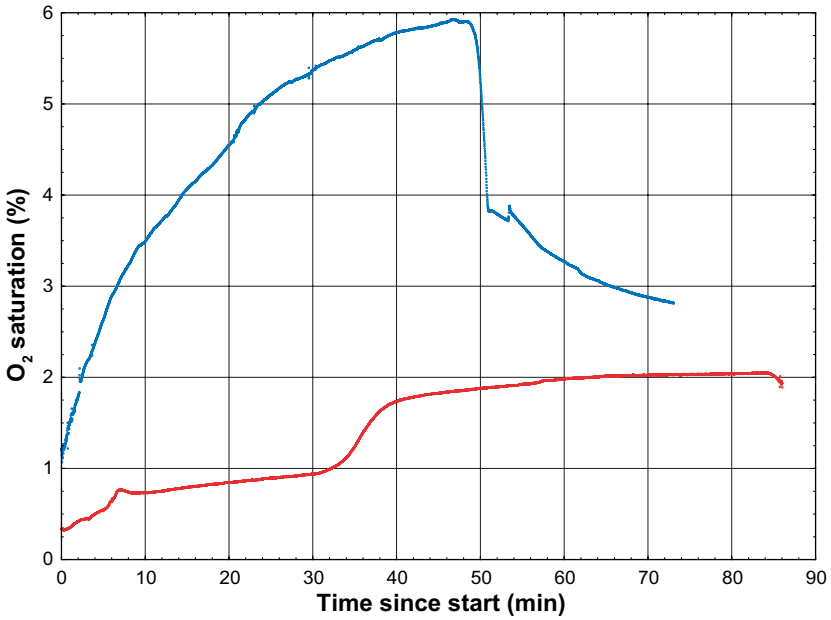

Figure 3-4. *O_2 saturation plotted against time for aerobic and anaerobic bentonite. The blue line represents aerobic bentonite and the red line represents anaerobic bentonite.*

4 Discussion

The Prototype repository is a field experiment located in the Äspö HLR, where the processes underway in a KBS-3 type nuclear waste repository are studied under near authentic conditions. The excavation of section 2 comprising deposition holes 5 and 6 enables the study of microorganisms that might be present and viable at the Prototype repository site in the buffer and backfill and near the copper canisters.

Since the construction of the Prototype, an extensive monitoring program has been conducted to follow developments at the site. Pore water from section 2 has been difficult to extract likely due to the experimental setup. Bacterial numbers as well as the ATP content of pore water have been low throughout the monitoring program (Eriksson 2007, Lydmark 2010, 2011).

The oxygen levels in the Prototype have decreased over time faster than would be expected from abiotic processes alone. This rapid decrease could be due to aerobic bacteria that consume oxygen in their respiration. However, since the Prototype is a drained repository, the oxygen decrement could also be due to the degassing of anaerobic groundwater that flows through the Prototype releasing, for example, nitrogen, which over time creates an anaerobic environment. This would not be the case in an actual repository. As the oxygen level decreases, a possibly problematic microbial group, the anaerobic SRB, could emerge. These microbes are not active in aerobic environments. SRB produce sulphide, a compound corrosive to copper. The numbers of SRB in the backfill and buffer pore water have so far usually been found to be quite low, approximately 1 SRB mL^{-1}, but they are occasionally higher. However, SRB such as *D. aespoeensis* have repeatedly been observed in Äspö groundwater from outside the Prototype repository in numbers of 10^4 to 10^6 cells mL^{-1} (Eydal et al. 2009).

A source of SRB is the bentonite itself. It has previously been reported that SRB exist and survive in the MX-80 bentonite used in the Prototype repository (Masurat et al. 2010a). However, studies of the viability of bacteria in bentonite have estimated that the numbers will stay low as long as the bentonite has a high swelling pressure, which results in too restricted a space for microorganisms to be able to migrate and grow efficiently (Pedersen 2010, Persson et al. 2011). However, this limited space for microorganisms could change due to the action of IRB, which can promote the reduction of structural Fe(III) in bentonite, an undesired process that can reduce the swelling capacity of the buffer. Consequently, it is important to evaluate the presence and numbers of IRB in the buffer materials.

Decommission analysis will indicate how well SRB and IRB have coped in recent years in the oxygenated environment, whether they can survive in a water-saturated repository, and also whether they will migrate through the backfill and buffer towards the canister after the oxygen is depleted in the Prototype.

4.1 Backfill

The results from 63 of the 143 randomly chosen backfill samples analysed for CHAB gave a good understanding of the cultivability of aerobic bacteria in the backfill material. As the results indicate, the best position for bacterial growth seems to be the boundary between the tunnel ceiling and the backfill material, where the highest bacterial numbers, i.e. both MPN and CHAB, were observed. The backfill–rock boundary area also seems to favour SRB over IRB (Figure 3-2), possibly because sulphate-rich, anaerobic groundwater seeps through cracks in the rock and comes into contact with the upper backfill layer, creating a favourable milieu for SRB. Geotechnical studies performed by Clay Technology AB on the water content of the backfill material also support this theory, since high water content correlates with high bacterial numbers (Olsson et al. 2013). On the other hand, backfill samples taken from the middle of the tunnel contained more IRB than SRB – bearing in mind that the data come from only five samples and are not statistically confirmed. The correlation between higher numbers of CHAB and SRB at the same sites could furthermore be explained by the fact that CHAB are responsible for the decrease in oxygen levels that creates the beneficial anaerobic habitats for SRB present at the same sites.

The Prototype repository tunnel backfill was prepared from 70% crushed rock and 30% sodium-exchanged bentonite material from Greece (Gunnarsson 2002). The purpose and function of the backfill in deposition tunnels is to sustain the multi-barrier principle by keeping the buffer in place and restricting groundwater flow through the deposition tunnels. In the actual repository, the backfill will (according to current plans) consist of bentonite blocks and pellets that will fill the tunnels completely from top to bottom. The proposed backfill design for the KBS-3 model is thus completely different from that used in the Prototype, so the CHAB and MPN analysis results presented here are not directly applicable to the final repository. However, the possible influx of nutrients from surrounding groundwater that favoured bacterial growth in the upper boundary area between the backfill and the rock in the Prototype could also occur in the KBS-3 model.

4.2 Buffer

4.2.1 Microbial composition in buffer

The numbers of SRB and CHAB were determined to be below detection. IRB were detected in varying numbers in the buffer. Most samples were below detection, but two samples from ring 5 in deposition hole 6 exhibited very high IRB growth. Both these samples were inoculated with bentonite from near the rock wall. One sample from the bulk of the same ring also produced a positive, but lower, MPN result. Samples taken from ring 5 near the canister all gave values below detection.

This creates a profile of bacterial numbers going from lower to higher the farther away from the canister a sample is taken. In the same manner as for the backfill, areas with high water content are also predicted to have high bacterial numbers. This correlates well with the water content of ring 5, which is among the highest (~26%) of all the analysed rings from the Prototype repository (Olsson et al. 2013). Ring 5 also has a higher water content nearer the rock wall than near the canister, especially at our sampling point around 255°, which also correlates well with the bacterial numbers. What is also worth mentioning about these results is that only 0.1 g of bentonite was used to inoculate the buffer cultures from deposition hole 6. When the amount of sample used for inoculation was increased nearly ten-fold to ~1 g for cultures from deposition hole 5, most did not reach the detection limit. Whether this result is only because the water content of the sampled rings from deposition hole 5 is lower than in deposition hole 6 is unclear, but the water content is definitely crucial for the bacterial numbers.

Another factor known to influence bacterial numbers is the density of the buffer. At ring 5 in deposition hole 6, IRB samples gave high bacterial numbers that could be correlated with a lower density of ~1,520 kg m^{-3} at that sampling site compared with 1,600–1,740 kg m^{-3} near the canister, where we could not detect any IRB. All density data are preliminary and provided by Clay Technology AB (Olsson et al. 2013).

In 2005, microbial composition experiments examining buffer material, similar to those conducted here for the Prototype, were conducted in the Canister Retrieval Test (CRT) project. The same trend, in which bacterial numbers decline with distance from the canister, could also be seen in the CRT experiments. The buffer water content of samples that exhibited growth in the CRT experiments was also around 26%, which closely matches our results from this experiment. However, sampled areas in the Prototype repository within ring 9 in deposition hole 6 and ring 5 in deposition hole 5 with a water content around 26% (and higher) and a low swelling pressure (~1,520 kg m^{-3}) displayed no signs of viable bacteria. In the CRT experiments, viable bacteria could also be found in buffer with a dry density over 2,000 kg m^3 (Lydmark and Pedersen 2011), something that we have been unable to find in Prototype buffer samples. The proposed theory is that no microorganisms will survive within the bentonite buffer as long as the density remains high.

In an actual repository, the water content near the canister will be low (~5% or lower for the first 1000 years) due to the heat produced by the radioactive material (Aoki et al. 2010). However, when the canister starts to cool, the water content of the bentonite near the canister may increase, producing a more favourable milieu for microbes such as IRB and possibly leading to potential problems with reduction of trivalent iron in montmorillonite, hence potentially affecting its properties and long-term stability (Stucki et al. 2002). This is a fairly recently described process that was not considered in projects such as the CRT or the Long-term Test of Buffer Material (LOT); it merits consideration in future studies.

To inquire into which species were cultivated in the IRB MPN, the *16S rDNA* gene was cloned, generating database matches of ~90% to known thermophilic bacteria. This is consistent with previous findings regarding thermophilic bacteria in bentonite clay (Svensson et al. 2011). Since the buffer in a repository will be exposed to high temperatures for up to 10,000 years, the abundance of thermophilic bacteria is extremely important, since this group of bacteria could persist for a long time in the buffer even at high temperatures. Furthermore, the classes found were Clostridia and Bacillus belonging to the phylum Firmicutes, many of which have the potential to form a dormant endospore structure. Endospores are resistant to high temperatures and to radiation and can withstand a lack of nutrients and water. As soon as the environment become more favourable, the endospore switches to a vegetative state. This implies that endospores may be present at the site; if conditions change and become more favourable, they may become viable bacteria. Most of the genera found were anaerobes associated with bacteria found in sediments, some of which are syntrophic with IRB. This suggests that IRB may be present, even though we could not detect them by cloning.

4.2.2 Gas content of buffer

The rate at which oxygen disappears from the buffer and the factors that are important in this process are central issues in the final repository debate. Here we have conducted two pilot experiments in which we measured the oxygen content of drill cores and the oxygen saturation in aerobic and anaerobic bentonite using microelectrodes installed in drilled holes filled with anaerobic water.

Drill cores

Figure 3-3 shows the oxygen contents of the five drill cores taken from deposition hole 5 in the Prototype repository. If the bentonite contained any oxygen, our theory was that it would diffuse out of the bentonite to the N_2 atmosphere in the gap between the holder wall and the drill core. However, for drill cores 2, 3, and 4 we experienced problems initially removing the oxygen, resulting in very high starting values on day 0. By day 41, the levels had dropped, probably due to oxygen having diffused into instead of out of the drill cores. For drill core 1, we observed a slight increase in oxygen content between days 41 and 82. However, the increase for the aerobic positive control was approximately the same as for drill core 1, suggesting that the increase probably came from vial leakage or oxygen contamination when injecting the sample into the chromatograph. All in all, this method was found to be too blunt for this type of application; therefore, we looked into the possibility of using microelectrodes, which are much more sensitive.

Microelectrodes

According to our hypothesis, any oxygen in the aerobic bentonite will diffuse into the oxygen-free water. This diffusion will cease once the oxygen saturation is the same in the water and the bentonite. During this diffusion process, the bentonite will absorb water and eventually dry out the drill-hole. When the drill-hole has completely dried out, the electrode will instead measure oxygen saturation in the surrounding atmosphere, which naturally will be the same as in the rest of the anaerobic box. The blue line in Figure 3-4 could describe such a process. Oxygen saturation in the water rises from 1% to 6% until the water in the hole has completely dried out, after which the electrode begins to measure oxygen saturation in the atmosphere; hence, it drops to about 3%, which was the oxygen saturation in the box at that time.

The reverse process would be expected for anaerobic bentonite, in which the oxygen saturation would first be unaffected, except for perhaps a small increase coming from the remaining oxygen in the box. When the water dries out, as in the previous case, the oxygen saturation will equal that of the surrounding atmosphere. The red line in Figure 3-4 follows such a pattern, in which the oxygen saturation levels out at approximately 0.5% and then rapidly increases to about 2%, which represents atmospheric oxygen saturation inside the box at the time of this experiment.

A clear difference in oxygen saturation could be seen between the aerobic and anaerobic bentonite, which suggests that this is an effective method for measuring oxygen in bentonite. Even though the data from this experiment come from only single measurements and are not statistically confirmed, we see good potential to develop this methodology further.

4.3 Canisters

SRB naturally occur in bentonite and groundwater, as demonstrated by previous studies, so main questions when decommissioning the Prototype are whether these SRB have survived in the boundary between the buffer and the copper canister and whether biofilm formation could be observed on the canister surface. One hypothesis is that SRB from the surrounding groundwater may migrate through the backfill and buffer towards the canister. However since the culturable numbers of these bacteria were low in the centre of the backfill and in the buffer, it seems as though migration from outside groundwater did not occur to any great extent. Further analysis of the growth of SRB, as indicated by the presence of their metabolic product, sulphide, did not find sulphide concentrations high enough to signify high SRB activity at the copper canister surface. This low cultivability might indicate that microbial activity was very low in the sample or that not enough material was sampled given the low bacterial numbers or even dormant populations to allow the culturing methods to recover the SRB.

Since cultivation could only barely detect viable bacteria at the site, molecular techniques were used for their potential to detect not only viable bacteria, but bacterial residues as well. Bacteria or bacterial residues could be detected by targeting the *16S rDNA* gene. Furthermore, by targeting *apsA*, a gene specific to SRB, we could also specifically detect SRB at the site.

The results indicate that bacterial cells, potentially *D. aespoeensis*, are present but not culturable, since we detected SRB on the deposition hole 5 canister using molecular methods but not using microbial cultivation methods. SRB have previously been reported to form biofilms on copper surfaces, although to a lesser extent than other known biofilm systems. (Persson et al. 2011). This implies that if SRB can find their optimal conditions, they could become viable and potentially produce sulphide, which is not favourable for canisters located in the KBS-3 concept repository.

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SKB's (Svensk Kärnbränslehantering AB) publications can be found at www.skb.se/publications.

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Appendix A

A1 Backfill samples

Sample	Micans number	Sampling date	Weight CHAB bentonite (g)	Weight IRB bentonite (g)	Weight SRB bentonite (g)	CHAB ml^{-1}	SD CHAB	CHAB gww^{-1}	SD CHAB	Length (m), *z*	Horizontal distance (m), *x*	Vertical distance (m), *y*
PXP000926	110974	2011−09−12	1.00	1.07	1.05	1.70E+03	1.59E+02	1.53E+04	1.43E+03	3,555.68	−1.842	1.612
PXP000927	110944	2011−09−01	1.00			5.40E+05	8.72E+04	4.86E+06	7.85E+05	3,555.233	−1.994	1.425
PXP000928	110980	2011−09−12	1.01			1.19E+03	3.24E+02	1.06E+04	2.89E+03	3,556.046	−1.459	2.01
PXP000929										3,556.053	−1.512	1.867
PXP000930										3,555.977	−1.555	1.787
PXP000931										3,555.705	−1.547	1.468
PXP000932	110981	2011−09−12	1.00			5.47E+02	1.53E+01	4.92E+03	1.38E+02	3,555.248	−1.62	1.239
PXP000933	110941	2011−08−31	0.99			8.42E+01	2.52E+01	7.65E+02	2.29E+02	3,556.182	−0.986	2.252
PXP000934										3,556.14	−0.997	2.152
PXP000935										3,556.079	−1.024	2.034
PXP000936	111000	2011−09−21	1.00			2.30E+02	2.65E+01	2.07E+03	2.39E+02	3,556.046	−1.068	1.78
PXP000937										3,555.669	−1.049	1.493
PXP000938	111001	2011−09−21	0.99			5.77E+02	2.10E+01	5.25E+03	1.91E+02	3,555.244	−1.104	1.242
PXP000939	111002	2011−09−21	0.99			6.83E+03	2.54E+03	6.21E+04	2.31E+04	3,556.256	−0.57	2.382
PXP000940										3,556.252	−0.572	2.307
PXP000941										3,556.225	−0.593	2.205
PXP000942	110982	2011−09−12	1.00			3.63E+03	1.55E+03	3.27E+04	1.40E+04	3,556.133	−0.533	1.938
PXP000943										3,555.964	−0.541	1.7
PXP000944	110945	2011−09−01	1.00			2.05E+03	5.73E+02	1.85E+04	5.16E+03	3,555.632	−0.553	1.469
PXP000945										3,555.209	−0.595	1.258
PXP000946	110942	2011−08−31	0.99			1.01E+04	2.31E+03	9.18E+04	2.10E+04	3,556.241	−0.003	2.469
PXP000947										3,556.201	−0.046	2.353
PXP000948	111003	2011−09−21	1.01			5.27E+02	1.25E+02	4.70E+03	1.11E+03	3,556.205	−0.031	2.243
PXP000949										3,556.171	−0.045	1.954
PXP000950										3,555.956	−0.022	1.713
PXP000951										3,555.604	−0.071	1.426

Sample	Micans number	Sampling date	Weight CHAB bentonite (g)	Weight IRB bentonite (g)	Weight SRB bentonite (g)	CHAB ml^{-1}	SD CHAB	CHAB gww^{-1}	SD CHAB	Length (m), *z*	Horizontal distance (m), *x*	Vertical distance (m), *y*
PXP000952	111004	2011-09-21	0.99			2.04E+03	2.07E+02	1.85E+04	1.88E+03	3,555.097	−0.08	1.228
PXP000953	110975	2011-09-12	1.04	1.02	1.08	1.30E+06	1.00E+05	1.13E+07	8.65E+05	3,556.129	0.449	2.398
PXP000954										3,556.086	0.465	2.291
PXP000955										3,556.051	0.472	2.194
PXP000956										3,556.057	0.474	1.966
PXP000957	110983	2011-09-12	1.00			2.90E+03	1.71E+03	2.61E+04	1.54E+04	3,555.903	0.48	1.787
PXP000958										3,555.583	0.477	1.413
PXP000959										3,555.1	0.434	1.222
PXP000960	110943	2011-08-31	1.01			3.70E+02	1.15E+02	3.30E+03	1.02E+03	3,555.906	0.958	2.223
PXP000961										3,555.853	0.935	2.082
PXP000962	110984	2011-09-12	1.02			2.87E+04	1.37E+04	2.53E+05	1.21E+05	3,555.867	0.949	1.981
PXP000963	110946	2011-09-01	1.01			6.17E+02	1.26E+02	5.50E+03	1.12E+03	3,555.559	0.953	1.432
PXP000964										3,555.044	0.954	1.155
PXP000965	110956	2011-09-06	0.99			2.53E+02	2.65E+01	2.30E+03	2.41E+02	3,555.852	1.445	1.986
PXP000966	111005	2011-09-21	0.99			3.23E+02	8.14E+00	2.94E+03	7.40E+01	3,555.823	1.402	1.797
PXP000967										3,555.789	1.41	1.687
PXP000968	110985	2011-09-15	1.00			6.83E+02	3.80E+02	6.15E+03	3.42E+03	3,555.509	1.427	1.398
PXP000969										3,555.082	1.436	1.171
PXP000970	110976	2011-09-12	1.07	1.01	1	3.37E+05	5.03E+04	2.83E+06	4.23E+05	3,555.235	1.959	1.415
PXP000971	110947	2011-09-01	1.01			7.07E+05	7.65E+04	6.30E+06	6.82E+05	3,555.153	2.026	1.285
PXP000972										3,555.051	2.097	1.197
PXP000973	110986	2011-09-15	0.99			4.50E+02	1.65E+02	4.09E+03	1.50E+03	3,554.79	−2.337	0.935
PXP000974										3,554.493	−2.419	0.583
PXP000975										3,554.108	−2.465	0.286
PXP000976										3,553.767	−2.509	0.051
PXP000977										3,554.751	−2.16	0.888
PXP000978										3,554.453	−2.269	0.532
PXP000979	110948	2011-09-01	1.01			7.00E+02	1.73E+02	6.24E+03	1.54E+03	3,554.098	−2.249	0.261
PXP000980										3,553.748	−2.235	0.021
PXP000981	110957	2011-09-06	1.00			1.20E+03	1.73E+02	1.08E+04	1.56E+03	3,554.813	−1.728	0.885
PXP000982										3,554.44	−1.742	0.529
PXP000983										3,554.098	−1.758	0.274
PXP000984	110987	2011-09-15	0.99			1.16E+03	3.12E+02	1.05E+04	2.84E+03	3,553.666	−1.772	−0.008

Sample	Micans number	Sampling date	Weight CHAB bentonite (g)	Weight IRB bentonite (g)	Weight SRB bentonite (g)	CHAB ml^{-1}	SD CHAB	CHAB gww^{-1}	SD CHAB	Length (m), *z*	Horizontal distance (m), *x*	Vertical distance (m), *y*
PXP000985										3,554.752	−1.151	0.903
PXP000986										3,554.476	−1.23	0.59
PXP000987	111007	2011−09−21	1.01			8.03E+02	2.70E+02	7.16E+03	2.41E+03	3,554.099	−1.214	0.314
PXP000988										3,553.628	−1.213	−0.017
PXP000989	110988	011−09−15	1.00			6.13E+02	3.06E+01	5.52E+03	2.75E+02	3,554.755	−0.665	0.937
PXP000990										3,554.409	−0.684	0.544
PXP000991	110989	2011−09−15	1.02			3.30E+02	4.00E+01	2.91E+03	3.53E+02	3,553.999	−0.722	0.262
PXP000992	110949	2011−09−01	1.01			7.87E+02	7.23E+01	7.01E+03	6.44E+02	3,553.612	−0.673	0.022
PXP000993										3,554.709	−0.103	0.948
PXP000994										3,554.411	−0.151	0.526
PXP000995	111008	2011−09−21	1.02			8.37E+02	8.08E+01	7.39E+03	7.13E+02	3,554.008	−0.123	0.177
PXP000996										3,553.583	−0.144	−0.048
PXP000997	110990	2011−09−15	1.01			8.27E+02	6.51E+01	7.37E+03	5.80E+02	3,554.757	0.444	0.779
PXP000998										3,554.364	0.422	0.491
PXP000999										3,553.996	0.404	0.165
PXP001000	110977	2011−09−12	1.01	1.03	1.01	2.03E+03	3.21E+02	1.81E+04	2.86E+03	3,553.579	0.409	−0.087
PXP001001										3,554.761	0.963	0.782
PXP001002										3,554.396	0.974	0.496
PXP001003	110950	2011−09−01	1.00			3.10E+02	7.94E+01	2.79E+03	7.15E+02	3,554	0.941	0.184
PXP001004	110991	2011−09−15	1.04			1.01E+03	1.30E+02	8.74E+03	1.13E+03	3,553.595	0.951	−0.119
PXP001005	110958	2011−09−06	1.00			8.80E+02	9.54E+01	7.92E+03	8.59E+02	3,554.831	1.482	0.827
PXP001006										3,554.462	1.482	0.545
PXP001007										3,554.031	1.478	0.222
PXP001008										3,553.594	1.518	−0.129
PXP001009										3,554.809	2.027	0.924
PXP001010	111010	2011−09−22	1.01			6.17E+02	7.77E+01	5.50E+03	6.92E+02	3,554.422	2.007	0.568
PXP001011										3,554.038	2.017	0.267
PXP001012										3,553.605	2.043	−0.127
PXP001013	111011	2011−09−22	0.99			2.37E+02	3.21E+01	2.15E+03	2.92E+02	3,554.844	2.231	0.963
PXP001014	110992	2011−09−15	1.01			9.47E+03	1.69E+03	8.44E+04	1.51E+04	3,554.415	2.332	0.642
PXP001015	110951	2011−09−01	1.01			5.23E+03	7.02E+02	4.66E+04	6.26E+03	3,553.922	2.41	0.288
PXP001016	111012	2011−09−22	1.00			4.30E+02	5.29E+01	3.87E+03	4.76E+02	3,553.577	2.402	−0.106
PXP001017	110993	2011−09−15	0.99			6.63E+02	1.81E+02	6.03E+03	1.65E+03	3,553.313	−2.485	−0.287

Sample	Micans number	Sampling date	Weight CHAB bentonite (g)	Weight IRB bentonite (g)	Weight SRB bentonite (g)	CHAB ml^{-1}	SD CHAB	CHAB gww^{-1}	SD CHAB	Length (m), *z*	Horizontal distance (m), *x*	Vertical distance (m), *y*
PXP001018	111013	2011-09-22	1.01			5.67E+02	2.77E+02	5.05E+03	2.47E+03	3,552.943	−2.407	−0.587
PXP001019										3,552.388	−2.279	−0.975
PXP001020										3,553.298	−2.221	−0.306
PXP001021										3,552.941	−2.185	−0.598
PXP001022	110952	2011-09-01	0.99			5.17E+02	1.94E+02	4.70E+03	1.76E+03	3,552.39	−2.099	−0.98
PXP001023										3,553.31	−1.778	−0.315
PXP001024										3,552.844	−1.727	−0.645
PXP001025										3,552.435	−1.702	−0.916
PXP001026	111014	2011-09-22	1.00			6.47E+02	3.57E+02	5.82E+03	3.21E+03	3,553.264	−1.221	−0.368
PXP001027										3,552.82	−1.2	−0.659
PXP001028	110978	2011-09-12	1.03	1.06	0.99	6.53E+02	2.01E+02	5.71E+03	1.76E+03	3,552.441	−1.19	−0.894
PXP001029	110994	2011-09-15	1.03			4.90E+02	9.54E+01	4.28E+03	8.34E+02	3,553.228	−0.631	−0.382
PXP001030										3,552.802	−0.673	−0.658
PXP001031										3,552.386	−0.65	−0.896
PXP001032										3,553.223	−0.108	−0.341
PXP001033	111015	2011-09-22	1.01			8.33E+02	5.77E+01	7.42E+03	5.14E+02	3,552.746	−0.111	−0.666
PXP001034										3,552.35	−0.114	−0.896
PXP001035										3,553.116	0.404	−0.409
PXP001036										3,552.74	0.387	−0.669
PXP001037	110953	2011-09-01	0.99			3.33E+02	5.77E+01	3.03E+03	5.25E+02	3,552.365	0.429	−0.934
PXP001038	111016	2011-09-22	0.99			5.73E+02	2.01E+02	5.21E+03	1.83E+03	3,553.192	0.938	−0.407
PXP001039										3,552.785	0.935	−0.704
PXP001040										3,552.412	0.929	−0.936
PXP001041	110995	2011-09-15	0.99			2.03E+03	8.33E+02	1.85E+04	7.57E+03	3,553.21	1.458	−0.429
PXP001042	110959	2011-09-06	1.02			4.35E+03	5.77E+01	3.84E+04	5.09E+02	3,552.813	1.46	−0.745
PXP001043	110996	2011-09-15	0.99			4.03E+02	6.11E+01	3.66E+03	5.55E+02	3,552.482	1.441	−0.968
PXP001044										3,553.227	2.033	−0.432
PXP001045										3,552.816	1.991	−0.788
PXP001046										3,552.521	1.94	−1
PXP001047										3,552.948	2.381	−0.472
PXP001048	110997	2011-09-15	1.02	1		7.67E+02	2.08E+02	6.77E+03	1.84E+03	3,552.69	2.367	−0.614
PXP001049										3,551.991	−2.169	−1.197
PXP001050	110960	2011-09-06	1.00			7.63E+02	5.51E+02	6.87E+03	4.96E+03	3,551.63	−1.99	−1.449

Sample	Micans number	Sampling date	Weight CHAB bentonite (g)	Weight IRB bentonite (g)	Weight SRB bentonite (g)	CHAB ml^{-1}	SD CHAB	CHAB gww^{-1}	SD CHAB	Length (m), *z*	Horizontal distance (m), *x*	Vertical distance (m), *y*
PXP001051	111017	2011-09-22	1.00			6.63E+02	9.29E+01	5.97E+03	8.36E+02	3,551.955	−1.727	−1.193
PXP001052										3,551.613	−1.681	−1.466
PXP001053										3,551.955	−1.208	−1.199
PXP001054										3,551.602	−1.207	−1.472
PXP001055										3,551.2	−1.242	−1.848
PXP001056	110998	2011-09-15	1.04			5.20E+02	2.65E+01	4.50E+03	2.29E+02	3,551.899	−0.674	−1.208
PXP001057										3,551.573	−0.679	−1.453
PXP001058	111018	2011-09-22	1.01			5.27E+02	8.03E+01	4.70E+03	7.15E+02	3,551.266	−0.691	−1.871
PXP001059										3,551.905	−0.104	−1.19
PXP001060										3,551.53	−0.112	−1.482
PXP001061	110954	2011-09-01	1.00			1.80E+04	1.73E+03	1.62E+05	1.56E+04	3,551.154	−0.086	−2.063
PXP001062										3,551.935	0.42	−1.229
PXP001063	110999	2011-09-15	1.04			1.36E+03	1.11E+02	1.18E+04	9.61E+02	3,551.48	0.431	−1.594
PXP001064										3,551.948	0.925	−1.267
PXP001065										3,551.574	0.896	−1.469
PXP001066										3,551.389	0.864	−1.736
PXP001067	110955	2011-09-01	1.15			6.00E+02	2.65E+02	4.70E+03	2.07E+03	3,552.03	1.427	−1.368
PXP001068	111019	2011-09-22	1.01			6.00E+02	1.73E+02	5.35E+03	1.54E+03	3,551.625	1.365	−1.603

Sample	IRB MPN cells mL^{-1}	IRB MPN lower limit	IRB MPN upper limit	IRB MPN cells g^{-1}	IRB MPN lower limit	IRB MPN upper limit	SRB MPN cells mL^{-1}	SRB MPN lower limit	SRB MPN upper limit	SRB MPN cells g^{-1}	SRB MPN lower limit	SRB MPN upper limit
PXP000926	2.30E+00	9.00E−01	8.60E+00	1.93E+01	7.57E+00	7.23E+01	2.70E+00	1.20E+00	6.70E+00	2.31E+01	1.03E+01	5.74E+01
PXP000953	3.00E+00	1.00E+00	1.20E+01	2.65E+01	8.82E+00	1.06E+02	8.00E+00	3.00E+00	2.50E+01	6.67E+01	2.50E+01	2.08E+02
PXP000970	4.00E−01	1.00E−01	1.70E+00	3.56E+00	8.91E−01	1.51E+01	2.30E+00	9.00E−01	8.60E+00	2.07E+01	8.10E+00	7.74E+01
PXP001000	8.00E+00	3.00E+00	2.50E+01	6.99E+01	2.62E+01	2.18E+02	2.20E+00	9.00E−01	5.60E+00	1.96E+01	8.02E+00	4.99E+01
PXP001028	3.00E+00	1.00E+00	1.20E+01	2.55E+01	8.49E+00	1.02E+02	1.40E+00	6.00E−01	3.50E+00	1.27E+01	5.45E+00	3.18E+01

A2 Buffer samples

A2.1 Deposition hole 6

Sample ID	Ring	Angle	Radius	Weight CHAB bentonite (gww)	Weight IRB bentonite (gww)	Weight SRB bentonite (gww)	CHAB gww^{-1}	SD CHAB	IRB MPN cells mL^{-1}	IRB MPN lower limit	IRB MPN upper limit	IRB MPN cells gww^{-1}	IRB MPN lower limit	IRB MPN upper limit	SRB MPN cells gww^{-1}	SRB MPN lower limit	SRB MPN upper limit
K6 Ring 9 Buffert 20 Kapsel 1	9	290	525	0.09	0.10	0.13	< 1.8E+04	–	< 0.2	–	–	< 18	–	–	< 18	–	–
K6 Ring 9 Buffert 20 Kapsel 2	9	290	525	0.12	0.14	0.12	< 1.8E+04	–	< 0.2	–	–	< 18	–	–	< 18	–	–
K6 Ring 9 Buffert 40 Kapsel 3	9	290	525	0.14	0.18	0.10	< 1.8E+04	–	< 0.2	–	–	< 18	–	–	< 18	–	–
K6 Ring 9 Buffert 40 Kapsel 4	9	290	525	0.13	0.09	0.14	< 1.8E+04	–	< 0.2	–	–	< 18	–	–	< 18	–	–
K6 Ring 9 Buffert 20 Berg 1	9	290	875	0.09	0.09	0.11	< 1.8E+04	–	< 0.2	–	–	< 18	–	–	< 18	–	–
K6 Ring 9 Buffert 20 Berg 2	9	290	875	0.14	0.10	0.09	< 1.8E+04	–	< 0.2	–	–	< 18	–	–	< 18	–	–
K6 Ring 9 Buffert 40 Berg 3	9	290	875	0.10	0.11	0.14	< 1.8E+04	–	< 0.2	–	–	< 18	–	–	< 18	–	–
K6 Ring 9 Buffert 40 Berg 4	9	290	875	0.10	0.14	0.12	< 1.8E+04	–	< 0.2	–	–	< 18	–	–	< 18	–	–
K6 Ring 9 Buffert 20 Bulk 1	9	290	625–775	0.12	0.11	0.10	< 1.8E+04	–	< 0.2	–	–	< 18	–	–	< 18	–	–
K6 Ring 9 Buffert 40 Bulk 2	9	290	625–775	0.12	0.10	0.10	< 1.8E+04	–	< 0.2	–	–	< 18	–	–	< 18	–	–
K6 Ring 7 Buffert 20 Kapsel 1	7	20	525	0.13			< 1.8E+04	–									
K6 Ring 7 Buffert 20 Kapsel 2	7	20	525	0.10			< 1.8E+04	–									
K6 Ring 7 Buffert 40 Kapsel 3	7	20	525	0.14			< 1.8E+04	–									
K6 Ring 7 Buffert 40 Kapsel 4	7	20	525	0.10			< 1.8E+04	–									
K6 Ring 7 Buffert 20 Berg 1	7	20	875	0.11			< 1.8E+04	–									
K6 Ring 7 Buffert 20 Berg 2	7	20	875	0.09			< 1.8E+04	–									
K6 Ring 7 Buffert 40 Berg 3	7	20	875	0.09			< 1.8E+04	–									
K6 Ring 7 Buffert 40 Berg 4	7	20	875	0.09			< 1.8E+04	–									
K6 Ring 7 Buffert 20 Bulk 1	7	20	775	0.12			< 1.8E+04	–									
K6 Ring 7 Buffert 40 Bulk 2	7	20	775	0.10			< 1.8E+04	–									
K6 Ring 5 Buffert 20 Kapsel 1	5	255	525	0.10	0.09	0.12	< 1.8E+04	–	< 0.2	–	–	< 18	–	–	< 18	–	–
K6 Ring 5 Buffert 20 Kapsel 2	5	255	525	0.12	0.12	0.09	< 1.8E+04	–	< 0.2	–	–	< 18	–	–	< 18	–	–
K6 Ring 5 Buffert 40 Kapsel 3	5	255	525	0.11	0.09	0.13	< 1.8E+04	–	< 0.2	–	–	< 18	–	–	< 18	–	–
K6 Ring 5 Buffert 40 Kapsel 4	5	255	525	0.12	0.11	0.11	< 1.8E+04	–	< 0.2	–	–	< 18	–	–	< 18	–	–
K6 Ring 5 Buffert 20 Berg 1	5	255	875	0.09	0.13	0.11	< 1.8E+04	–	< 0.2	–	–	< 18	–	–	< 18	–	–
K6 Ring 5 Buffert 20 Berg 2	5	255	875	0.10	0.09	0.11	< 1.8E+04	–	< 0.2	–	–	< 18	–	–	< 18	–	–
K6 Ring 5 Buffert 40 Berg 3	5	255	875	0.09	0.14	0.11	< 1.8E+04	–	≥ 160	–	–	10,286	–	–	< 18	–	–
K6 Ring 5 Buffert 40 Berg 4	5	255	875	0.12	0.11	0.13	< 1.8E+04	–	160	60	530	13,091	4,909	43,364	< 18	–	–
K6 Ring 5 Buffert 20 Bulk 1	5	255	775	0.09	0.13	0.14	< 1.8E+04	–	< 0.2	–	–	< 18	–	–	< 18	–	–
K6 Ring 5 Buffert 40 Bulk 2	5	255	775	0.13	0.11	0.10	< 1.8E+04	–	3	1	12	245	82	982	< 18	–	–

Sample ID	Ring	Angle	Radius	Weight CHAB bentonite (gww)	Weight IRB bentonite (gww)	Weight SRB bentonite (gww)	CHAB gww^{-1}	SD CHAB	IRB MPN cells mL^{-1}	IRB MPN lower limit	IRB MPN upper limit	IRB MPN cells gww^{-1}	IRB MPN lower limit	IRB MPN upper limit	SRB MPN cells gww^{-1}	SRB MPN lower limit	SRB MPN upper limit
K6 Ring 3 Buffert 20 Kapsel 1	3	210	525	0.10			< 1.8E+04	–									
K6 Ring 3 Buffert 20 Kapsel 2	3	210	525	0.11			< 1.8E+04	–									
K6 Ring 3 Buffert 40 Kapsel 3	3	210	525	0.11			< 1.8E+04	–									
K6 Ring 3 Buffert 40 Kapsel 4	3	210	525	0.09			< 1.8E+04	–									
K6 Ring 3 Buffert 20 Berg 1	3	210	875	0.09			< 1.8E+04	–									
K6 Ring 3 Buffert 20 Berg 2	3	210	875	0.10			< 1.8E+04	–									
K6 Ring 3 Buffert 40 Berg 3	3	210	875	0.11			< 1.8E+04	–									
K6 Ring 3 Buffert 40 Berg 4	3	210	875	0.13			< 1.8E+04	–									
K6 Ring 3 Buffert 20 Bulk 1	3	210	775	0.11			< 1.8E+04	–									
K6 Ring 3 Buffert 40 Bulk 2	3	210	775	0.11			< 1.8E+04	–									
K6 Ring 1 Buffert 20 Kapsel 1	1	60	525	0.12			< 1.8E+04	–									
K6 Ring 1 Buffert 20 Kapsel 2	1	60	525	0.10			< 1.8E+04	–									
K6 Ring 1 Buffert 40 Kapsel 3	1	60	525	0.10			< 1.8E+04	–									
K6 Ring 1 Buffert 40 Kapsel 4	1	60	525	0.09			< 1.8E+04	–									
K6 Ring 1 Buffert 20 Berg 1	1	60	875	0.09			< 1.8E+04	–									
K6 Ring 1 Buffert 20 Berg 2	1	60	875	0.12			< 1.8E+04	–									
K6 Ring 1 Buffert 40 Berg 3	1	60	875	0.09			< 1.8E+04	–									
K6 Ring 1 Buffert 40 Berg 4	1	60	875	0.15			< 1.8E+04	–									
K6 Ring 1 Buffert 20 Bulk 1	1	60	775	0.10			< 1.8E+04	–									
K6 Ring 1 Buffert 40 Bulk 2	1	60	775	0.10			< 1.8E+04	–									

A2.2 Deposition hole 5

Sample ID	Ring	Angle	Radius	Weight CHAB bentonite (g)	Weight IRB bentonite (g)	Weight SRB bentonite (g)	CHAB gww^{-1}	SD CHAB	IRB MPN cells mL^{-1}	IRB MPN lower limit	IRB MPN upper limit	IRB MPN cells g^{-1}	IRB MPN lower limit	IRB MPN upper limit	SRB MPN cells g^{-1}	SRB MPN lower limit	SRB MPN upper limit
K5 Ring 9 Buffert 20 Kapsel 1	9	25	525	0.97	0.99	1.50	< 1.8E+03	–	< 0.2	–	–	< 1.8	–	–	< 1.8	–	–
K5 Ring 9 Buffert 20 Kapsel 2	9	25	525	0.99	1.03	0.99	< 1.8E+03	–	< 0.2	–	–	< 1.8	–	–	< 1.8	–	–
K5 Ring 9 Buffert 40 Kapsel 3	9	25	525	0.98	0.99	1.02	< 1.8E+03	–	7.00E−01	2.00E−01	2.10E+00	6.36E+00	1.82E+00	1.91E+01	< 1.8	–	–
K5 Ring 9 Buffert 40 Kapsel 4	9	25	525	1.00	1.00	1.22	< 1.8E+03	–	< 0.2	–	–	< 1.8	–	–	< 1.8	–	–
K5 Ring 9 Buffert 20 Berg 1	9	25	875	1.13	1.03	0.98	< 1.8E+03	–	< 0.2	–	–	< 1.8	–	–	< 1.8	–	–
K5 Ring 9 Buffert 20 Berg 2	9	25	875	0.98	1.01	1.14	< 1.8E+03	–	< 0.2	–	–	< 1.8	–	–	< 1.8	–	–
K5 Ring 9 Buffert 40 Berg 3	9	25	875	1.01	1.01	1.04	< 1.8E+03	–	< 0.2	–	–	< 1.8	–	–	< 1.8	–	–
K5 Ring 9 Buffert 40 Berg 4	9	25	875	1.01	1.02	1.01	< 1.8E+03	–	< 0.2	–	–	< 1.8	–	–	< 1.8	–	–
K5 Ring 9 Buffert 20 Bulk 1	9	25	625	0.99	1.00	1.02	< 1.8E+03	–	< 0.2	–	–	< 1.8	–	–	< 1.8	–	–
K5 Ring 9 Buffert 40 Bulk 2	9	25	625	1.01	1.85	1.08	< 1.8E+03	–	< 0.2	–	–	< 1.8	–	–	< 1.8	–	–
K5 Ring 7 Buffert 20 Kapsel 1	7	160	525	1.37			< 1.8E+03	–									
K5 Ring 7 Buffert 20 Kapsel 2	7	160	525	0.99			< 1.8E+03	–									
K5 Ring 7 Buffert 40 Kapsel 3	7	160	525	1.05			< 1.8E+03	–									
K5 Ring 7 Buffert 40 Kapsel 4	7	160	525	1.11			< 1.8E+03	–									
K5 Ring 7 Buffert 20 Berg 1	7	160	875	1.01			< 1.8E+03	–									
K5 Ring 7 Buffert 20 Berg 2	7	160	875	0.99			< 1.8E+03	–									
K5 Ring 7 Buffert 40 Berg 3	7	160	875	1.05			< 1.8E+03	–									
K5 Ring 7 Buffert 40 Berg 4	7	160	875	1.23			< 1.8E+03	–									
K5 Ring 7 Buffert 20 Bulk 1	7	160	625	1.08			< 1.8E+03	–									
K5 Ring 7 Buffert 40 Bulk 2	7	160	625	1.03			< 1.8E+03	–									
K5 Ring 5 Buffert 20 Kapsel 1	5	70	525	1.05	1.57	0.98	< 1.8E+03	–	< 0.2	–	–	< 1.8	–	–	< 1.8	–	–
K5 Ring 5 Buffert 20 Kapsel 2	5	70	525	1.86	1.06	1.38	< 1.8E+03	–	< 0.2	–	–	< 1.8	–	–	< 1.8	–	–
K5 Ring 5 Buffert 40 Kapsel 3	5	70	525	0.94	1.25	1.05	< 1.8E+03	–	< 0.2	–	–	< 1.8	–	–	< 1.8	–	–
K5 Ring 5 Buffert 40 Kapsel 4	5	70	525	1.23	0.99	1.21	< 1.8E+03	–	< 0.2	–	–	< 1.8	–	–	< 1.8	–	–
K5 Ring 5 Buffert 20 Berg 1	5	70	875	0.98	1.01	1.03	< 1.8E+03	–	< 0.2	–	–	< 1.8	–	–	< 1.8	–	–
K5 Ring 5 Buffert 20 Berg 2	5	70	875	0.98	1.23	1.08	< 1.8E+03	–	< 0.2	–	–	< 1.8	–	–	< 1.8	–	–
K5 Ring 5 Buffert 40 Berg 3	5	70	875	0.99	1.11	1.00	< 1.8E+03	–	< 0.2	–	–	< 1.8	–	–	< 1.8	–	–
K5 Ring 5 Buffert 40 Berg 4	5	70	875	0.98	0.97	0.99	< 1.8E+03	–	< 0.2	–	–	< 1.8	–	–	< 1.8	–	–
K5 Ring 5 Buffert 20 Bulk 1	5	70	625	0.98	0.96	1.04	< 1.8E+03	–	< 0.2	–	–	< 1.8	–	–	< 1.8	–	–
K5 Ring 5 Buffert 40 Bulk 2	5	70	625	1.03	1.02	0.98	< 1.8E+03	–	< 0.2	–	–	< 1.8	–	–	< 1.8	–	–

Sample ID	Ring	Angle	Radius	Weight CHAB bentonite (g)	Weight IRB bentonite (g)	Weight SRB bentonite (g)	CHAB gww^{-1}	SD CHAB	IRB MPN cells mL^{-1}	IRB MPN lower limit	IRB MPN upper limit	IRB MPN cells g^{-1}	IRB MPN lower limit	IRB MPN upper limit	SRB MPN cells g^{-1}	SRB MPN lower limit	SRB MPN upper limit
K5 Ring 3 Buffert 20 Kapsel 1	3	115	525	1.01			< 1.8E+03	–									
K5 Ring 3 Buffert 20 Kapsel 2	3	115	525	1.02			< 1.8E+03	–									
K5 Ring 3 Buffert 40 Kapsel 3	3	115	525	0.98			< 1.8E+03	–									
K5 Ring 3 Buffert 40 Kapsel 4	3	115	525	1.02			< 1.8E+03	–									
K5 Ring 3 Buffert 20 Berg 1	3	115	875	1.19			< 1.8E+03	–									
K5 Ring 3 Buffert 20 Berg 2	3	115	875	0.99			< 1.8E+03	–									
K5 Ring 3 Buffert 40 Berg 3	3	115	875	1.05			< 1.8E+03	–									
K5 Ring 3 Buffert 40 Berg 4	3	115	875	0.98			< 1.8E+03	–									
K5 Ring 3 Buffert 20 Bulk 1	3	115	625	1.13			< 1.8E+03	–									
K5 Ring 3 Buffert 40 Bulk 2	3	115	625	1.01			< 1.8E+03	–									
K5 Ring 1 Buffert 20 Kapsel 1	1	25	525	1.03			< 1.8E+03	–									
K5 Ring 1 Buffert 20 Kapsel 2	1	25	525	1.00			< 1.8E+03	–									
K5 Ring 1 Buffert 40 Kapsel 3	1	25	525	1.00			< 1.8E+03	–									
K5 Ring 1 Buffert 40 Kapsel 4	1	25	525	1.16			< 1.8E+03	–									
K5 Ring 1 Buffert 20 Berg 1	1	25	875	1.11			< 1.8E+03	–									
K5 Ring 1 Buffert 20 Berg 2	1	25	875	1.08			< 1.8E+03	–									
K5 Ring 1 Buffert 40 Berg 3	1	25	875	1.02			< 1.8E+03	–									
K5 Ring 1 Buffert 40 Berg 4	1	25	875	1.03			< 1.8E+03	–									
K5 Ring 1 Buffert 20 Bulk 1	1	25	625	1.00			< 1.8E+03	–									
K5 Ring 1 Buffert 40 Bulk 2	1	25	625	1.04			< 1.8E+03	–									

A3 Canister samples

A3.1 Depostion hole 6

Sample	Sample type	Number of replicates	Analysis	Sampling method	Sampling vessel
Negative control	Sterile scalpel	2	DNA extraction	Scalpel	PowerSoil maxi tube
Sample	Bentonite in contact with canister surface	4	DNA extraction	Scalpel	PowerSoil maxi tube
Negative control	Sterile scalpel	2	DNA extraction clay	Scalpel	15 mL Falcon tube
Sample	Bentonite in contact with canister surface	4	DNA extraction clay	Scalpel	15 mL Falcon tube
Negative control	Sterile swab	2	DNA extraction	Swab	PowerSoil tube
Sample	Canister surface material	4	DNA extraction	Swab	PowerSoil tube
Negative control	Sterile scalpel	2	20°C SRB culture	Scalpel	Tube with 9 mL SRB medium
Sample	Bentonite in contact with canister surface	10	20°C SRB culture	Scalpel	Tube with 9 mL SRB medium
Spiked control	Sample + 0.1 mL *D. aespoeensis* culture	2	20°C SRB culture	Scalpel	Tube with 9 mL SRB medium
Positive control	Sample + Swab soaked in water from KJ0052F01	2	20°C SRB culture	Scalpel	Tube with 9 mL SRB medium
Negative control	Sterile swab	2	20°C SRB culture	Swab	Tube with 9 mL SRB medium
Sample	Canister surface material	10	20°C SRB culture	Swab	Tube with 9 mL SRB medium
Spiked control	Sample + 0.1 mL *D. aespoeensis* culture	2	20°C SRB culture	Swab	Tube with 9 mL SRB medium
Positive control	Swab soaked in water from KJ0052F01	2	20°C SRB culture	Swab	Tube with 9 mL SRB medium
Negative control	Sterile scalpel	2	40°C SRB culture	Scalpel	Tube with 9 mL SRB medium
Sample	Bentonite in contact with canister surface	10	40°C SRB culture	Scalpel	Tube with 9 mL SRB medium
Spiked control	Sample + 0.1 mL *D. aespoeensis* culture	2	40°C SRB culture	Scalpel	Tube with 9 mL SRB medium
Positive control	Sample + Swab soaked in water from KJ0052F01	2	40°C SRB culture	Scalpel	Tube with 9 mL SRB medium
Negative control	Sterile swab	2	40°C SRB culture	Swab	Tube with 9 mL SRB medium
Sample	Canister surface material	10	40°C SRB culture	Swab	Tube with 9 mL SRB medium
Spiked control	Sample + 0.1 mL *D. aespoeensis* culture	2	40°C SRB culture	Swab	Tube with 9 mL SRB medium
Positive control	Swab soaked in water from KJ0052F01	2	40°C SRB culture	Swab	Tube with 9 mL SRB medium

A3.2 Deposition hole 5

Sample	Sample type	Number of replicates	Analysis	Sampling method	Sampling vessel
Negative control	Sterile swab	2	DNA extraction	Swab	PowerSoil tube
Sample	Canister surface material	10	DNA extraction	Swab	PowerSoil tube
Spiked control	Sample + 0.1 mL *D. aespoeensis* culture	2	DNA extraction	Swab	PowerSoil tube
Positive control	Sample + 0.1 mL KA3110A water	2	DNA extraction	Swab	PowerSoil tube
Negative control	Sterile scalpel	2	20°C SRB culture	Scalpel	Tube with 9 mL SRB medium
Sample	Bentonite in contact with canister surface	5	20°C SRB culture	Scalpel	Tube with 9 mL SRB medium
Spiked control	Sample + 0.1 mL *D. aespoeensis* culture	2	20°C SRB culture	Scalpel	Tube with 9 mL SRB medium
Positive control	Sample + 1 mL KA3110A water	2	20°C SRB culture	Scalpel	Tube with 9 mL SRB medium
Negative control	Sterile swab	2	20°C SRB culture	Swab	Tube with 9 mL SRB medium
Sample	Canister surface material	5	20°C SRB culture	Swab	Tube with 9 mL SRB medium
Spiked control	Sample + 0.1 mL *D. aespoeensis* culture	2	20°C SRB culture	Swab	Tube with 9 mL SRB medium
Positive control	Sample + 1 mL KA3110A water	2	20°C SRB culture	Swab	Tube with 9 mL SRB medium
Negative control	Sterile scalpel	2	20°C SRB culture	Scalpel	Tube with 9 mL SRB medium
Sample	Canister surface material	5	20°C SRB culture	Scalpel	Tube with 9 mL SRB medium
Spiked control	Sample + 0.1 mL *D. aespoeensis* culture	2	20°C SRB culture	Scalpel	Tube with 9 mL SRB medium
Positive control	Sample + 1 mL KA3110A water	2	20°C SRB culture	Scalpel	Tube with 9 mL SRB medium
Negative control	Sterile scalpel	2	40°C SRB culture	Scalpel	Tube with 9 mL SRB medium
Sample	Bentonite in contact with canister surface	5	40°C SRB culture	Scalpel	Tube with 9 mL SRB medium
Spiked control	Sample + 0.1 mL *D. aespoeensis* culture	2	40°C SRB culture	Scalpel	Tube with 9 mL SRB medium
Positive control	Sample + 1 mL KA3110A water	2	40°C SRB culture	Scalpel	Tube with 9 mL SRB medium
Negative control	Sterile swab	2	40°C SRB culture	Swab	Tube with 9 mL SRB medium
Sample	Canister surface material	5	40°C SRB culture	Swab	Tube with 9 mL SRB medium
Spiked control	Sample + 0.1 mL *D. aespoeensis* culture	2	40°C SRB culture	Swab	Tube with 9 mL SRB medium
Positive control	Sample + 1 mL KA3110A water	2	40°C SRB culture	Swab	Tube with 9 mL SRB medium
Negative control	Sterile scalpel	2	40°C SRB culture	Scalpel	Tube with 9 mL SRB medium
Sample	Canister surface material	5	40°C SRB culture	Scalpel	Tube with 9 mL SRB medium
Spiked control	Sample + 0.1 mL *D. aespoeensis* culture	2	40°C SRB culture	Scalpel	Tube with 9 mL SRB medium
Positive control	Sample + 1 mL KA3110A water	2	40°C SRB culture	Scalpel	Tube with 9 mL SRB medium
Method control	KA3110A water on canister	2	DNA extraction	Swab	PowerSoil tube
Method control	Culture of *D. aespoeensis* on canister	2	DNA extraction	Swab	PowerSoil tube
Method control	KA3110A water on canister	2	20°C SRB culture	Scalpel	Tube with 9 mL SRB medium
Method control	Culture of *D. aespoeensis* on canister	2	20°C SRB culture	Scalpel	Tube with 9 mL SRB medium
Method control	KA3110A water on canister	2	40°C SRB culture	Scalpel	Tube with 9 mL SRB medium
Method control	Culture of *D. aespoeensis* on canister	2	40°C SRB culture	Scalpel	Tube with 9 mL SRB medium
Method control	KA3110A water on canister	2	20°C SRB culture	Swab	Tube with 9 mL SRB medium
Method control	Culture of *D. aespoeensis* on canister	2	20°C SRB culture	Swab	Tube with 9 mL SRB medium
Method control	KA3110A water on canister	2	40°C SRB culture	Swab	Tube with 9 mL SRB medium
Method control	Culture of *D. aespoeensis* on canister	2	40°C SRB culture	Swab	Tube with 9 mL SRB medium

A4 Titanium sample collectors in the outer section

Depositon hole			Instrument position in block				
No	Mark	Block	Direction	*α* degree	*r* mm	*z* mm	Remark
5	KB501	C1	A	0	262	450	
5	KB502	C1	C	180	262	450	
5	KB503	R2	A	0	685	1,450	
5	KB504	R2	B	90	685	1,450	
5	KB505	R2	C	180	685	1,450	
5	KB506	R2	D	270	685	1,450	
5	KB507	R8	A	0	685	4,450	
5	KB508	R8	B	90	685	4,450	
5	KB509	R8	C	180	685	4,450	
5	KB510	R8	D	270	685	4,450	
5	KB511	R10	A	0	262	5,450	
5	KB512	R10	C	180	262	5,450	
5	KB513	C4	A	0	875	6,950	with tube
5	KB514	C4	A	0	875	6,950	with tube
6	KB601	C1	A	0	262	450	
6	KB602	C1	C	180	262	450	
6	KB603	R2	A	0	685	1,450	
6	KB604	R2	B	90	685	1,450	
6	KB605	R2	C	180	685	1,450	
6	KB606	R2	D	270	685	1,450	
6	KB607	R9	A	0	685	4,950	
6	KB608	R9	B	90	685	4,950	
6	KB609	R9	C	180	685	4,950	
6	KB610	R9	D	270	685	4,950	
6	KB611	R10	A	0	262	5,450	
6	KB612	R10	C	180	262	5,450	
6	KB613	C4	A	0	875	6,950	with tube
6	KB614	C4	A	0	875	6,950	with tube

Backfill		Instrument position			
Section	Mark	*x* m	*y* m	*z* m	Remark
Inner part	KFA01	0	2.4	3,556	with tube
Inner part	KFA02	0	−2.4	3,556	with tube
Between dep. holes 5 and 6	KFA03	0	2.4	3,548	with tube
Between dep. holes 5 and 6	KFA04	0	−2.4	3,548	with tube