Microbial Analysis of Highly Compacted Bentonite Samples from Two Large In Situ Tests at the Äspö Hard Rock Laboratory, Sweden

NWMO TR-2014-15 July 2014

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Atomic Energy of Canada Limited

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ABSTRACT

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Abstract
Two Long-Term Test (LOT) bentonite samples and nine Canister Retrieval Test (CRT) bentonite samples from the Äspö Hard Rock Laboratory were analyzed at Atomic Energy of Canada Limited (AECL) to establish the culturability and viability of naturally present bacteria in these compacted, 100% bentonite samples. The purpose of this work was to assess the potential for microbially influenced corrosion (MIC) of used fuel containers in a future repository. Culturability and viability were assessed as a function of sample temperature, water activity (a_w) and dry density. Results showed a reduction in both culturability (by several orders of magnitude) and viability (by a factor of 1.5 to 3) with a decrease in water activity (a_w) and an increase in dry density. Higher temperature also had a negative effect on both culturability and viability. Culture results in samples directly at the bentonite-rock and bentonite-canister interfaces were generally higher than in corresponding bulk samples. This suggests that interfaces are more conducive to microbial activity, possibly related to the availability of more space (lower dry density). Viability in all samples was always orders of magnitude higher than culturability, suggesting that the majority of cells naturally present in the bentonite were in the viable-but-not-culturable state and were, therefore, likely metabolically not very active in situ. In addition, culturabilty at the canister-bentonite interface was very low and possibly spurious. DNA analysis yielded positive identification of only two species, an aerobic, possibly thermophilic, chemolithotrophic beta-Proteobacterium (*Tepidimonas* spp.) and an obligate anaerobic fermenting spore-forming Gram-positive bacterium (*Clostridium* spp). These results suggest survival of thermophilic and spore-forming organisms in this environment. Similar (but more extensive) analyses on equivalent samples were also conducted in Sweden and there was generally good agreement between the Swedish and AECL results.

The overall conclusion from these analyses is that there is a potential for MIC in highly compacted bentonite buffer in a future repository because of the continued presence of low numbers of viable (but largely not culturable) cells in many of the samples analyzed. However, it is unlikely that this potential will be realized as long as high dry density and low water activity conditions are maintained in highly compacted 100% bentonite buffer in a future repository. Such conditions will limit in situ microbial activity to insignificant levels.

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1. INTRODUCTION

This report describes the results of the microbial analyses of a number of highly compacted 100% Wyoming MX-80 bentonite samples that were retrieved from two large-scale engineering tests carried out at SKB's Äspö Hard Rock Laboratory (HRL). The analyses were carried out at Atomic Energy of Canada Limited (AECL) (Whiteshell Laboratories) in 2006. One of the ultimate objectives of sampling large in situ experiments is to determine the extent of survival and activity of bacteria in bentonite under repository-relevant conditions, in order to assess the potential for adverse microbial effects such as microbially influenced corrosion (MIC) of used fuel containers, in a future repository.

The objective of the results recorded in this report is, therefore, to aid in building an NWMO data base of microbial characteristics of highly compacted MX-80 bentonite samples retrieved from large scale experiments carried out under in situ and repository-relevant conditions. Currently ongoing NWMO-funded laboratory studies with highly compacted Wyoming MX-80 bentonite at the University of Saskatchewan aim to determine microbial survival and activity in highly compacted bentonite under reducing conditions. The data in this report can be used as a benchmark to compare and test the validity of current experimental and analytical techniques. Previous large scale in situ tests at AECL's Underground Research Laboratory (URL) were carried out largely with a buffer mixture of 50% bentonite and 50% sand (e.g., Stroes-Gascoyne et al. 1996, 1997) and the microbial analyses of samples of these tests are, therefore, not directly comparable to tests with 100% bentonite. One exception was the Buffer Coupon Long-Term Test (LOT) which unfortunately was terminated after 18 months due to the pending URL closure (Dixon et al. 2004), which, therefore, did not really provide long-term data on microbes in compacted 100% bentonite.

The LOT and CRT tests carried out at SKB's Äspö HRL both provide this longer term perspective, especially the LOT test. The two tests involved are described below.
The microbial analyses reported herein were carried out by AECL for NWMO. Equivalent samples were analyzed by SKB and are documented in their reports (e.g., LOT: Karnland, 1998; Karnland et al., 2000; Karnland and Sanden, 2000; Karnland et al., 2009, 2011; Wersin, 2013; e.g., CRT: Goudarzi et al., 2004, 2005a, b, 2006; Luo, 2006; Johannesson, 2007; Nirvin, 2007; Eng, 2008; Dueck et al., 2011; Lydmark and Pedersen 2011).

Karnland et al. (2009) and Lydmark and Pedersen (2011) report the microbial analyses of the LOT and CRT tests, respectively, carried out in Sweden. The results of microbial analysis carried out on equivalent samples at AECL are compared with the Swedish results in this report.

1.1 Description of the Long-Term Test of Buffer Material (LOT)

The LOT test series involving MX-80 bentonite at the Äspö HRL aims at validating models and hypotheses concerning physical properties in a bentonite buffer material and of related processes regarding microbiology, radionuclide transport, copper corrosion and gas transport under conditions similar to those in a Swedish-type (SKB-3) repository (SKB 2005). The expression "long term" refers to a time span long enough to study the buffer performance at full water saturation, but obviously not long term compared to the lifetime of a repository. The specific objectives of the LOT test series are (SKB 2005):

- collecting data for validation of models concerning buffer performance under quasi-steady-state conditions after water saturation, e.g., swelling pressure, cation exchange capacity and hydraulic conductivity;
- checking existing models for buffer-degrading processes, e.g., illitisation and salt enrichment;
- collecting information concerning survival, activity and migration of bacteria in the buffer;
- checking calculated data concerning copper corrosion, and information regarding type of corrosion;
- measuring gas penetration pressure and gas transport capacity; and
- collecting information which may facilitate the realisation of the full scale test series with respect to clay preparation, instrumentation, data handling and evaluation.

The LOT test series consisted of a number of emplaced parcels, which each contained a heater in a central copper tube, pre-compacted clay buffer (MX-80; Volclay Limited, Liverpool, England; SKB's reference material), instruments, and parameter-controlling equipment. Each parcel was emplaced in a vertical borehole with a diameter of 300 mm and a depth of about 4 m. The tests consisted of experiments under realistic repository conditions (S-type tests; up to 90^{o}C) and controlled adverse conditions (A-type tests, up to 130^{o}C). Table 1 (SKB 2005) gives a description of the entire test series (and its 2014 status) while Figure 1 illustrates the layout and set-up of an S-type parcel.

The tests were started in 1998 (Karnland 1998) and several of the parcels have been retrieved, analyzed and the results reported (Table 1) (Karnland et al. 2000, pilot parcels S1 and A1; Karnland et al. 2011, test parcel A0 and Karnland et al. 2009, test parcel A2). In pilot parcel S1, survival of ten different species of laboratory-grown bacteria was tested under repository-relevant conditions, and results have been reported (Motamedi 1999, Pedersen et al. 2000). Only spore-forming bacteria (including a spore-forming sulphate reducing bacterium (SRB)) were found to be able to survive in the harsh conditions of test parcel S1 (Table 1), and it was concluded that the risk of MIC would be very low under such conditions.

Two bentonite samples were shipped to AECL from different regions of LOT test parcel A2 (Table 1), in order to determine the survival of naturally present microbes in the bentonite under adverse conditions after five years of exposure, a time frame not easily attained in small scale laboratory tests. Samples from the same region were analyzed for microbial occurrence by SKB (Karnland et al. 2009) and a comparison between the results obtained by AECL and SKB is included in this report.

1.2 Description of the Canister Retrieval Test

One of the objectives of the Canister Retrieval Test (CRT) was to demonstrate the recovery of an emplaced copper canister from fully saturated compacted 100% bentonite that has reached its maximum swelling pressure. In addition, the CRT was also used to study the thermal, hydraulic and mechanical (THM) evolution of the bentonite from start until full water saturation. The results of monitoring and manual excavation with laboratory testing of parts of the bentonite were intended to increase the understanding of the THM processes in a deposition borehole. The CRT was located in the main test area at the -420 m level of the Äspö HRL. The test period was separated into three stages (Goudarzi et al. 2005a and Thorsager et al. 2002):

Stage I Drilling of the deposition hole and installation of instrumented bentonite blocks and the canister containing heaters.

Stage II Saturation of the bentonite and evolution of the thermal regime with measurement of THM processes.

Stage III a) Excavation of the upper half of the bentonite and extensive sampling and laboratory testing of dry density, water content and other properties of the buffer, including microbial characterisation, the subject of this report.

b) Recovery of the canister from the lower half of the bentonite using a saltwater dissolution method.

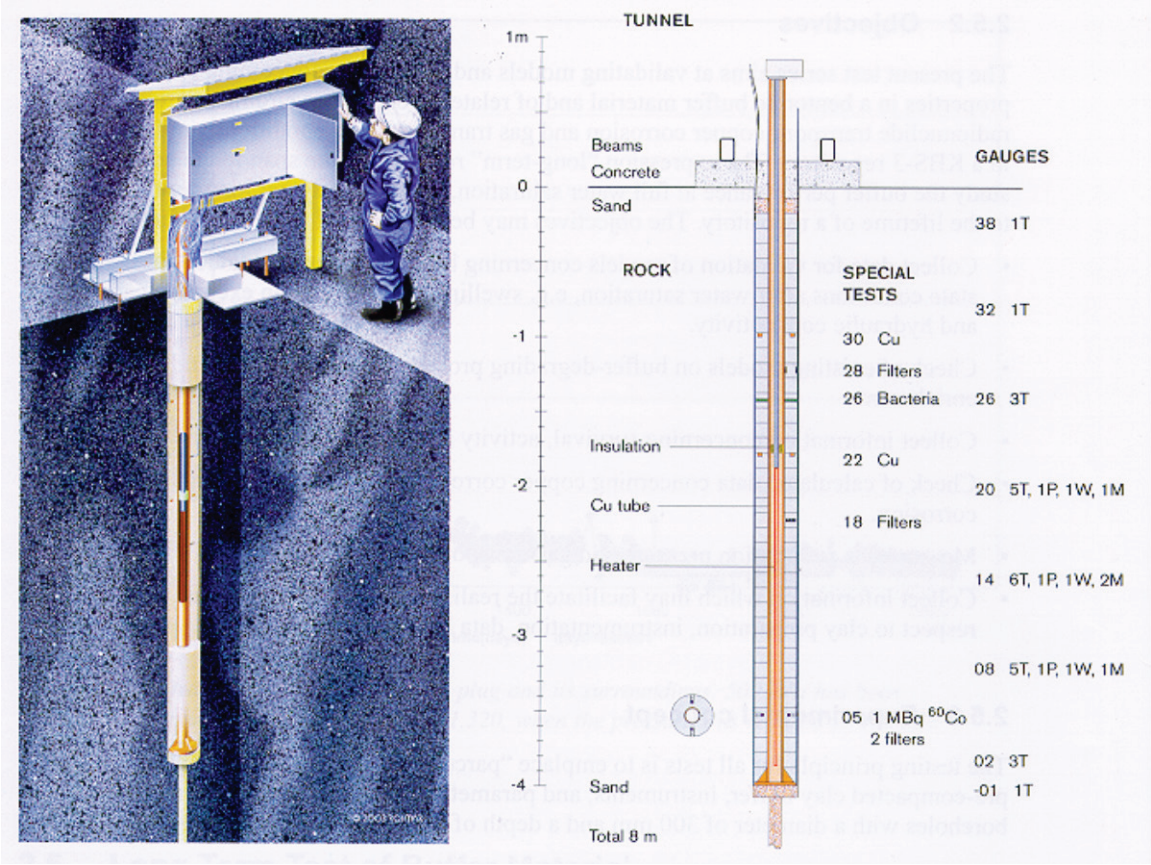


Figure 1: Illustration of the Experimental Set-up in the Long-Term Test (LOT) of Buffer Material (left) and a Cross-section View of an S-type Parcel (right)

Table 1: Layout of the Long-Term Test (LOT) Series

(from SKB 2005)

Type	No.	Max T, °C	Controlled Parameter	Time, years	Remark 1	Remark 2
A	1	130	T, [K^+], pH, am	1	Pilot Test	Karnland et al. 2000
A	0	120-150	T, [K^+], pH, am	1	Main Test	Karnland et al. 2011
A	2	120-150	T, [K^+], pH, am	5	Main Test	Karnl;and et al. 2009
A	3	120-150	T	5	Main Test	Ongoing
S	1	90	T	1	Pilot Test	Karnland et al. 2000
S	2	90	T	5	Main Test	Ongoing
S	3	90	T	>>5	Main Test	Ongoing

A = Adverse conditions
S = Standard conditions
T = Temperature
[K^+] = Potassium concentration
pH = High pH from cement
am = Accessory minerals added

The bentonite used for the CRT was MX-80 (Volclay Limited, Liverpool, England), SKB's reference material. It was installed in the form of blocks (and ring-shaped blocks) consisting of highly compacted Na-bentonite, with a diameter of 1.65 m and a nominal height of 0.5 m. Instruments for measuring temperature, relative humidity, total pressure and pore pressure were installed in many of the bentonite blocks. When the stack of blocks was 6 m high, the copper canister equipped with electrical heaters was lowered down in the centre. Cables to heaters, and instrumentation installed in the rock were connected, and additional bentonite blocks were emplaced until the hole was filled to 1 m from the tunnel floor. The borehole was sealed with a concrete plug and a steel plate was used as cover. The plug was secured against heave caused by the swelling clay by nine cables anchored to the rock. Artificial addition of water to saturate the bentonite as quickly as possible after emplacement was provided evenly around the bentonite blocks by means of permeable mats attached to the rock wall. The design of the mats was such that they would not jeopardise the ultimate retrieval of the canister. The tunnel hosting the borehole was left open for access and inspections of the plug support. The experimental set-up is shown in Figure 2.

The CRT was divided into five different components with respect to its bentonite content (Figure 3):

1. the 1-cm-wide empty radial gap between the cylindrical canister surface and the bentonite rings,
2. the 29-cm-thick bentonite rings surrounding the canister,
3. the large bentonite blocks below and above the canister,

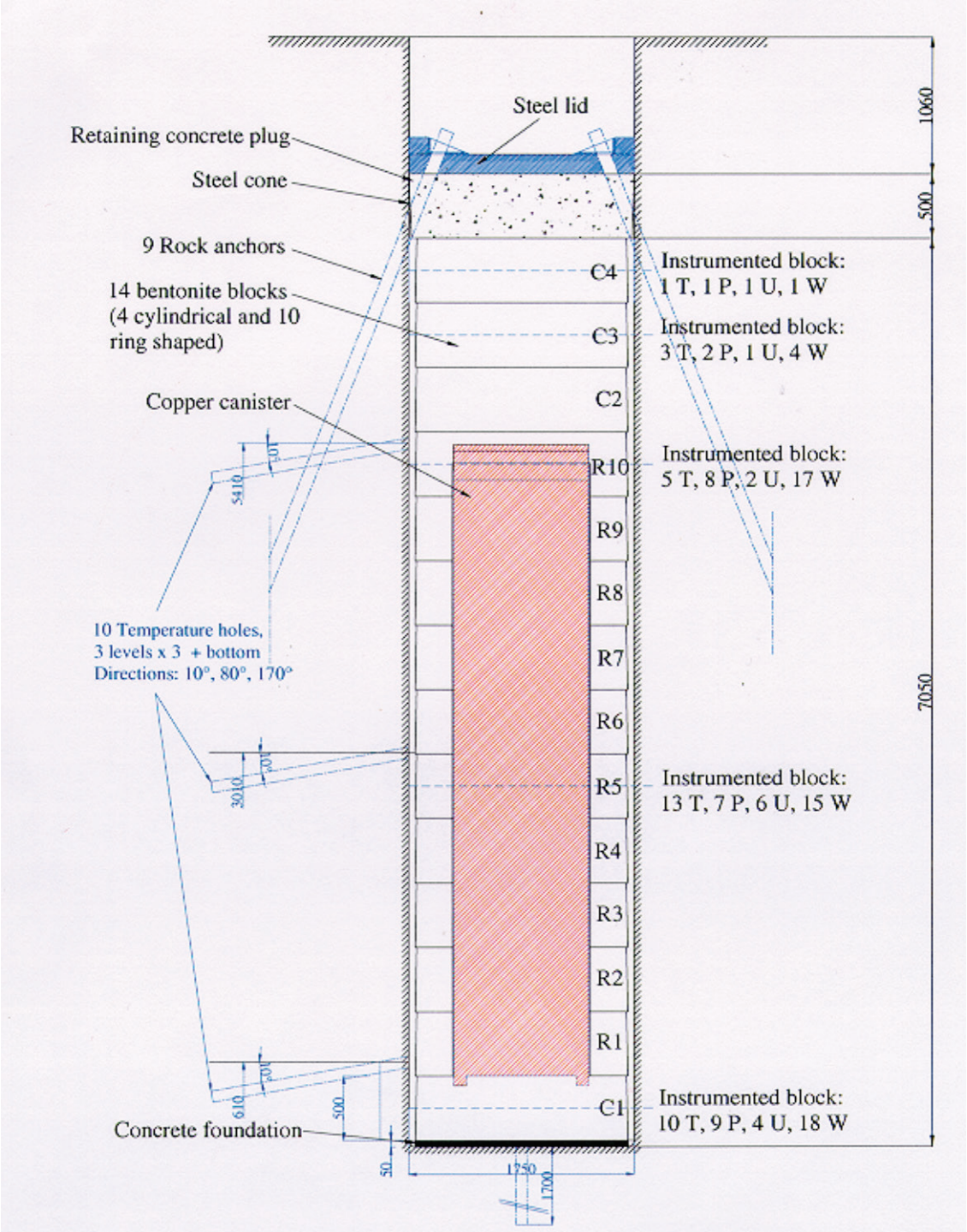


Figure 2: Schematic View of the CRT Showing the Experimental Layout Eng, 2008)

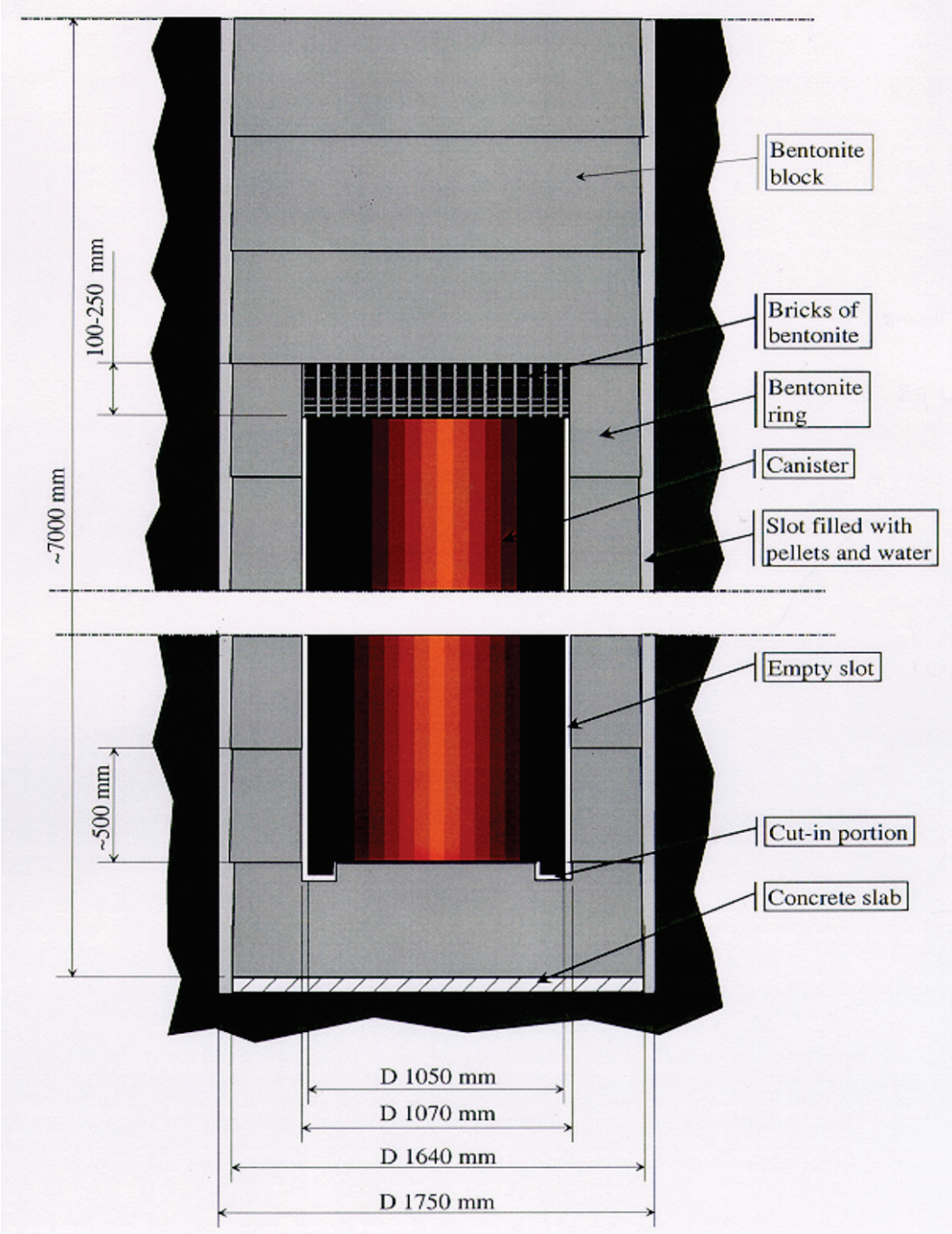


Figure 3: Schematic Drawing of the CRT Canister Hole with Bentonite Blocks with Dimensions in mm

4. the 5-cm-wide space between the bentonite rings (and large blocks) and the rock surface, which was filled with bentonite pellets and water, and

5. the space between the top of the canister, the upper bentonite ring, and the lower large bentonite block, which was filled with small brick-shaped bentonite blocks.

Ten bentonite rings (Figure 2), with an inside diameter of 1.07 m, an average outside diameter of 1.64 m, and a height of 0.50 m, were placed in the borehole. This left a 1-cm gap between the canister and the bentonite rings (Figure 3), which allowed for easy emplacement of the canister. The rings had an initial water content (after addition of water) of 17% and an initial dry density of 1790 kg/m^3. For compaction reasons, the outer surface of the rings was slightly tapered. The diameter at the upper part of the surface was 1.630 m and the diameter at the lower part of the surface was 1.650 m. Four bentonite blocks with an average diameter of 1.65 m and a height of 0.5 m were emplaced, one below the canister and three above the canister (Figure 2). The initial water ratio of these blocks was the same as the water ratio of the rings, i.e. 17%, but the initial dry density was only 1710 kg/m^3 in order to yield the same average density as in the rings (when the 1-cm gap is included). These blocks were also tapered with an upper diameter of 1.631 m and a lower diameter of 1.651 m.

The 10 bentonite rings (Figure 2) had a total height of just over 5 m, which was about 25 cm higher than the canister. This remaining space was filled with bentonite bricks (Figure 3) with dimensions of 115x234x64 mm and with a dry density of about 1800 kg/m^3. The bricks had an initial water content of 17%, and the average dry density including the gaps between the bricks was 1620 kg/m^3. Höganäs Bjufv AB in Bjufv produced the bentonite bricks for the CRT.

Production of the bentonite components is described in detail elsewhere (Goudarzi et al. 2005 and Thorsager et al. 2002). In summary, the bentonite was mixed with water at Hackman-Rörstrand in Lidköping and transported in “big bags” to Hydrowell AB in Ystad for pressing. Table 2 shows the achieved basic properties of each block compacted for the CRT. The first block (which was placed at the bottom of the deposition hole) was named CRT C1 and the ring-shaped blocks were named CRT R1 to CRT R10 (with CRT1-R1 placed above the bottom (C1) block). The cylindrical blocks placed above the canister were named CRT C2 to CRT C4 (Figure 2).

Figure 3 shows that there was a gap with an average radial width of 5 cm between the bentonite rings and blocks and the rock surface. The purpose of this gap was to permit the easy installation of the bentonite blocks. In order to increase the average density of the bentonite, the gap was filled with bentonite pellets and (in order to increase the wetting rate) also with water. Bentonite pellets for the CRT were produced by Sahut-Conceur in France. The pellets were pillow-shaped with dimensions 16x16x8 mm. They were compacted in double rollers under high pressure. The average dry density of a single bentonite pellet was 1800 kg/m^3 but the average dry density including voids after filling was estimated to be 1130 kg/m^3 according to tests performed before the field installation. The natural initial water content of the bentonite pellets was 10%, and after the voids were filled with water, the average water content of the pellets was about 45%. The pellets were placed in the gap after all the bentonite blocks had been emplaced and the gap was drained before and during the filling procedure. The voids between the pellets in the gap were filled with water. To assure that all the voids between the pellets would be filled, the water was fed into the gap from the bottom of the deposition hole. Four tubes were used to fill the gap with water. They were gradually withdrawn as water was added in order to prevent the swelling bentonite from sealing the water transport pathways.

The total volume of water needed was estimated to be about 800 litres but in fact about 950 L of water was added. Site water from bore hole HD0025 in the D-tunnel was used for this purpose.

Table 2: Blocks Compacted for the Canister Retrieval Test

(A. Eng, Pers. Comm. 2006)

Block Name	Date of Compaction (yy-mm-dd)	Water Ratio	Compacted Density (kg/m^3)	Dry Density (kg/m^3)	Weight (kg)	Average Height (mm)	Degree of Sat.	Void Ratio
CRT NR R1	99-11-04	0.173	2091.9	1783.4	1280.0	506.5	0.859	0.558
CRT NR R6	99-11-04	0.171	2102.7	1795.6	1282.0	505.0	0.867	0.548
CRT NR R8	99-11-08	0.171	2099.8	1793.2	1286.0	507.1	0.865	0.551
CRT NR R9	99-11-08	0.171	2098.3	1791.9	1288.0	508.0	0.861	0.551
CRT NR R7	99-11-09	0.172	2099.5	1791.4	1290.0	508.7	0.866	0.552
CRT NR R3	99-11-09	0.167	2102.7	1801.8	1280.0	503.9	0.855	0.543
CRT NR R2	99-11-10	0.172	2095.4	1787.9	1288.0	508.5	0.861	0.555
CRT NR R4	99-11-10	0.170	2116.3	1808.8	1290.0	504.6	0.879	0.537
CRT NR R5	99-11-11	0.175	2086.5	1775.7	1278.0	506.9	0.859	0.565
CRT NR R10	00-01-10	0.171	2069.1	1767	1272.0	509.2	0.830	0.574
CRT NR C4	00-01-12	0.173	2016.4	1719	2156.0	505.4	0.780	0.617
CRT NR C3	00-01-12	0.171	2004.8	1712	2094.0	493.7	0.761	0.623
CRT NR C2	00-01-13	0.170	2003.1	1712.1	2104.0	496.7	0.759	0.624
CRT NR C1	00-01-14	0.173	1987.7	1694.5	2128.0	506.3	0.751	0.641

The total weight of pellets in the filled gap was 2576 kg. Assuming a gap width of 0.055 m, an average bore hole diameter of 1.762 m, which was measured, and a height of 7.07 m, the average estimated dry density in the gap after filling was 1020 kg/m^3, which was lower than expected (1130 kg/m^3), probably due to the fact that all cables were located in the gap. With this actual dry density of the pellets, the blocks, together with the pellets filling the gap between the blocks and the wall of the deposition hole, had an average density at saturation of about 2015 kg/m^3.

The supply of water from the rock was judged to be insufficient for quickly saturating the bentonite. Therefore, filter mats were installed on the rock surface of the CRT deposition hole to facilitate and speed up saturation of the bentonite. Strips of matting with a width of 10 cm and a length of 6.25 m were attached with uniform spacing to the rock wall around the bore

hole. In order to smooth the rock surface and prevent damage to the mats, a fine layer of cement was applied between the mats and the rock before installation. The filter strips were made of four layers of porous plastic filters with an average pore size of 40 μm. The filters were tested in the laboratory before installation in order to check their function and permeability. The tests were performed by enclosing the filters in a rigid box under swelling pressure from water-saturated bentonite of the same density as the CRT buffer. The tests were run for several months and the hydraulic conductivity of the filter strips remained at a value of about 10^{-4} m/s. The filters were only applied from the bottom of the hole up to the middle of the next-uppermost block (C3), i.e. 0.75 m from the plug (Figure 2). The filters were not applied to the entire length of the borehole in order to not interfere with the bentonite seal against the cement plug and in order to not intersect the cable slots in the rock.

The CRT deposition borehole was covered with a retaining plug since the test tunnel was left open. The plug consisted of a concrete cone placed on the bentonite layer and a steel lid, which was pre-stressed by rock ties. Together, this structure allowed all parts to be situated below the cast concrete floor in the tunnel. The purpose of the plug was to prevent the blocks of bentonite from swelling uncontrollably and to simulate a real storage situation, where the deposition holes are covered with backfill that allows for some upward swelling of the compacted bentonite. The plug was, therefore, designed to allow for controlled upward displacement and to withstand a uniform pressure of 9 MPa. The steel lid was made of steel of grade S 355JR. The diameter of the steel lid was 1738 mm. The thickness of the steel lid was 150 mm.

A full size canister with a copper outer shell and a steel insert, taken from the test production facility, was used in the CRT. Instead of fuel assemblies, there were three heaters in each of the 12 assembly compartments. In addition, a 14 cm superstructure with a lid was applied on top of the canister in order to connect and lead all cables into the canister. The canister was installed after all bentonite rings except the upper one (R10, Figure 2) were in place. A specially developed canister deposition machine was used to emplace the canister.

The following measurements were taken during the duration of the CRT:

- temperature (in the bentonite, inside and on the surface of the canister and in the rock),
- total pressure in the bentonite (i.e., swelling pressure + pore water pressure),
- pore water pressure in the bentonite,
- relative humidity in the bentonite,
- forces on the retaining plug,
- plug displacement measurement, and
- water inflow into the permeable mats.

The slot between the rock and the bentonite blocks was filled with bentonite pellets and water on 001026. This date, therefore, marked the start of the CRT. A one-metre water head (from a water supply tank) was connected to the filters on 001102. The heating of the canister started with an initially applied constant power of 700 W on 001027 (i.e., one day after test start). The power was raised to 1700 W on 001113. The power was further raised to 2600 W on 010213. At the end of 2001 two of the 36 electrical heaters had failed due to short circuit to earth and no power was generated during one day between November 5 and 6, 2001 (day 375). The heaters were also shut off during one week between March 4 and 11 2002 (days 495 to 502) for control measurements. The water pressure in the mats was increased stepwise to 800 kPa in the period 5/9 – 10/10 2002 (days 687-713). The power of the heaters in the canister was

reduced to 2100 W on day 683 (10/9 –02) and to 1600 W on day 1135 (4/12 –03). The later reduction was done after another heater failure that took place on day 1134. There were additional problems with the heaters resulting in failure of heaters and short power interruptions. The latest occurred on 2005-02-20 (day 1578) and 2005-03-10 (day 1596), after which only 4 heaters were still functioning. Consequently the power in the canister had to be reduced from 1600 W to about 1150 W on day 1596 (10/3- 05). The water pressure in the mats attached to the deposition hole wall was temporarily reduced to 100 kPa during the period 5/12 2002 – 9/1 2003 (days 770-805) and to 400 kPa during the period 9/1 2003 – 23/1 2003 (days 805-819). The water pressure was reduced to atmospheric in March 2005 in order to try to keep the last heaters alive.

On October 11 (day 1811) the power was switched off in order to prepare for the dismantling, excavation and retrieval of the test, which started at the beginning of 2006 (January).

2. SAMPLES RECEIVED FROM THE LOT CRT TEST FOR ANALYSIS AT AECL

Samples for microbial analyses were taken from both the LOT and CRT experiments by experienced personnel and further processed at the University of Goteborg (U of G). After the LOT and CRT bentonite samples arrived at the U of G from Äspö HRL, sub-samples were taken using sterile (and anaerobic) methods for analysis at U of G (LOT samples) or at Microbial Analytics Sweden AB (Micans) (CRT samples). Additional subsamples were taken and wrapped in sterilized foil, bagged in airtight plastic bags and shipped on ice by courier from Sweden to Canada. Upon arrival at Whiteshell Laboratories (WL), the samples were placed immediately in the anaerobic glove box for sampling, using sterilized knives and scalpels. The time between the samples leaving U of G and arriving at WL ranged from three to five days, and analyses were initiated on the day of arrival at WL.

Two bentonite samples were received from the LOT test and nine bentonite samples from different regions of the CRT test at AECL. These samples were analyzed to determine the survival of naturally present microbes in the bentonite under repository-relevant conditions. A similar experiment, involving buffer (i.e., a 50% bentonite, 50% sand mixture) was analyzed in the Canadian Nuclear Fuel Waste Management Program in 1994 at AECL's URL (Stroes-Gascoyne et al. 1996, 1997). However, the current NWMO concept design of a repository in granitic host rock calls for compacted 100% bentonite around the waste containers. Therefore, the results and conclusions from the microbial analyses of the LOT and CRT samples will be applicable to both the Swedish and Canadian Programs.

Two bentonite samples were received from parcel A2 of the LOT test (Table 1). One sample came from block 38 (Top LOT, wet) and one sample came from block 19/20 (Mid LOT, dry). Figure 4 gives a schematic of Parcel A2, while Figure 5 gives temperature data for this parcel. Table 3 gives temperature data for block 38 and block 20. Table 4 gives details of the sub-sampling at AECL of these two samples.

Nine bentonite samples were received from various blocks of the CRT, i.e., the C2 layer above the container (two samples, one from near the rock, one from the center of the block), the R10

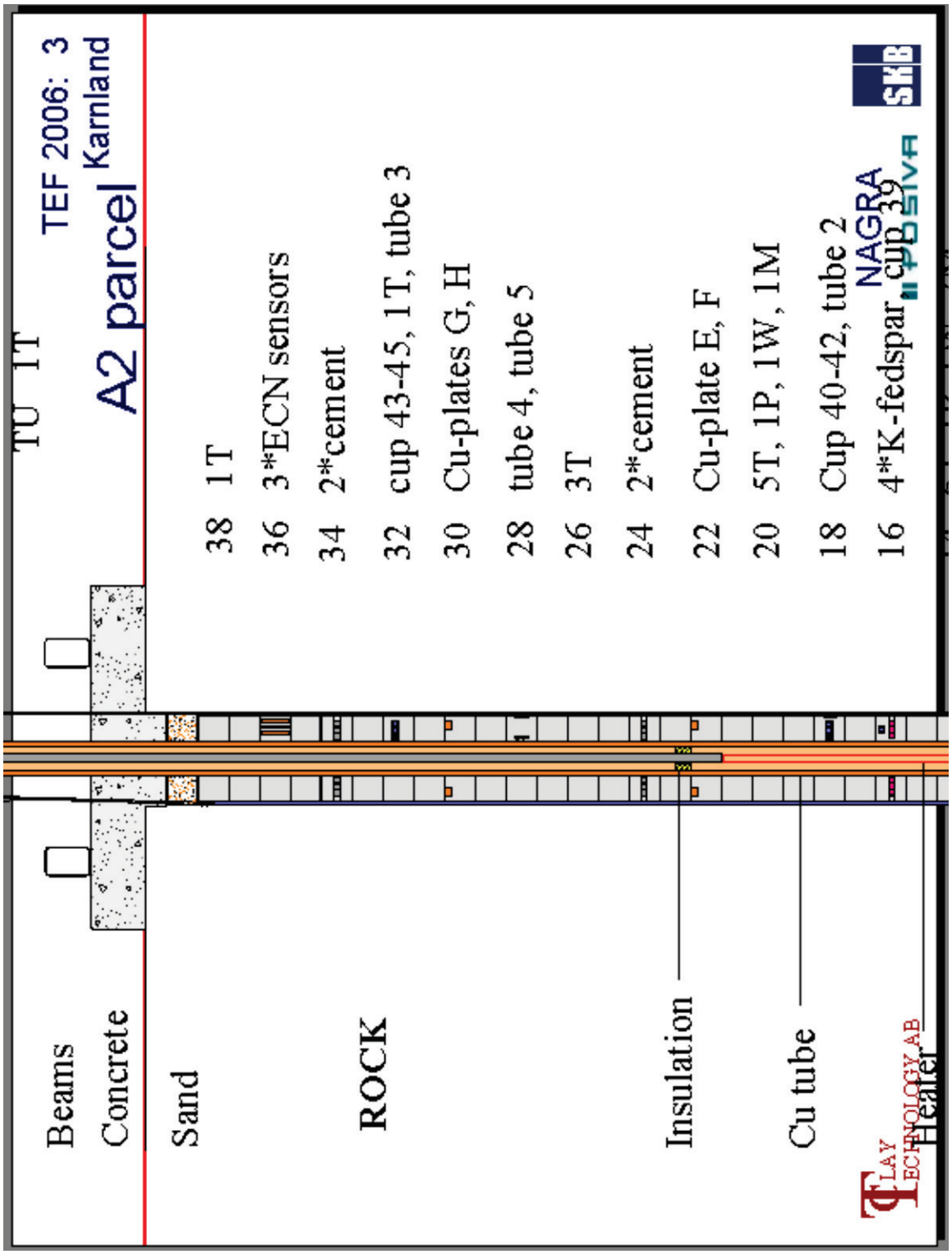


Figure 4: Layout and Details of the LOT A2 Parcel; Samples were Received from Blocks 38 and 19/20

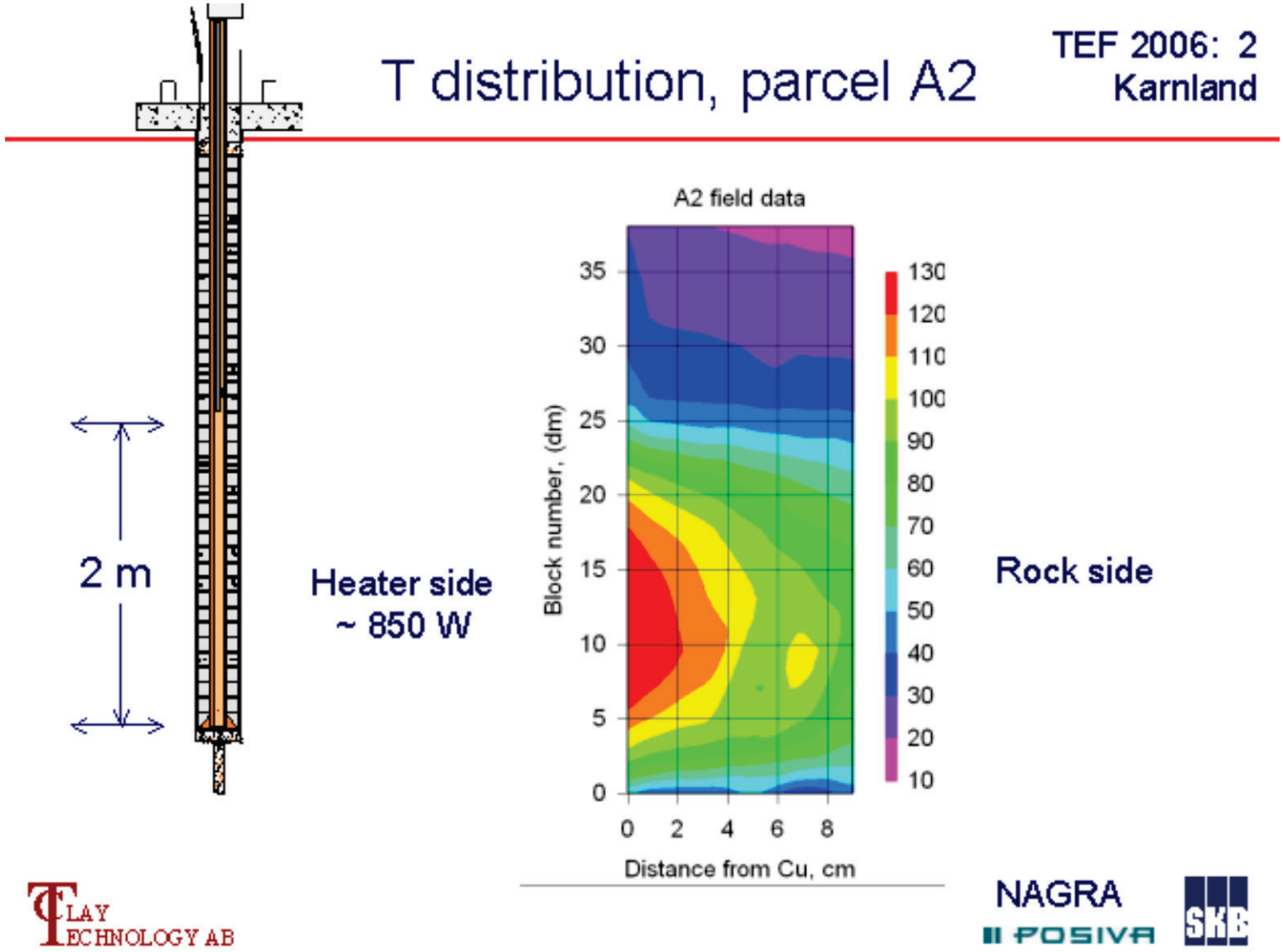


Figure 5: Temperature Distribution in the LOT A2 Parcel

ring in touch with the canister lid (one sample), the R9 ring (two samples, one from the rock-bentonite interface, one from the bentonite-copper canister interface), the R8 ring (two samples, one from the rock-bentonite interface, one from the bentonite-copper canister interface) and the R6 ring (two samples, one from the rock-bentonite interface, one from the bentonite-copper canister interface). Table 5 gives approximate temperature ranges for block C1, ring R5 and ring R10 of the CRT. These data were derived from several graphs in the SKB report by Goudarzi et al. (2005a). No temperature data were provided or available for the C2, R9, R8, and R6 samples. Therefore, temperatures were derived from the layers corresponding closest to those where the samples came from, as given in Table 6. For the C2 samples, temperature data for R10 (Table 5) were used. For the R10 samples, R10 data (Table 5) were available. For the R9, R8 and R6 samples, temperature data for R5 (Table 5) were used. Table 6 gives details of the microbial sub-sampling of these nine samples. Figures 6, 7 and 8 (provided by SKB) give detailed information on moisture content, degree of saturation and dry density in block C2 and Rings R7 and R10, respectively. These data could be used directly for the samples from layers C2 and R10. For the samples from layers R9, R8 and R6, the data for R7 (Figure 8) were used.

Table 3: Temperature Data for LOT Samples

(Karnland, Pers. Comm. 2006)

Position Block No.[#]	0	1	3	5	7	9
38	19	19	19	19	19	19
32	26	26	26	26	26	26
26	43	43	41	40	39	38
20	101	101	91	81	73	67
14	131	124	110	100	92	85
8	124	124	113	101	92	82
2	83	83	77	71	67	62
0	0	0	0	0	0	0

[#] NOTE: Samples were received from Block 38 and Block 19/20

Table 4: Details of LOT Samples Analyzed at AECL

Sample #	Sent	Location	Temperature (range) °C	Position of Sub-Sample for Microbial Analysis at AECL
1679a	230106	TopLOT, wet Block 38	Cool ~ 19°C	10 – 20 cm into clay sample
1679b	(repeat)			
1680a	230106	MidLOT, dry Block 19/20	Hot ~ 80 - 100°C	10 – 20 cm into clay sample
1680b	(repeat)			

Table 5: Temperature Data for Layers R10, R5 and C1 of the CRT

(Goudarzi et al. 2005a (IRP-05-15))

Ring 5	Directions ABC Temperature Range °C		Direction D Temperature Range °C	
Time Period	**Rock**	**Canister**	**Rock**	**Canister**
0 – 100 d	20 – 48	20 – 56	20 – 47	20 - 58
100 – 700 d	48 – 73	56 – 83	47 – 68	58 – 87
700 – 1150 d	73 – 64	83 – 74	68 – 63 (60)	87 – 78 (75)
1150 – 1580 d	64 – 56	74 – 63	63 – 54	78 – 64
1580 – end	56 – 48	63 – 54	54 – 47	64 – 55

Ring 10	Directions AD Temperature Range °C	
Time Period	**Rock**	**Canister**
0 – 100 d	20 – 32.5	20 – 37.5
100 – 700 d	32.5 – 48.5 (45)	37.5 – 58 (56)
700 – 1150 d	45 – 43 (46)	56 – 50 (54)
1180 – 1580 d	46 – 41	54 – 47
1580 – end	41 – 37	47 – 41

Block C1	Directions ABCD Temperature Range °C	
Time Period	**Rock**	**Canister**
0 – 100 d	20 – 36	20 – 55
100 – 700 d	36 – 54	55 – 80
700 – 1150 d	54 – 50 (52)	80 – 70 (73)
1150 – 1580 d	52 – 46	73 – 60
1580 – end	46 – 41	60 – 51

Table 6: Details of CRT Samples Analyzed at AECL

Sample #	Sent	Location	Temperature* (range) °C	Position of Sub-samples for Microbial Analysis
1681a	080206	C2 Layer above container	20 - 48.5[1]	C1 closest to rock (but not in contact)
1681b				Duplicate sample
1682a	080206	C2 Layer above container	20 - 58[1]	D3 closest to center
1682b				Duplicate sample
1683	150206	R10 Sample in touch with Cu canister (heater) lid	20 - 58[2]	Cu-bentonite interface scraping
1684	150206	" "	20 - 58[2]	Sample ¾" into 1683
1685	150206	" "	20 - 58[2]	Dark areas of 1683 (Cu-bentonite interface)
1686	270206	R9 Rock-bentonite interface	20 - 73[3]	Rock-bentonite interface scraping
1687	270206		20 -73	Sample 1" into 1686
1688	270206	" "	20 - 87[3]	Cu-bentonite interface scraping
1689	270206	R9 Cu-bentonite interface (50-70° angle)	20 - 87	Sample 1" into 1688
		" "		
1690	060306	R8 Rock-bentonite interface (rocks embedded in clay)	20 - 73[3]	Rock-bentonite interface scraping
1691	060306	" "	20 - 73	Sample 1" into 1690
1692	060306	R8 Cu-bentonite interface	20 - 87[3]	Cu-bentonite interface scraping
1693	060306	" "	20 - 87	Sample 1.5" into 1692
1694	230306	R6 Cu-bentonite interface	20 - 87[3]	Cu-bentonite interface scraping
1695	230306	" "	20 - 87	Sample 2-4" into 1694
1696	230306	R6 Rock-bentonite interface	20 - 73[3]	Rock-bentonite interface scraping
1697	230306	" "	20 - 73	Sample 1" into 1696

* Temperature ranges estimated from Goudarzi et al. (2005a) (IPR-05-15) (see Table 5)
[1] Data from R10 ring (Table 5)
[2] Data from R10 ring (Table 5)
[3] Data from R5 ring (Table 5)

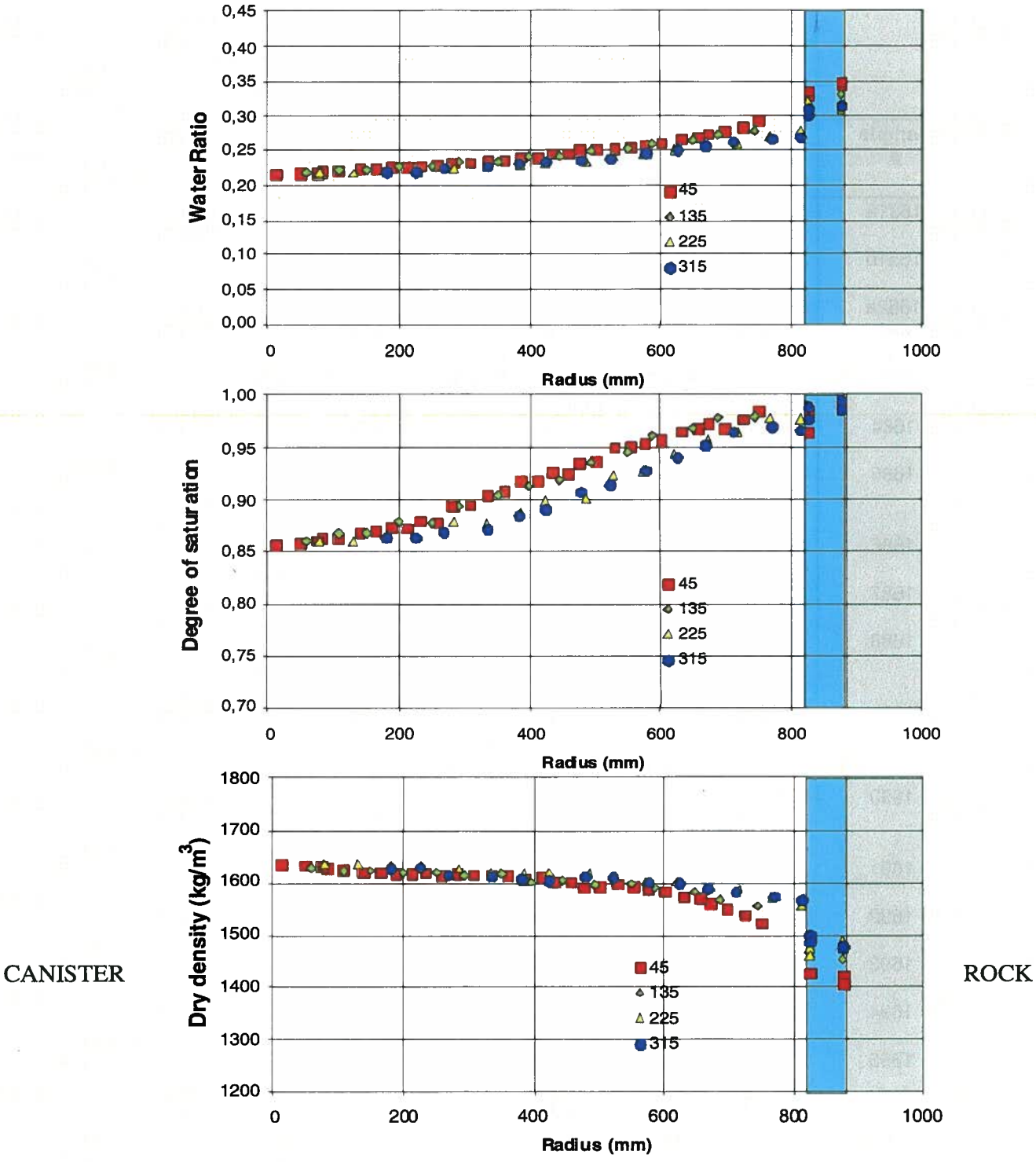


Figure 6: Moisture Content, Saturation and Dry Density Data for CRT Block C2

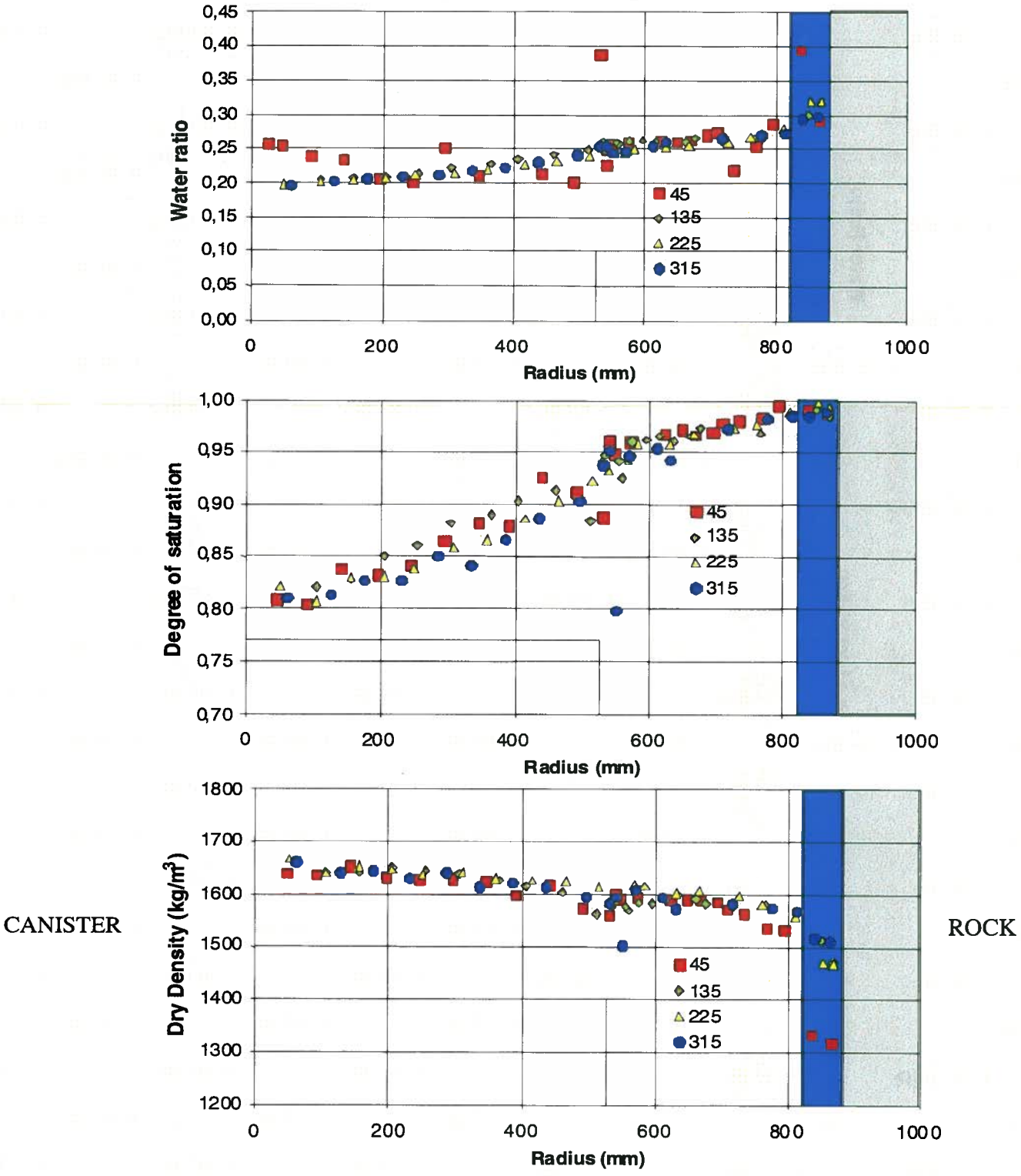


Figure 7: Moisture Content, Saturation and Dry Density Data for CRT Block R10

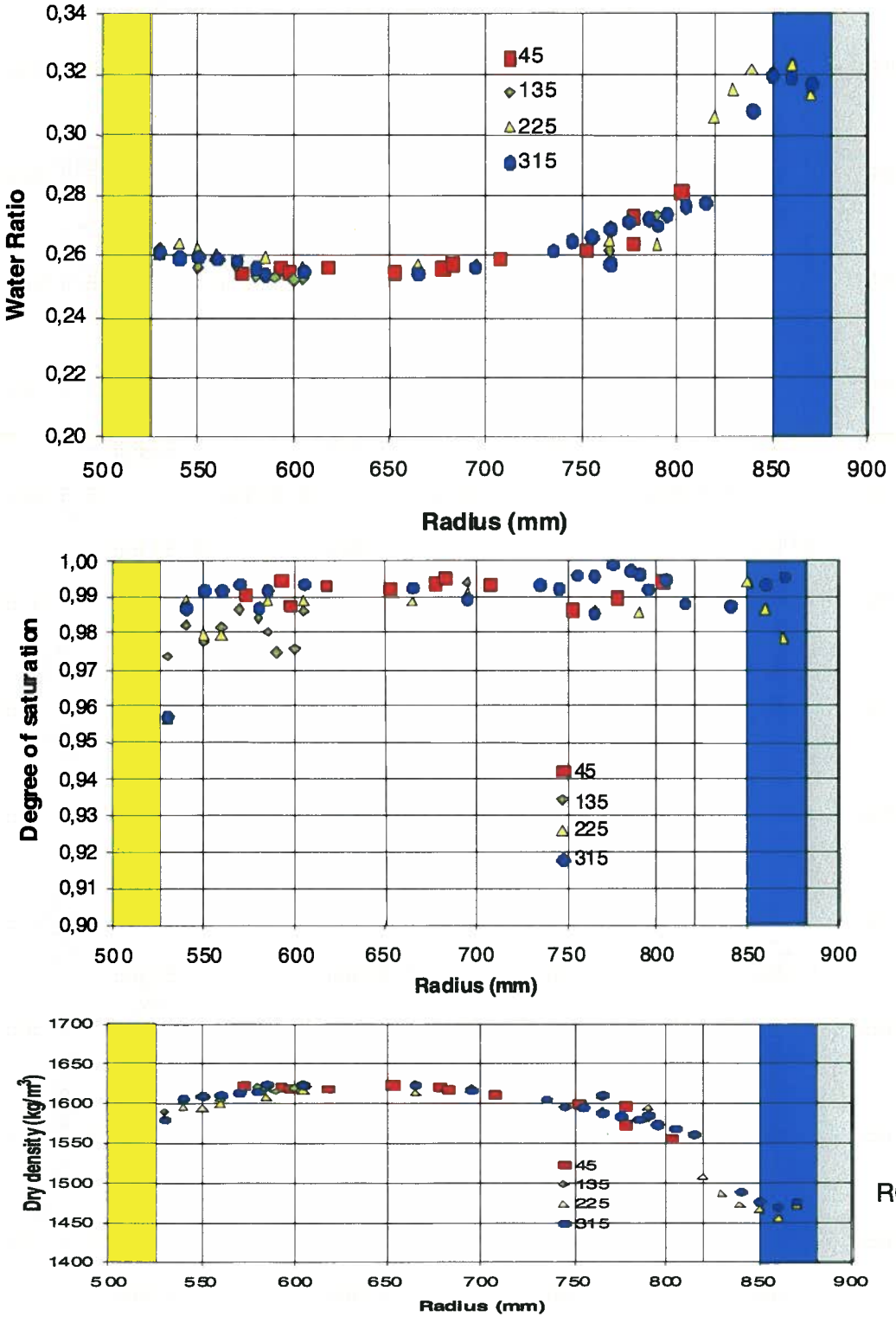


Figure 8: Moisture Content, Saturation and Dry Density Data for CRT Block R7

At the time the samples were taken, shipped to and analyzed at AECL-WL in 2006, SKB had not released their findings in published reports and, therefore, the results obtained at AECL were not recorded in a published NWMO report until present. There now exist a number of SKB reports on the CRT (Goudarzi et al. 2004, 2005a,b, 2006; Luo 2006; Johannesson 2007; Nirvin 2007; Eng, 2008; Dueck et al. 2011 and Lydmark and Pedersen 2011) that can be accessed for test information and results. A number of SKB reports also exist on the LOT experiment (Karnland 1998, Karnland et al. 2000; Karnland et al. 2009; Karnland et al. 2011). The report by Lydmark and Pedersen (2011) reports the microbial results obtained by Micans for the CRT samples, while the report by Karnland et al. (2009) includes the microbial analyses results obtained by U of G for the LOT samples.

This report contains the results of the microbial analysis of the samples received by AECL. These samples were equivalent to those analysed in Sweden. This report, therefore, also contains a comparison of the AECL microbial results with the SKB results for the CRT (Lydmark and Pedersen 2011) and LOT (Karnland et al. 2009) tests.

3. ANALYSES OF THE LOT AND CRT SAMPLES

3.1 At AECL

Upon arrival at WL, each sample was analyzed the same day, using the following methods:

Water content was measured by drying a weighed sub-sample at 110^{o}C to constant weight.

Dry density was measured by cutting several (five to seven) cubes from each sample, measuring the dimensions of the cubes (for volume calculations), weighing, and drying the cubes at 110^{o}C to constant weight, from which water content and dry density values were calculated.

Water activity (a_w) was measured on each sample using a Decagon^{TM} WP4 Dewpoint PotentiaMeter (Decagon Devices, Pullman, WA). The instrument was calibrated using a range of KCl solutions provided by Decagon Devices.

Aerobic and anaerobic heterotrophic bacteria were cultured by plating on R2A medium (Reasoner and Geldreich 1985), followed by incubation for one week at 30^{o}C for aerobes and four weeks at room temperature (~25^{o}C) for anaerobes. Sulphate-reducing bacteria (SRB) were cultured in anaerobic modified Postgate's B medium (Atlas 1993) for four weeks at 30^{o}C, using the Most Probable Number (MPN) method. Interfaces were sampled by scraping material off the surface with sterilized scalpels. Bulk samples were taken by scooping one to two inches into the clay mass using sterilized spoons.

Enumeration of viable cells by plate counting generally results in values that are too low. Relative plate count efficiencies in most cases amount to only about 0.1 to 5% of the microorganisms counted microscopically, due to the selectivity of media and the inability of many cells to form colonies (Riis et al. 1998). The analysis of phospholipid fatty acids (PLFA), which are important components of all cellular membranes, is a powerful tool in the quantitative determination of viable biomass and community structure in environmental samples, without the need for culturing (e.g., Tunlid and White 1991). Subsamples of all LOT and CRT samples were, therefore, frozen and shipped on ice to Microbial Insights, Rockford, Tennessee, USA, for

biomass, community structure and nutritional status analysis, through extraction and analysis of PLFA. Cell equivalents were calculated by using an established PLFA content of *E. coli*, depending on the environmental conditions. For the calculations in this report, a conversion factor of 2 x 10^4 cells per picomole of PLFA was used. Different types of PLFA signal the presence of different groups of microorganisms, and this information is used in the community structure analysis. Table 7 gives the physiological groups and their corresponding PLFA structure groups as they relate to general classifications, to assist in the interpretation of the PLFA results.

PLFA analysis also provides information about the physiological status of the Gram-negative Proteobacteria present. The membrane of a microbe adapts to the changing conditions of its environment, and these changes are reflected in its PLFA. Toxic compounds or adverse environmental conditions may disrupt the membrane and some bacteria respond by making *trans* fatty acids instead of the usual *cis* fatty acids (Guckert et al. 1986) in order to strengthen the cell membrane, making it less permeable. Many Proteobacteria respond to a lack of available substrate or to high toxicity conditions by making cyclopropyl (Guckert et al. 1986) or mid-chain branched fatty acids (Tsitko et al. 1999), which point to less energy expenditure and a slowed growth rate. The physiological status ratios for “decreased membrane permeability” (i.e., the *trans*/*cis* ratio) or “slowed growth” (cy/cis ratio) are based on dividing the amount of fatty acid induced by the environmental conditions by the amount of its biosynthetic precursor. Ratios for “slowed growth” and for “decreased membrane permeability” of the cell membrane provide information on the “health” of the Gram-negative community, and reflect how the community is responding to the conditions present in the environment. It should be noted that caution is warranted when interpreting these ratios from only one sampling event. The most effective way to use the physiological status indicators is in long term monitoring and comparing how these ratios increase or decrease over time. A marked increase in either of these ratios suggests a change in environment, which is less favourable to the Gram-negative Proteobacteria population. The ratio for “slowed growth” is a relative measure, and does not directly correspond to log or stationary phases of growth but is useful as a comparison of growth rates among sampling locations and also over time. An increase in this ratio (i.e., slower growth rates) suggests a change in conditions, which are not as supportive of rapid, “healthy” growth of the Gram-negative population, often due to a reduction in available substrate. A larger ratio for “decreased membrane permeability” suggests that the environment has become more unfavourable or toxic to the Gram-negative population, requiring energy expenditure to produce *trans* fatty acids in order to make the cell membranes more rigid.

All LOT and CRT samples were also subjected to DNA extraction followed by amplification using the polymerase chain reaction (PCR) and to denaturing graduating gel electrophoresis (DGGE). These analyses were carried out by Microbial Insights, Rockford, Tennessee, USA.

3.2 In Sweden

A substantial number of microbial analyses on LOT and CRT samples were carried out for SKB by the U of G (LOT samples) and by Microbial Analytics Sweden AB (Micans) (CRT samples). Results can be found in Karnland et al. (2009) for the LOT samples and in Lydmark and Pedersen (2011) for the CRT samples.

Table 7: PLFA Structure Groups as they Relate to General Classifications

Physiologic Group	PLFA Structure Group	General Classification
Firmicutes (F)	Terminally Branched, Saturated (Ter Br Sats)	Representative of Gram-Positive bacteria. Indicative of fermenting bacteria, mainly Clostridia/Bacteriodes-like.
Proteobacteria (P)	Monoenoics (Monos)	Found in Gram-Negative bacteria, which are fast growing, utilize many carbon sources, and adapt quickly to a variety of environments.
Anaerobic Metal Reducers (A)	Branched Monoenoics (Br Monos)	Commonly found in the cell membranes of microaerophilic and obligate anaerobes such as sulphate- or iron-reducing bacteria.
SRB/Actinomycetes (S)	Mid-chain Branched, Saturated (Mid Br Sats)	Common in Actinomycetes (spp.), SRB and certain Gram-Positive Bacteria.
General (G)	Normal Saturated (N Sats)	Found in both the prokaryotic and eukaryatic kingdoms. High proportions of these PLFA often indicate less diverse populations.
Eukaryotes (E)	Polyenoics	Found in organisms such as fungi, protozoa, algae, higher plants and animals.

Both the LOT and CRT samples analyzed by Micans were located essentially adjacent to those analyzed by AECL. Samples from blocks 38 (Top LOT) and 19/20 (Mid LOT) from LOT A2 parcel were analyzed. CRT samples from layers C2, R10, R9 and R6 were analyzed. It is not clear why samples from R8 were not analyzed by Micans. (AECL received and analyzed samples from layer R8 in addition to those mentioned above (see Table 6).)

Micans took samples at 1 cm intervals (from 0 to 9 cm, 10 samples) across the radius of the LOT samples (Karnland et al. 2009), whereas AECL sampled in two places. Micans sampled at 18 locations across the radius of the CRT R2 layer, at five locations in the CRT R10 layer, at 33 locations in the CRT R9 layer and at 10 locations in the CRT R6 layer (Lydmark and Pedersen 2011). AECL took four samples in each of the CRT R2, R9, R8 and R6 layers and three samples in the CRT R10 layer. The Micans samples covered the rock-bentonite interface, the Cu-bentonite interface and the bulk bentonite in between. The AECL samples were taken at and in the vicinity of the rock-bentonite and Cu-bentonite interfaces, but not in the bulk bentonite between the two interfaces.

The Micans analyses included enrichment cultures, which were examined for the presence of viable heterotrophic and autotrophic sulphate-reducing bacteria (SRB) and autotrophic acetate-producing bacteria (acetogens, AA). Enrichment cultures differ from enumeration-type cultures in that they give only qualitative results for the presence of viable cells, and are usually carried out on samples, which are expected to contain low levels of microbes. A small amount of bentonite was added to two types of liquid growth media and incubated with ample time (6 weeks at 30^{o}C) to give any viable cells present an optimal chance to metabolize, grow and divide. The cultures were analyzed for sulphides and acetate and by microscopy, in order to determine if they contained viable SRB (two types) and AA.

Enumerations (quantitative) for SRB, heterotrophic aerobic bacteria and AA were also carried out, using an approximately 12-16 h old enrichment culture as inoculum. The media used can be found in Karnland et al. (2009) for the LOT samples and in Lydmark and Pedersen (2011) for the CRT samples.

All bentonite samples were also subjected to an analysis for Adenosine-Tri-Phosphate (ATP), which has been proposed as a measure of viable cells (Eydal and Pedersen 2007).

4. RESULTS

4.1 Results Obtained by AECL

Table 8 gives results for the average water content and the measured dry densities of the two LOT samples, as measured from a number of small cubes cut from the larger sample. However, based on the theoretical relationship between the water content and dry density in compacted Wyoming MX-80 bentonite (shown in Figure 9), the measured dry density values in the LOT samples appear too high by a considerable amount. This is likely due to inaccurate (imprecise) measurements of the volume of the small cubes from which these dry density values were derived (a measuring error of a few mm can quickly lead to an error in volume of 20-40%). Therefore, it was decided to calculate the dry densities for these samples using the measured water content and the theoretical relationship between water content and compacted 100% bentonite in Figure 9, assuming 100% saturation in the samples. Note that if either of the

LOT samples were under-saturated, this calculation would lead to a small overestimation of the dry density in that sample.

Table 8: Dry Density Data (AECL) for LOT Samples

Sample	Water Content AECL (measured) (% of dry weight)	Dry Density AECL (measured) (g/cm^3)	Dry Density AECL (calculated)* (g/cm^3)
Block 38 (1679)	39.8 (average of 6)	1.77	1.30
Block 19/20 (1680)	31.2 (average of 5)	1.85	1.45

* Calculated from measured water content, assuming 100% saturation

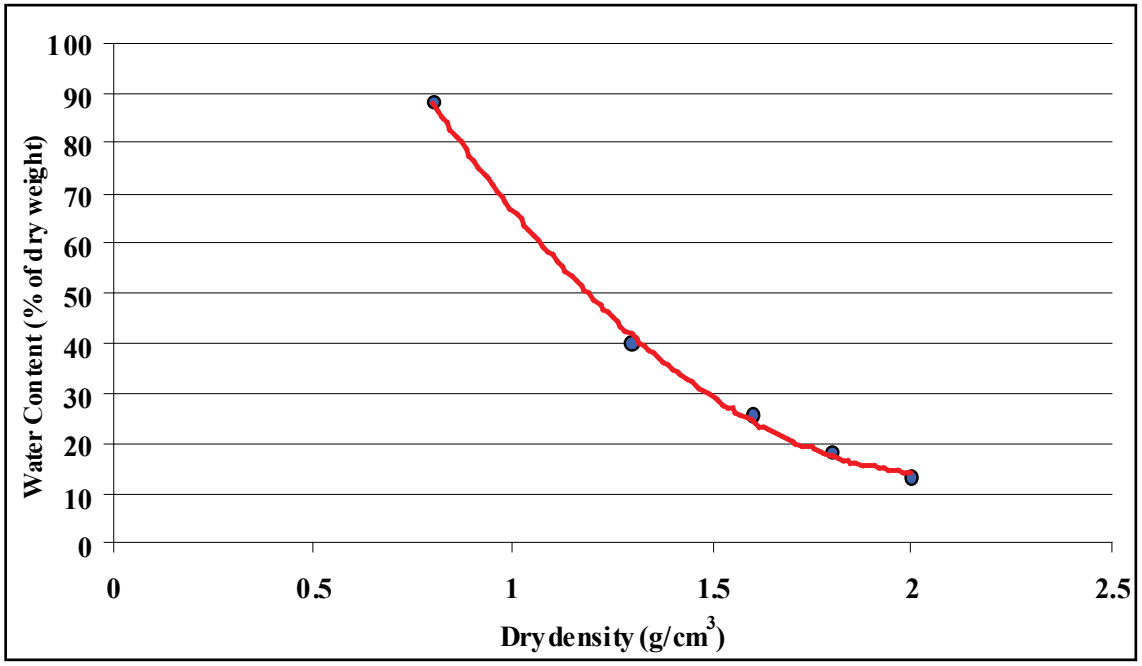


Figure 9: Theoretical Relationship between Saturated Water Content (% of dry weight) and Dry Density in Compacted 100% Bentonite

Measured water content, measured a_w, calculated dry density, and the culture results for aerobes, anaerobes and SRB, as well as the PLFA-based community size data for the two LOT samples (in duplicate, see Table 4) are given in Table 9.

Table 10 gives the AECL results for measured water content and measured dry density in the nine CRT samples. Included in this table are the SKB results for water content and dry density, derived from Figure 6 (C2 samples), Figure 7 (R10 samples) and Figure 8 (R9, R8 and R6 samples). Note that Figure 8 gives data for block R7 only; it is assumed here that these data also reflect the conditions in blocks R9, R8 and R6, from which samples were received.

Table 9: Water Content, Water Activity, PLFA and Culture Results (AECL) for LOT Samples

Sample #	Water Content % of dry wt.	a_w	Calculated Dry Density g/cm^3	Aerobes CFU/g dry wt.	Anaerobes CFU/g dry wt.	SRB MPN/g dry wt.	Total Cells (PLFA) Cells/g dry wt.
1679a	41.1%	0.980	1.30	$(6.81\pm0.57)\times10^3$	$(8.60\pm0.39)\times10^2$	3.7	8.18×10^5
1679b	38.7%	0.977		$(9.84\pm1.06)\times10^3$	$(3.27\pm1.09)\times10^1$	3.9	
1680a	31.3%	0.954	1.45	$(4.01\pm6.94)\times10^0$	0 (< 11)	< 3.6	2.67×10^5
1680b	30.3%	0.949		0 (< 11)	0 (< 11)	< 3.4	

Table 10: Dry Density Data (SKB, AECL) for CRT Samples

Sample	Water Content SKB* (% of dry weight)	Water Content AECL (measured) (% of dry weight)	Dry Density SKB* (g/cm^3)	Dry Density AECL (measured) (g/cm^3)	Dry Density AECL (calculated)** (g/cm^3)
C2 near rock (1681)	26.5 – 29.5	27.5 (average of 6)	1.54	1.74	1.54
C2 near canister (1682)	22.0	23.0 (average of 5)	1.64	1.97	1.64
R10 near canister lid (1683)	20 – 26	24.2 (average of 7)	1.56	2.34	1.60
R9 near rock (1686)	30 – 32	27.7 (average of 5)	1.46 – 1.51	1.89	1.55
R9 near canister (1688)	26	25.4 (average of 5)	1.58 – 1.61	1.79	1.58
R8 near rock (1690)	30 – 32	31.2 (average of 5)	1.46 – 1.51	1.72	1.45
R8 near canister (1692)	26	24.5 (average of 5)	1.58 – 1.61	1.78	1.62
R6 near rock (1696)	30 – 32	29.9 (average of 5)	1.46 – 1.51	2.11	1.47
R6 near canister (1694)	26	24.7 (average of 5)	1.58 – 1.61	1.77	1.61

*SKB data are derived from Figure 6 (C2 Sample), Figure 7 (R10 Samples) and Figure 8 (R9, R8, R6 Samples);
NOTE that Figure 8 gives data for block R7; it is assumed here that these data also reflect conditions in Blocks R9, R8 and R6.
**Calculated from measured water content data, assuming 100% saturation

Table 10 shows that the water content values measured by AECL are in good agreement with the SKB data derived from Figures 6, 7 and 8. However, as was the case for the LOT samples, it appears that the AECL-measured dry density values for the CRT samples are too high by a considerable amount, likely due to imprecise volume measurements of the small cubes from which the dry density data were determined. Therefore, dry density data were calculated from the AECL-measured water content values in Table 10, using the theoretical relationship in Figure 9 and assuming 100% saturation.

Figure 8 shows that the bentonite was fully saturated near the rock and almost saturated (99%) near the canister for ring-shaped block R7 (assumed to be representative of blocks R9, R8 and R6). Therefore, the calculated dry densities (based on water content) in the samples from these blocks are likely adequate. Also, Table 10 shows that the thus calculated dry densities are in good agreement with the SKB-provided dry density values derived from Figure 8 for these samples. Figures 6 and 7 show that blocks C2 and R10 were saturated near the rock but up to 15 – 20% undersaturated near the center of the block (C2) or near the canister (R10) (note that the R10 sample received came from the bentonite just above the canister). Therefore, the calculated dry densities for the samples from these locations may be overestimated. However, note that the relationship in Figure 9 is not linear and that an underestimation in saturated water content of 15-20% would lead to only an approximately 7% overestimation in dry density in the samples from these locations. Also, Table 10 shows that the thus calculated dry densities are in good agreement with the SKB-provided dry density values derived from Figures 6 and 7 for these samples. Therefore, the calculated dry densities for the samples from blocks C2 and R10 are likely acceptable.

Measured water content, measured a_w, calculated dry density, and the culture results for aerobes, anaerobes and SRB, as well as the PLFA-based community size data for the nine CRT samples are given in Table 11. Both a_w and swelling pressure were proposed in earlier work as the parameter that controls microbial activity and survival in compacted bentonite (e.g., Stroes-Gascoyne et al. 1997; Hedin 2006). Further laboratory studies have shown a distinct effect of a_w, swelling pressure and dry density on microbial culturability in compacted 100% bentonite (Stroes-Gascoyne et al. 2006, 2010). One of the LOT samples and all but one of the CRT samples had been exposed to temperatures in excess of 50^{o}C. Therefore, culturability in the LOT and CRT samples was evaluated against sample temperature, a_w and dry density (no swelling pressure data were provided for these samples). In Figures 10, 11 and 12, the average culture results for each sample (Table 9 (LOT) and Table 11 (CRT)) are shown as a function of the maximum sample temperature (Table 4 (LOT) and Table 6 (CRT)), the average a_w value in each sample (Table 9 (LOT) and Table 11 (CRT)), and the calculated dry density in each sample (Table 9 (LOT) and Table 11 (CRT)), respectively, in order to determine if there are any trends in culturability as a function of these parameters (i.e., temperature, a_w and dry density). Note that average culturability was used in these evaluations, i.e., the average of two measurements (LOT samples and CRT C2 samples) or the average of the surface and bulk measurement (R10, R9, R8 and R6 samples).

Results from the PLFA analyses are given in Tables 12 (LOT) and 13 (CRT), respectively. In addition to the PLFA-based viable biomass data and the average culture-based biomass data (from Tables 9 and 11, respectively), Tables 12 and 13 also give PLFA-based ratios that assess the physiological status of the communities present as well as the community structure (as explained in Table 7) in the LOT and CRT samples, respectively.

Table 11: Water Content, Water Activity, PLFA and Culture Results (AECL) for CRT Samples

Sample #	Water Content % of dry wt.	a_w	Calculated Dry Density g/cm^3	Aerobes CFU/g dry wt.	Anaerobes CFU/g dry wt.	SRB MPN/g dry wt.	Total Cells (PLFA) cells/g dry wt.
1681a C_2 (Rock)	27.6	0.932	1.54	$(3.85\pm6.67)\times10^{0}$	$(3.85\pm6.67)\times10^{0}$	< 3.5	5.41×10^{5}
1681b C_2 Duplicate	27.8	0.936		$(2.89\pm0.96)\times10^{1}$	$(1.28\pm1.47)\times10^{1}$	< 2.9	
1682a C_2 (Centre)	22.7	0.874	1.64	$(9.95\pm17.2)\times10^{1}$	$(2.98\pm2.58)\times10^{1}$	4.5	3.56×10^{5}
	22.8	0.880		$(1.21\pm1.21)\times10^{1}$	$(1.21\pm1.21)\times10^{1}$	< 3.6	
1682b C_2 Duplicate							
1683 R10 (Cu-B int.)	28.1	0.891		$(3.53\pm6.11)\times10^{0}$	$(7.05)\pm(6.11)\times10^{0}$	<3.2	
1684 R10 (Bulk 1683)	24.3	0.889	1.60	0 (< 11.1)	0 (< 11.1)	< 3.3	5.21×10^{5}
1685 R10 (Dark, Cu-B, int. 1683)	24.4	0.895		0 (< 12.5)	0 (< 12.5)	< 3.8	
1686 R9 (R-B int.)	29.4	0.944		$(1.29\pm0.41)\times10^{2}$	$(8.92\pm7.73)\times10^{0}$	< 4.01	9.11×10^{5}
1687 R9 (Bulk 1686)	28.8	0.942	1.55	$(3.12\pm5.40)\times10^{0}$	0 (< 9.1)	<2.80	
1688 R9 (Cu-B int.)	23.0	0.888		0 (< 14.3)	$(2.34\pm0.81)\times10^{1}$	< 4.08	6.88×10^{5}
1689 R9 (Bulk 1688)	27.3	0.935	1.58	0 (< 10)	$(6.48\pm11.2)\times10^{0}$	< 2.92	
1690 R8 (R-B int.)	26.9	0.948		$(2.25\pm0.56)\times10^{4}$	$(2.24\pm0.56)\times10^{1}$	< 2.88	9.44×10^{5}
1691 R8 (Bulk 1690)	32.3	0.957	1.45	0 (< 7.7)	0 (< 7.7)	< 2.28	
1692 R8 (Cu-B int.)	23.0	0.882		$(5.12\pm8.88)\times10^{0}$	0 (< 14.3)	<4.61	7.34×10^{5}
1693 R8 (Bulk 1692)	26.8	0.930	1.62	0 (< 7.7)	0 (< 7.7)	< 2.36	
1694 R6 (Cu-B int.)	22.5	0.866		$(2.34\pm1.89)\times10^{2}$	$(4.92\pm5.88)\times10^{2}$	< 11.1	2.75×10^{5}
1695 R6 (Bulk 1694)	25.9	0.919	1.61	$(4.42\pm7.66)\times10^{0}$	0 (< 12.5)	< 3.98	
1696 R6 (R-B int.)	29.7	0.944		$(2.34\pm0.10)\times10^{4}$	$(2.65\pm0.23)\times10^{2}$	< 3.76	9.48×10^{5}
1697 R6 (Bulk 1696)	31.1	0.950	1.47	0 (< 8.3)	$(8.10\pm0.00)\times10^{1}$	< 2.24	

B = Bentonite
Cu = Copper canister
R = Rock
int. = interface

Table 12: PLFA Results (AECL) for LOT Samples

Sample*	Biomass Cells/g Dry wt.	Physiological Status (Proteobacteria only)		Community Structure (% of total PLFA)						Culturable Organisms		
		R1	R2	F	P	A	S	G	E	Aerobes CFU/g	Anaer-robes CFU/g	SRB MPN/g
1679 TopLOT Block 38	$8.18x10^5$	1.40	0.49	10.29	42.59	1.79	8.00	34.63	2.70	$8.3x10^3$	$4.5x10^2$	4
1680 MidLot Block 19/20	$2.67x10^5$	0.00	0.00	26.59	12.32	0.00	3.91	55.49	1.70	BDL	BDL	BDL

* For detailed sample description see Table 4
R1 = "slowed growth" (starvation)
R2 = "decreased membrane permeability" (toxicity)
BDL = below detection limit
F : Firmicutes (Ter Br Sats)
P : Proteobacteria (Monos)
A : Anaerobic metal reducers (Br Monos)
S : SRB/Actinomycetes (Mid Br Sats)
G : General (N sats)

E : Eukaryotes (polyenoics)

Table 13: PLFA Results (AECL) for CRT Samples

Sample*	Biomass Cells/g Dry wt.	Physiological Status (Proteobacteria only)		Community Structure (% of total PLFA)						Culturable Organisms		
		R1	R2	F	P	A	S	G	E	Aerobes CFU/g	Anaer-obes CFU/g	SRB MPN/g
1681 C2 near rock	$5.41x10^5$	0.93	0.37	2.56	38.26	0.00	3.31	40.91	14.96	$1.6x10^1$	BDL	BDL
1682 C2 near center	$3.56x10^5$	1.03	0.60	3.31	43.74	0.00	5.15	34.38	13.45	BDL	$2.1x10^1$	BDL
1683 R10 near Cu lid	$5.21x10^5$	2.27	0.71	2.93	17.30	6.33	10.65	59.93	2.86	BDL	BDL	BDL
1686 R9 near rock	$9.11x10^5$	3.24	0.30	4.07	20.21	0.00	1.83	57.66	16.22	$6.6x10^1$	BDL	BDL
1688 R9 near canister	$6.88x10^5$	1.20	1.32	3.74	27.38	3.84	3.97	46.25	14.82	BDL	$1.5x10^1$	BDL
1690 R8 near rock	$9.44x10^5$	7.42	1.36	16.24	25.33	2.23	9.91	45.21	1.09	$1.1x10^4$	$1.1x10^1$	BDL
1692 R8 near canister	$7.34x10^5$	4.34	1.73	11.44	27.80	0.00	0.00	58.60	2.16	BDL	BDL	BDL
1694 R6 near canister	$2.75x10^5$	0.45	0.00	4.82	32.58	0.00	9.75	47.99	4.88	$1.2x10^2$	$2.5x10^2$	BDL
1696 R6 near rock	$9.48x10^5$	19.46	2.09	17.33	37.83	0.00	4.71	37.41	2.73	$1.2x10^4$	$1.7x10^2$	BDL

* For detailed sample description see Table 6
R1 = "slowed growth" (starvation)
R2 = "decreased membrane permeability" (toxicity)
BDL = below detection limit
F : Firmicutes (Ter Br Sats)
P : Proteobacteria (Monos)
A : Anaerobic metal reducers (Br Monos) S : SRB/Actinomycetes (Mid Br Sats) G : General (N sats) E : Eukaryotes (polyenoics)

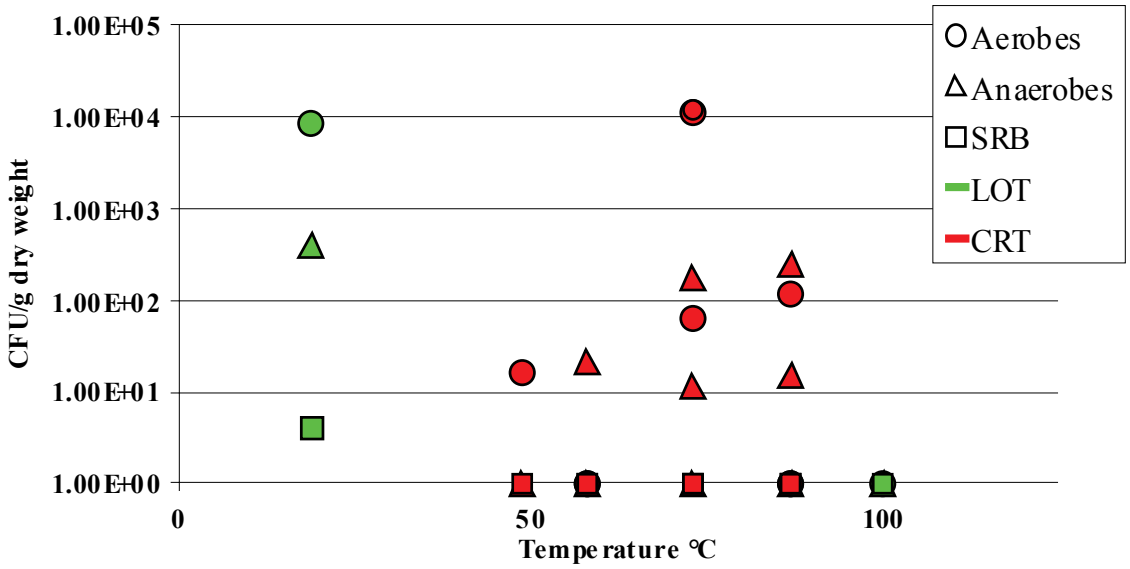


Figure 10: Culturability of Aerobes, Anaerobes and SRB as a Function of Temperature in LOT and CRT Samples

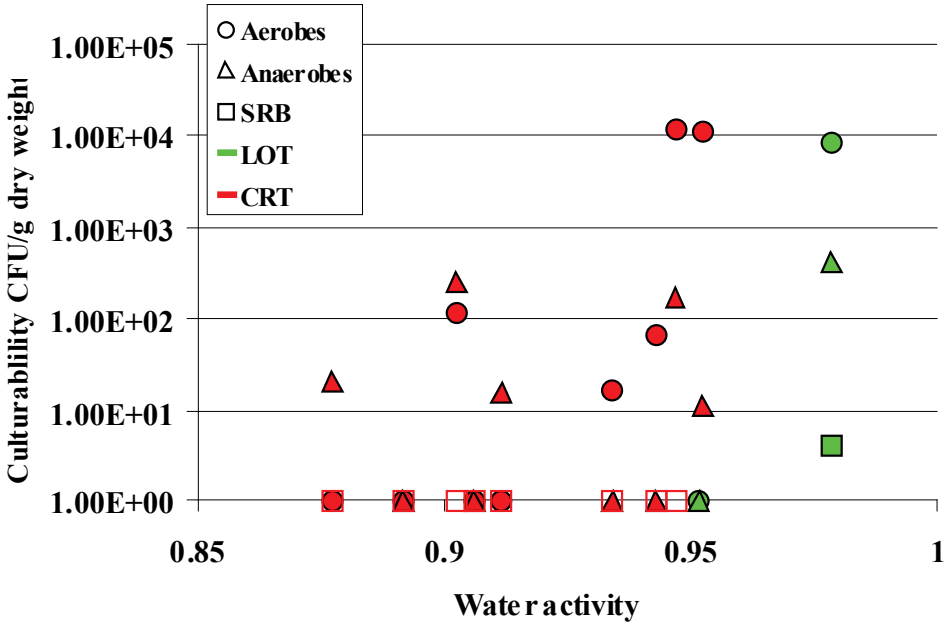


Figure 11: Culturability of Aerobes, Anaerobes and SRB as a Function of Water Activity in LOT and CRT Samples

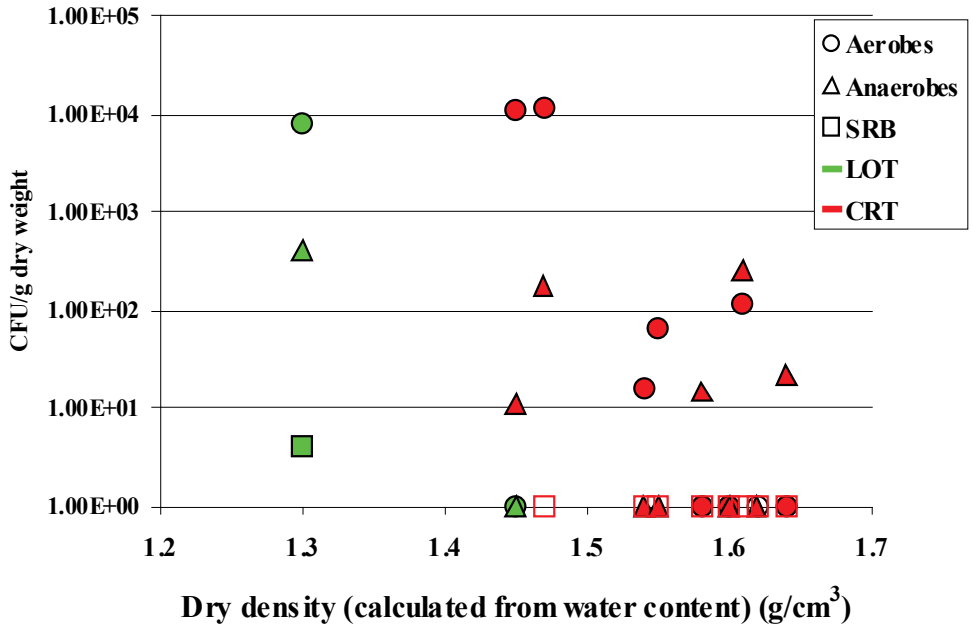


Figure 12: Culturability of Aerobes, Anaerobes and SRB as a Function of Calculated Dry Density in LOT and CRT Samples

In Figures 13, 14 and 15, the PLFA-based viable biomass data (Tables 12 and 13) are shown as a function of the maximum sample temperature (Table 4 (LOT) and Table 6 (CRT)), the average a_w value in each sample (Table 9 (LOT) and Table 11 (CRT)), and the calculated dry density in each sample (Table 9 (LOT) and Table 11 (CRT)), in order to determine if any trends exist in the PLFA-based viable biomass with respect to these parameters (i.e., temperature, a_w and dry density).

In Figure 16, the (average) culturable and PLFA-based viable biomass in the LOT and CRT samples are compared. Figure 17 shows the community structure, as derived from the PLFA analyses, in the LOT and CRT samples.

Figure 18 compares the PLFA-based physiological status ratios "slowed growth" and "decreased membrane permeability" (for Proteobacteria) in the LOT and CRT samples (see Tables 12 and 13). Note that in some samples the "reduced membrane permeability" ratio was zero. This is due to the fact that the specific fatty acids that make up this ratio were not detected in that sample, likely due to the low biomass present. This does not mean that there was no membrane stress in these samples.

Only three CRT samples (1690, 1692 and 1694) gave sufficient PCR product to attempt DGGE analysis. Average culturability in samples 1690, 1692 and 1694 ranged from below detection limit (BDL) in 1692 to 2.5 x 10^2 CFU/g in 1694 and 1.1 x 10^4 CFU/g in 1690. Other samples had similar average culturabilities (e.g., 1696) but did not produce sufficient PCR product for DGGE analysis. Extracting DNA from clay-based materials is notoriously difficult (e.g., Stroes-

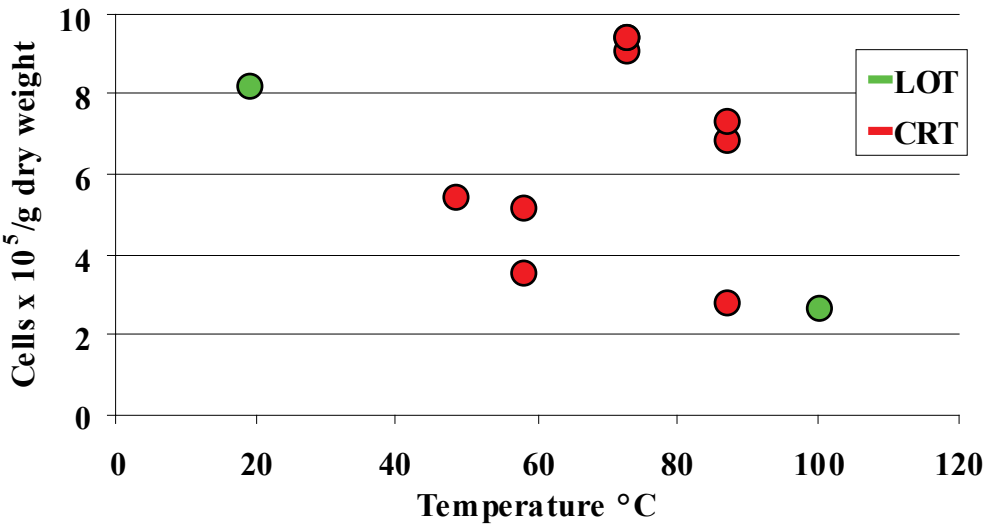


Figure 13: PLFA-based Viable Biomass as a Function of Temperature in LOT and CRT Samples

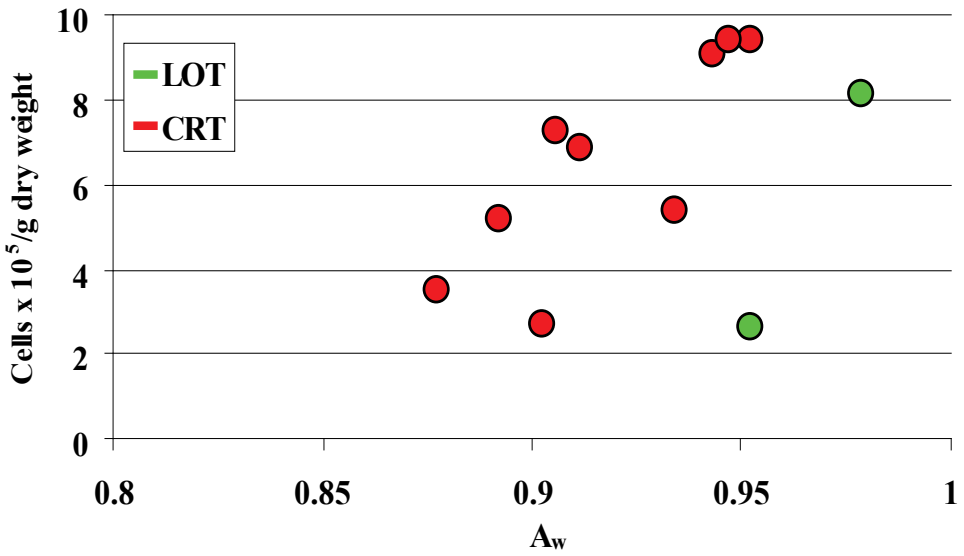


Figure 14: PLFA-based Viable Biomass as a Function of Water Activity in LOT and CRT Samples

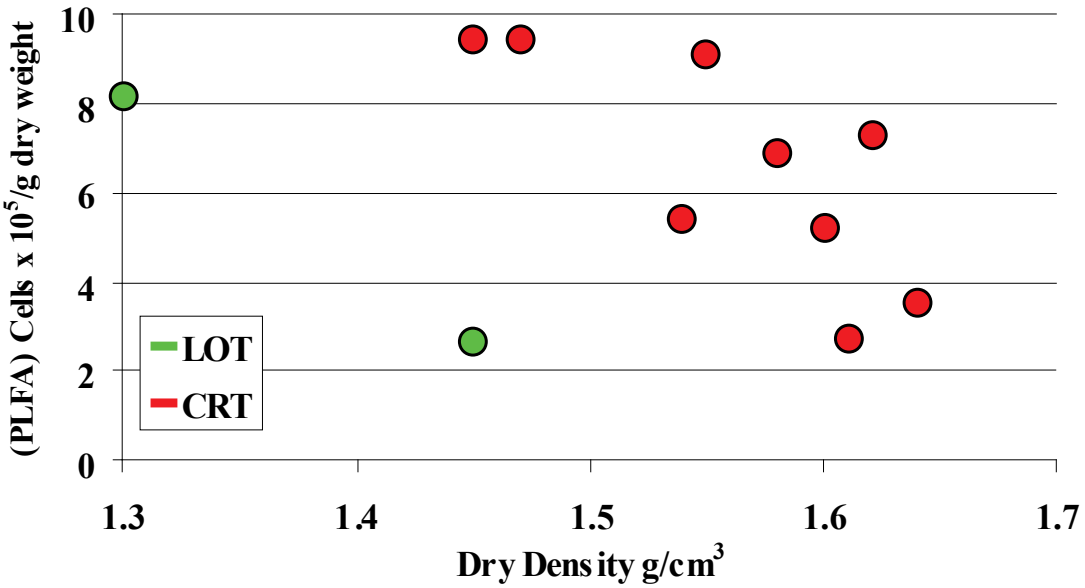


Figure 15: PLFA-based Viable Biomass as a Function of Calculated Dry Density in LOT and CRT Samples

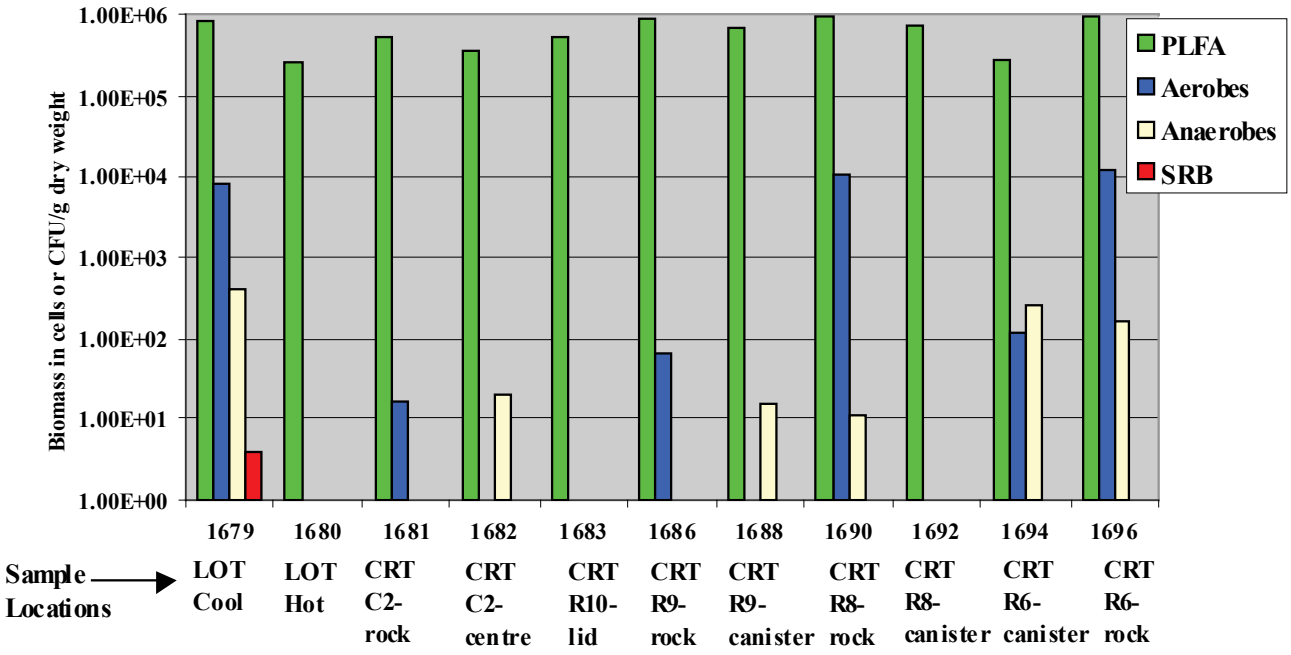


Figure 16: Comparison of Culturability and PLFA-based Viability in LOT and CRT Samples

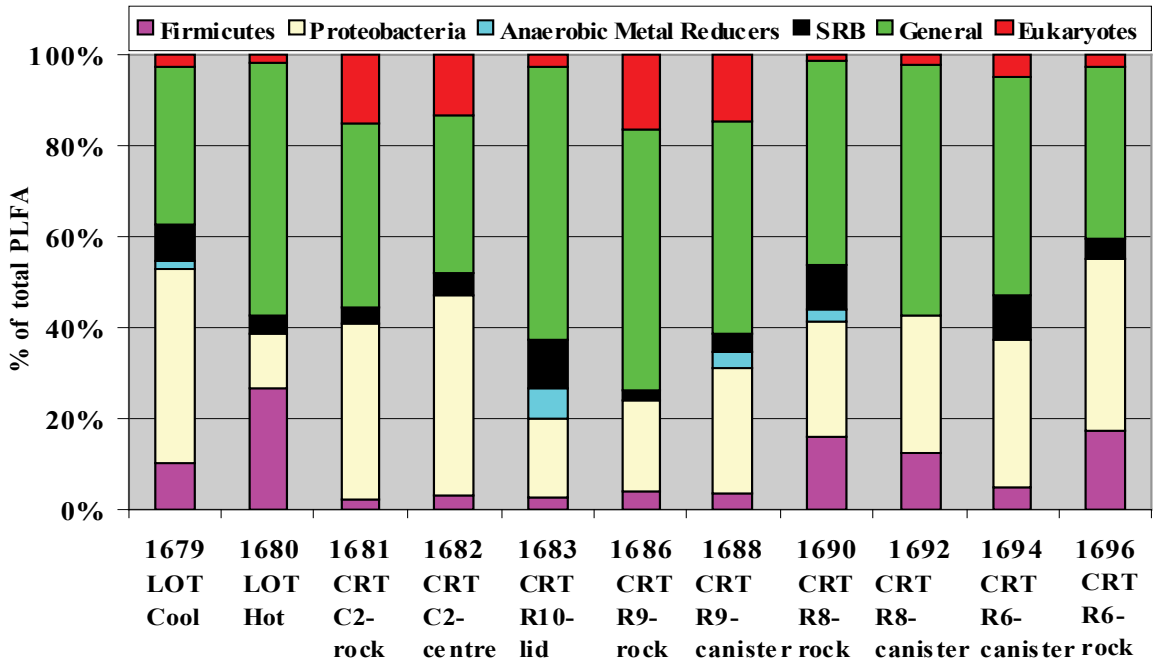


Figure 17: PLFA-based Community Structure Results for LOT and CRT Samples

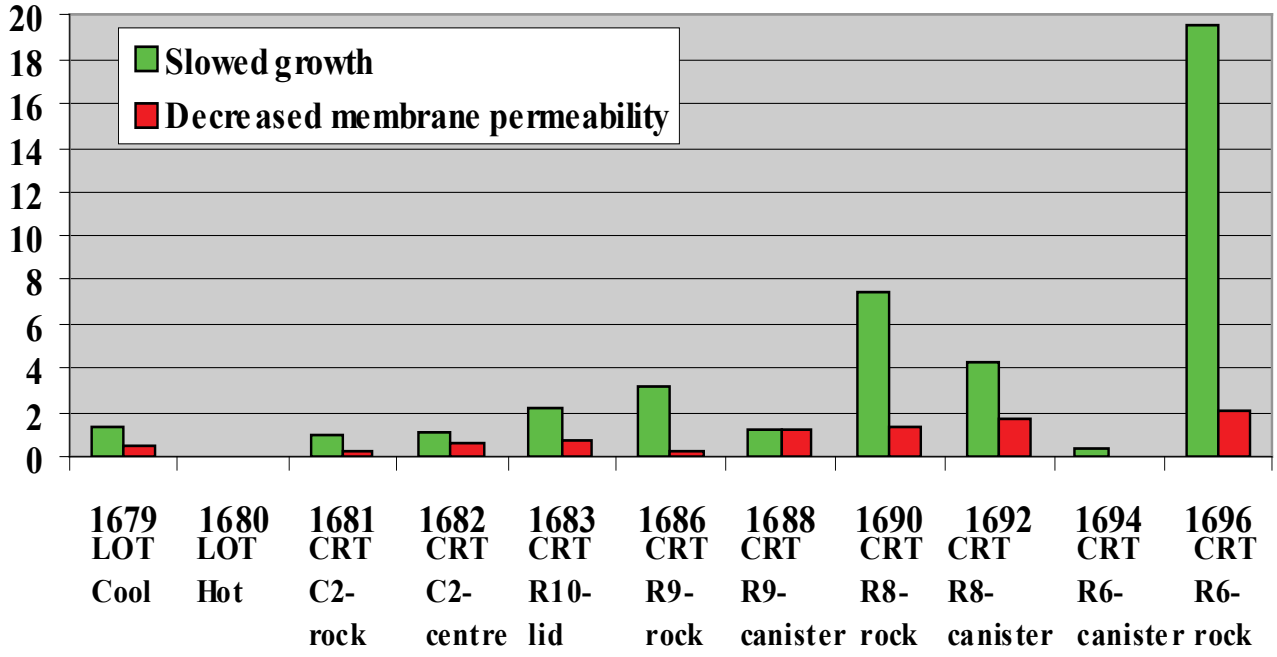


Figure 18: Comparison of Ratios for Slowed Growth and Decreased Membrane Permeability in LOT and CRT Samples

Gascoyne et al. 2007). Figure 19 gives the (limited) DGGE profiles of these samples, while Table 14 gives the identifications resulting from the DGGE profiles.

Figures 20 and 21 compare the aerobic culturability as a function of water activity in the LOT and CRT samples respectively with results from laboratory experiments (Stroes-Gascoyne et al. 2006).

4.2 Results Obtained by Sweden

All U of G results for the LOT samples can be found in Karnland et al. (2009) while all Micans results for the CRT samples are recorded in Lydmark and Pedersen (2011).

Tables 15 and 17 show a comparison between Sweden and AECL for sample location, dry density and water content in the LOT and CRT samples, respectively. Tables 16 and 18 compare enumeration results. To facilitate this comparison in Table 18, the numerous Swedish samples have been condensed to three regions, the rock-bentonite interface, the bulk bentonite and the Cu-bentonite interface. The Swedish enumeration results are expressed in ranges for these regions.

5. DISCUSSION

5.1 Results Obtained by AECL

5.1.1 Effects of Temperature on Culturability and Viability

Figures 10 (average culture results) and 14 (PLFA results) show that for the two LOT samples there is a distinct effect of temperature on culturability and viability, with the low temperature LOT sample showing close to 10^4 Colony-Forming Units (CFU)/g dry weight aerobes, 4 x 10^2 CFU/g anaerobes and a small but positive number of SRB at 19°C and negative culture results in the ~100°C sample. The viability as indicated by PLFA content was reduced by a factor of 3 in the ~100°C sample, compared to the 19°C sample.

For the CRT samples, the trend of average culturability (Figure 10) and viability (Figure 14) as a function of temperature is not very clear. However, for the samples taken from the bentonite rings around the container (i.e., the R9, R8 and R6 samples), average culturability and viability is generally higher near the rock-bentonite interface (~73°C) than near the canister (~87°C) (Table 13). In addition, the results in Table 11 show that samples taken directly at the rock-bentonite interface (i.e., 1686, 1690 and 1696) contain considerably more culturable aerobes than the samples taken slightly further into the bulk of the bentonite (i.e., 1687, 1691 and 1697). Similar trends (although with much lower aerobic culturability) were obtained for the samples taken directly at the bentonite-canister interface (i.e., 1688, 1692 and 1694) compared to those slightly further into the bulk of the bentonite (i.e, 1689, 1693 and 1695). It appears, therefore, that interfaces are conducive to higher culturability.

Table 14: Sequence Results from Bands Excised from Figure 19

Band	Similar Genus*	Similarity Index	GenBank Accession* Number	Electron Donors	Electron Acceptors	Description of Organism
8.1	*Tepidimonas* spp.	0.968	AB206468, AY594193	SO,$S_2O_3^{2-}$	Sulphur, O_2	Aerobic (chemolithoheterotroph)
10.1	*Clostridium* spp.	0.849	AB237714	H_2, acetate	acetate, proline	Obligate anaerobe, spore-formation, no sulfate reduction and Gram positive cell walls. Clostridia live by fermentation

*Identifications are based on DNA sequences in the Ribosomal Database Project (RDP)

Table 15: Comparison of LOT Results from Sweden and AECL

	Sweden					AECL			
LOT Layer	**Sample Location (cm)**	**Type of Sample**	**Density (kg/m^3)**	**Water Content (%)**	**Dry Density* (kg/m^3)**	**Sample Location (cm)**	**Type of Sample**	**Water Content (%)**	**Dry Density** (kg/m^3)**
Top LOT	0	Cu-B							
Block 38	1	Bulk	1864	38	1355				
T = 19°C	2	Bulk							
	3	Bulk	1879	38	1359				
	4	Bulk							
	5	Bulk	1859	37	1353				
	6	Bulk							
	7	Bulk	1862	39	1337	~ 7	Bulk	39.8	1300
	8	Bulk				19°C			
	9	R-B	1847	41	1312				
Mid LOT	0	Cu-B							
Block 19/20	1	Bulk	1978	29	1538				
101°C (0, 1)	2	Bulk							
91°C (3)	3	Bulk	1982	29	1542				
	4	Bulk							
81°C (5)	5	Bulk	1969	29	1506				
	6	Bulk							
73°C (7)	7	Bulk	1955	30	1473	~ 7	Bulk	31.2	1450
	8	Bulk				80-100°C			
67°C (9)	9	R-B	1928	33	1441				

R-B = Rock-Bentonite Interface
Cu-B = Copper-Bentonite Interface

*Calculated from density and water content
**Calculated from water content assuming 100% saturations

Table 16: Comparison of Culture Results for LOT Samples from Sweden and AECL

(from Karnland et al. 2009)

	Sweden						AECL					
LOT Layer	**Sample Location (cm)**	**Sample Type**	**Aerobes CFU/g x10^3**	**SRB MPN/g**	**AA MPN/g**	**ATP amole/g**	**Sample Location (cm)**	**Sample Type**	**Aerobes CFU/g x10^3**	**Anaerobes CFU/g x10^2**	**SRB MPN/g**	**PLFA Cell Equiv./g x10^5**
Top LOT Block 38	0	Cu-B	4 ± 0.4	7(3-41)	10(2-36)	<10^5						
	1	Bulk	0.6 ± 0.3	-	15(3-54)	<10^5						
	2	Bulk	58 ± 7	11(2-39)	6(3-37)	<10^5						
	3	Bulk	22 ± 14	-	35(2-54)	<10^5						
	4	Bulk	0.7 ± 0.3	-	-	<10^5						
	5	Bulk	13 ± 5	22(3-53)	17(4-64)	<10^5						
	6	Bulk	3 ± 2	-	-	<10^5						
	7	Bulk	9 ± 5	5(2-30)	7(3-42)	<10^5	~ 7	Bulk	8.3	4.5	4	8.2
	8	Bulk	13 ± 9	-	27(3-64)	<10^5						
	9	R-B	8 ± 4	-	-	<15						
Mid LOT Block 19/20	0	Cu-B	-	-	-	<10^5						
	1	Bulk	0.09±0.08	-	-	<10^5						
	2	Bulk	-	-	-	<10^5						
	3	Bulk	-	-	-	<10^5						
	4	Bulk	49 ± 34	-	-	1 ±0.2 x10^6						
	5	Bulk	-	-	-	<10^5						
	6	Bulk	-	-	-	<10^5						
	7	Bulk	0.12±0.08	-	-	<10^5	~ 7	Bulk	BDL	BDL	BDL	2.7
	8	Bulk	-	-	-	<10^5						
	9	Bulk				<10^4-10^6						

R – B = Rock-Bentonite Interface
Cu-B = Copper-Bentonite Interface
Bulk = Bulk Bentonite
Cell Equiv. = Cell Equivalent

SRB = Sulphate-reducing Bacteria
AA = Autotrophic Acetogens
ATP = Adenosine-Tri-Phosphate

CFU = Colony Forming Units
MPN = Most Probable Number (99% confidence limit)
PLFA = Phospholipid Fatty Acid

Table 17: Comparison of CRT Results from Sweden and AECL

(from Lydmark and Pedersen 2011)

MICANS (SKB)						AECL (OPG)			
ORT Layer	**Distance from Center (cm)**	**Type of Sample**	**Density (kg/m^3)**	**Water Content (%)**	**Dry Density* (kg/m^3)**	**Distance from Center (cm)**	**Type of Sample**	**Water Content (%)**	**Dry Density** (kg/m^3)**
C_2	88-86	R-B	2016	26	1600	~ 86	R-B	27.5	1540
33-55°C	60-59	Bulk	2009	24	1620				
	33-32	Bulk	2013	22	1650	~ 30	Bulk	23	1640
R10 (33-55°C)	53	Cu-B	1980	22	1623	~ 30-50	Cu-B	24.2	1600
R9	88	R-B	1914	32	1450	88-87	R-B	27.7	1550
33-55°C	88-70	Bulk	1909	29	1480				
	70	Bulk	2025	25	1620				
	65-55	Bulk	2028	26	1610				
	54	Cu-B	2016	26	1600				
	53	Cu-B	1995	27	1571				
	53	Cu-B	2016	26	1600	53-54	Cu-B	25.4	1580
R8						88-87	R-B	31.2	1450
						53-54	Cu-B	24.5	1620
R6	88-85	R-B	1970	29	1527	88-87	R-B	29.5	1470
50-85°C	55-53	Cu-B	1985	26	1575	53-57	Cu-B	24.7	1610

R-B = Rock-Bentonite Interface
Cu-B = Copper-Bentonite Interface
Bulk = Bulk Bentonite
*Calculated from density and water content
**Calculated from water content assuming 100% saturation

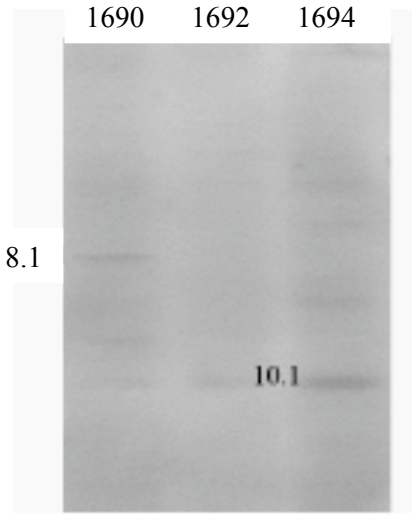
Table 18: Comparisons of CRT Culture Results for Samples from Sweden and AECL

(from Lydmark and Pedersen 2011)

Sweden							AECL					
CRT Layer	**Distance from Center (cm)**	**Type of Sam-ple**	**Aerobes CFU/g range**	**SRB MPN/g range**	**AA MPN/g range**	**ATP amole/g**	**Dis-tance from Center (cm)**	**Type of Sam-ple**	**Aerobes CFU/g Range**	**Anaerobes CFU/g Range**	**SRB MPN/g**	**PLFA Cell Equiv. /g**
C_2	88-86	R-B	bdl to $(1.3\pm0.9)\times10^4$	bdl-24 (bdl-89)	bdl-42 (bdl-124)	$<10^5$	~ 86	R-B	$(4\pm7)\times10^0$-$(3\pm1)\times10^1$	$(4\pm7)\times10^0$-$(1.3\pm1.5)\times10^1$	<4	5.4×10^5
	60-32	Bulk	bdl to $(4.7\pm3.3)\times10^3$	bdl-14 (bdl-76)	bdl-63 (bdl-154)	$<10^5$	~ 30	Bulk	$(1.2\pm1.2)\times10^1$-$(1.0\pm1.7)\times10^2$	$(3.0\pm2.6)\times10^1$-$(1.2\pm1.2)\times10^1$	<4	3.6×10^5
R10	53	Cu-B	bdl to $(3\pm0.6)\times10^2$	bdl	bdl	$<10^5$	~30–50	Cu-B	$(3.5\pm6.1)\times10^0$-<12.5	$(7\pm6)\times10^0$-<12.5	<3	5.2×10^5
R9	88-87	R-B	bdl to $(1.6\pm2.2)\times10^3$	bdl-10	bdl-19 (bdl-71)	$<10^5$	88-87	R-B	$(3\pm5)\times10^0$-$(1.3\pm0.4)\times10^2$	$(9\pm8)\times10^0$-<9	<3	9.1×10^5
	80-55	Bulk	bdl to $(1.0\pm1.3)\times10^3$	bdl	bdl	$<10^5$						
	54-53	Cu-B	bdl to $(1.6\pm1.3)\times10^3$	bdl	bdl-19 (bdl-71)	$<10^5$	53-54	Cu-B	<14.3	$(2\pm1)\times10^1$-$(6\pm11)\times10^0$	<4	6.9×10^5
R8							88-87	R-B	$(2.2\pm0.6)\times10^4$-<7.7	$(2.2\pm0.6)\times10^1$-<7.7	<3	9.4×10^5
							53-54	Cu-B	$(5\pm9)\times10^0$-<7.7	<7.7	<3	7.3×10^5
R6	88-85	R-B	bdl to $(5\pm4)\times10^2$	bdl	bdl-8	$<10^5$	88-87	R-B	$(2.3\pm0.1)\times10^4$ -<8.3	$(8.1\pm0)\times10^1$ -$(2.7\pm0.2)\times10^2$	<3	9.5×10^5
	55-53	Cu-B	bdl to $(7\pm5)\times10^2$	bdl	bdl	1.4×10^6	53-57	Cu-B	$(4.4\pm7.7)\times10^0$-$(2.3\pm1.9)\times10^2$	$(4.9\pm5.9)\times10^2$ -<12.5	<7	2.8×10^5

bdl = below detection limit of the culture method
R-B = Rock-Bentonite Interface
Cu-B = Copper-Bentonite Interface
Bulk = Bulk Bentonite
Cell Equiv. = Cell Equivalents
SRB = Sulphate-Reducing Bacteria
AA = Autotrophic Acetogens
ATP = Adenosine-Tri-Phosphate
CFU = Colony Forming Units
MPN = Most Probable Number (99% confidence limit)
PLFA = Phospholipid Fatty Acids

DGGE Profile for samples 1690, 1692, 1694



Notes:

1. Only samples 1690, 1692 and 1694 produced sufficient PCR product to attempt DGGE profiles.
2. Bacteria must constitute at least 1-2% of the total bacterial community to form a visible band.
3. Labelled bands were excised and sequenced (results in Table 14).

Figure 19: DGGE Profiles of Amplified DNA from a Portion of the 16S rRNA Gene in Three CRT Samples

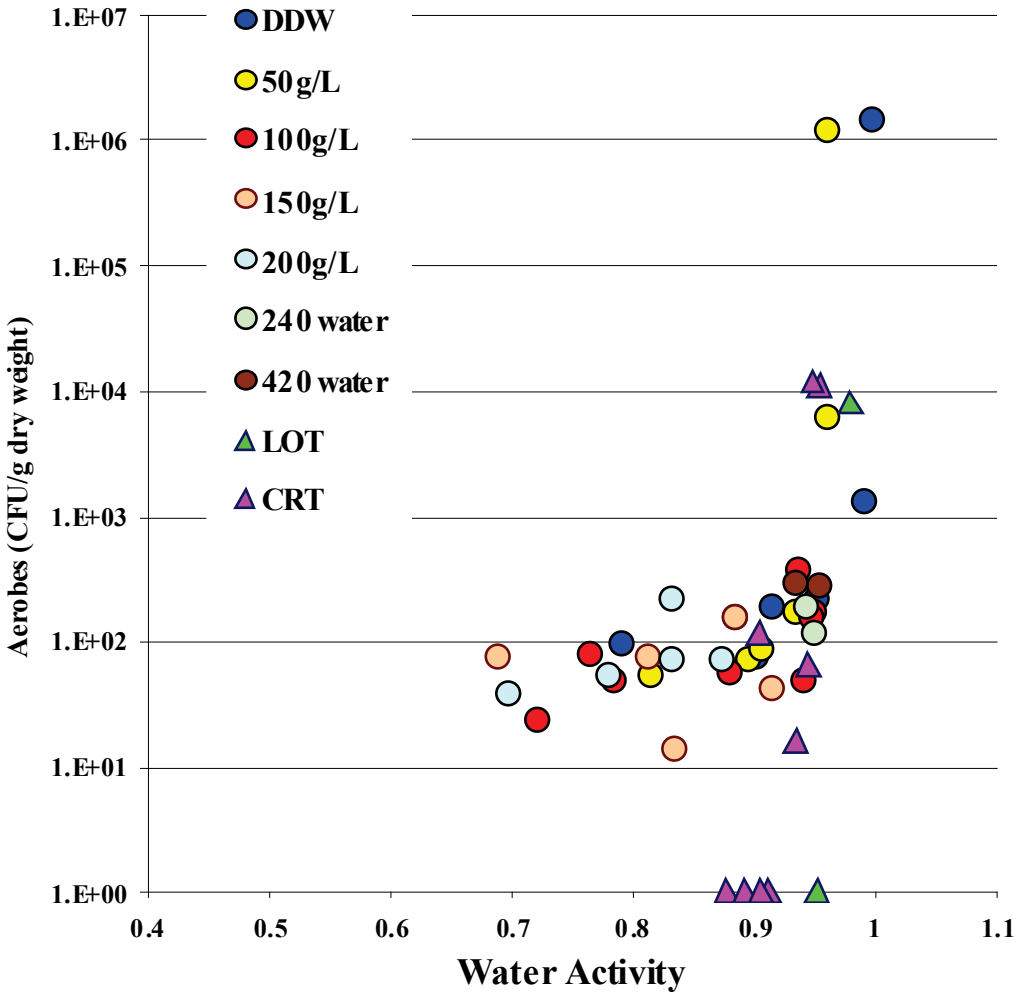


Figure 20: Aerobic Culturability as a Function of Water Activity; Comparison of LOT and CRT Results with Laboratory Experiments

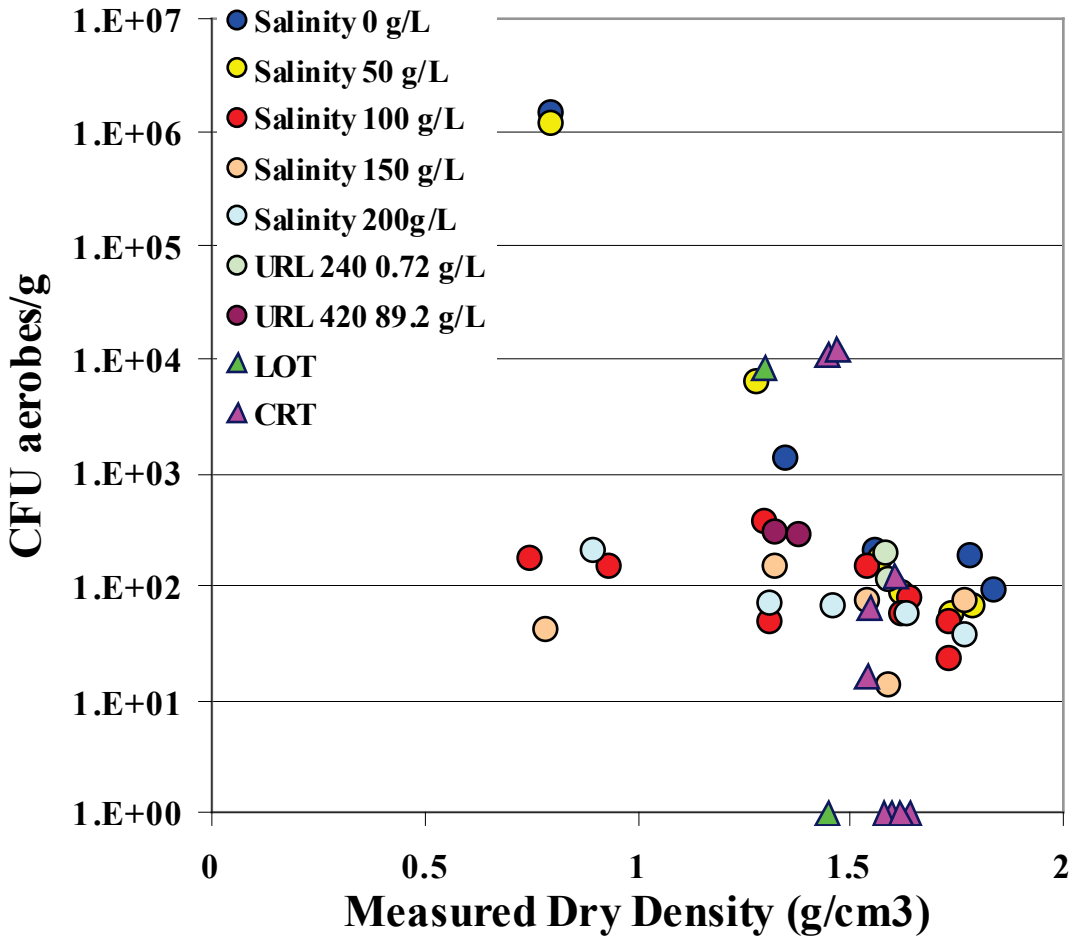


Figure 21: Aerobic Culturability as a Function of Dry Density; Comparison of LOT and CRT Results with Laboratory Experiments

The samples from the layers above the container (i.e., the R10 and C2 samples, at ~50°C) showed mostly comparable or lower average culturability and viability than the hotter R samples. Possible reasons for this may include slightly lower a_w and higher dry density values in the layers above the container.

5.1.2 Effects of Water Activity on Culturability and Viability

Figures 11 (average culture results) and 14 (PLFA results) show that for the two LOT samples, a_w is close to 1.00 in the cool sample with highest (average) culturability and viability and about 0.95 in the hot sample that showed no culturability and reduced viability. It is likely that the high temperature of ~100°C is the overriding cause of the zero culturability in the second LOT sample, rather than just the reduced a_w value.

For the CRT samples, a trend between average a_w values and average culturability and viability appears present, with culturability and viability values the highest around a_w of 0.94 to 0.95 and

lower in samples with lower a_w values. However, while in many samples culturability is non-existent, some samples maintain culturability at levels between 10^1 and 2 x 10^2 organisms (Figure 11) for average a_w values as low as 0.888, comparable to results obtained for the same type of compacted bentonite in laboratory studies, as shown in Figure 20 (Stroes-Gascoyne et al., 2006). Figure 14 shows that with decreasing average a_w values in the samples, the PLFA-based viability is reduced by a factor of 1.5 to 3. Another way of interpreting these data is using the non-averaged a_w and culture results in Table 11. It is then noticed that right at the rock-bentonite interface the measured a_w was slightly lower than in the corresponding bulk sample, yet with higher culturability at the interface. Correspondingly, at the bentonite-canister interface, a_w is considerably lower than in the corresponding bulk of the bentonite, yet again with low but (in the case of sample 1694 especially) higher culturability than in the corresponding bulk samples. This again suggests that interfaces are especially conducive to microbial culturability, despite adverse conditions. It could also perhaps suggest that the surfaces of the bentonite samples were somehow contaminated during sample retrieval at the Äspö HRL. However, this appears unlikely because of the absence of a systematic trend and the absence of any culturability in several surface samples (e.g., 1685 in R10 and 1688 in R9).

5.1.3 Effects of Dry Density of Culturability and Viability

Figures 12 (average culture results) and 15 (PLFA results) show that for the hot LOT sample, average culturability was non-existent at a relatively low dry density of 1.45 kg/dm^3 and viability was down by a factor of three compared to the cooler LOT sample. However, this sample was subjected to 100^{o}C and is likely responding more to temperature than to dry density. The cooler LOT sample had a dry density of 1.30 kg/dm^3 and significant culturability (~10^4 CFU/g). The CRT samples appear to show a trend with dry density in that the highest average culturability (~10^4 CFU/g dry weight) and viability (~9.5 x 10^5 cells/g dry weight) occurred in samples with the lowest dry densities (1.45-1.47 kg/dm^3). However, even at dry densities as high as 1.63 kg/dm^3, some culturability (~10^1 to 10^2 CFU/g dry weight) remains in a number of samples, whereas viability is reduced by a factor of up to about three. These culture results are comparable to results from laboratory studies with the same type of compacted bentonite, as shown in Figure 21 (Stroes-Gascoyne et al., 2006).

Table 2 gives the as compacted dry densities for the CRT blocks at 85% saturation. The values ranged from 1.71 kg/dm^3 for the C2 block, 1.77 kg/dm^3 for the R10 block to 1.79 kg/dm^3 for the R9, R8 and R7 blocks. Upon saturation, concurrent swelling occurred into free space (i.e., the 5-cm wide pellet- and water-filled gap between the rock and the bentonite and the 1-cm wide empty gap between the canister and the bentonite). This reduced dry densities to around 1.45 – 1.54 kg/dm^3 near the rock interface and to 1.58 – 1.64 kg/dm^3 near the canister interface (Table 10).

It is possible that the few remaining culturable organisms in some samples, despite having been subjected to high temperatures, low a_w values and high dry density, are spore formers for which conditions in the compacted bentonite of the CRT samples were not adverse enough to lose culturability entirely. Figure 16 compares culturability levels with viability levels in all samples. Viability is orders of magnitude higher in all bentonite LOT and CRT samples. Although viability appears to drop with increasing adverse conditions with respect to temperature, a_w and dry density, the overall drop is only about a factor of three, far less than the drop in culturability, which ranged from two to four orders of magnitude. This suggests that the adverse effects (of high temperature, low a_w and high dry density) have forced the bacteria in

the bentonite into a viable but (largely) non-culturable (VBNC; e.g., McDougald et al. 1998) state with some loss of viability. Prolonged adverse conditions would slowly reduce viability further. McDougald et al. (1998) have proposed that during the initial stages of adverse effects in their environment, cells lose culturability while maintaining intact membranes and RNA and DNA. Such cells thus maintain the potential for resuscitation (i.e., they are still viable). In the latter stages of the VBNC state, cells gradually experience degradation of nucleic acids and thus gradually lose the potential for resuscitation (i.e., a gradual decline into non-viability). How long this process would take depends on many factors and could stretch over many years.

However, it is not likely that VBNC cells maintain a high level of in situ metabolism and, therefore, these cells can be considered dormant and hardly active in situ, unless the adverse conditions change, as would be the case when a (sudden) drop in dry density occurs, for instance when the bentonite swells into an empty space. Such a drop in dry density would be accompanied by an increase in a_w in the bentonite, and both factors appear to have a positive effect on culturability and viability. Stroes-Gascoyne et al. (2011) were in fact able to show that viability of microbial cells naturally present in small compacted bentonite plugs increased as the clay plugs were allowed to swell into void space, which reduced dry density and increased water activity.

5.1.4 Community Structure and Physiological Status

Figure 17 presents the PLFA-based community structure in the LOT and CRT samples. It is clear that there appear to be some shifts in the populations, but generally population structure is fairly similar in samples from the same bentonite blocks in the CRT. This could perhaps be related to the pre-experiment manufacturing method of the blocks. It is not clear what source (or sources) of water were used in the mixing of the bentonite prior to pressing of the blocks (Section 1.2). For example, Eukaryotes (organisms such as aerobic fungi, algae, protozoa and higher plants and animals) form a considerable part of the population make-up in the two samples from the C2 and R9 layers, but only a small part in the deeper R8 and R6 layers. Concurrently Firmicutes (Gram-positive, hardier, mostly anaerobic organisms) are low in the shallower C2, R10 and R9 layers and considerably higher in the deeper R8 and R6 layers, further into the borehole. The largest groups of PLFA in virtually all samples are, as expected, those typical of the Gram-negative Proteobacteria and especially the normal saturated lipids that are found in both the Prokaryotic and Eukaryotic kingdoms. A high proportion of normal saturated lipids often indicates a less diverse population, which suggests a less-than-optimal environment in the bentonite.

Figure 18 shows lipid ratios indicative of “slowed growth” and “decreased membrane permeability” for all LOT and CRT samples. Although the absolute values of the ratios are not necessarily meaningful, the shifts in ratios when comparing samples from the same origin (i.e., compacted bentonite in this case) can indicate changes in the physiological status of the organisms present. Figure 18 shows an increasing trend with depth in the CRT samples, both in the “slowed growth” ratio and to a lesser extent in the “reduced membrane permeability” ratio. This could indicate that with increasing burial time and depth, the cells become increasingly more stressed to the point eventually of cell dormancy (non-culturability) and ultimately cell death (non-viability), as discussed.

5.1.5 DNA-based Identifications

Only samples 1690, 1692 and 1694 yielded sufficient PCR product to produce a very limited DGGE profile as shown in Figure 19. The production of PCR product appears not directly related to the culturability or PLFA content of the samples. Table 14 gives the species identified from the DGGE profile (Figure 19). Only two species resulted: Band 8.1 in sample 1690 corresponds to *Tepidimonas* spp., an aerobic chemolithotrophic organism. *Tepidimonas* spp. is a beta-Proteobacterium and appears to belong to a relatively new genus. A variety of new species of this genus have been found recently, and all of these appear to be (slightly) thermophilic (e.g., Moreira et al. 2000, Freitas et al. 2003, Ko et al. 2005, Albuquerque et al. 2006). All CRT samples from the CRT R blocks were subjected to temperatures in excess of 70^{o}C and it is, therefore, possible that the *Tepidimonas* spp. found in sample 1690 has thermophilic characteristics.

Band 10.1, present in all three samples but strongest in sample 1694, corresponds to *Clostridium* spp., an obligate anaerobic, fermenting spore-forming Gram-Positive organism, frequently found in soils. Clostridia are also indicated by the presence of fatty acids indicative of Firmicutes in these samples. In a previous study by Motamedi (1999) and Pedersen et al. (2000) in which parcel S1 of the LOT test was doped with a variety of laboratory-grown bacteria, only spore-forming bacteria were found to survive the compacted and heated bentonite environment. Therefore, the positive identification of the spore-forming bacterium *Clostridium* spp in the CRT confirms survival of spore-formers in compacted bentonite under repository-relevant conditions. Spore-formers are inactive as long as they are in the spore state, and would pose, therefore, no MIC threat to the used fuel containers in a repository, unless conditions change and they are revived to their vegetative state.

5.2 Comparison of Results Obtained by AECL and Sweden

Tables 15 and 17 compare sample location, water content and dry density data obtained by Sweden and AECL. Generally there is excellent to good agreement between the two laboratories for water content and dry density in corresponding locations, i.e., the values agree within 1 to 3 % for the LOT samples and between 2 to 11% for the CRT samples.

The Swedish enrichment cultures (results not shown) indicated elevated sulphide and acetate concentrations in most of the Top LOT (wet) samples analyzed and all these cultures contained bacteria with a variety of morphologies as seen by microscopy, indicating the presence of viable SRB and AA throughout the Top LOT sample. In the Mid LOT (dry) bentonite, no acetate was found in any of the samples. Only three of the Mid LOT samples contained a low concentration of sulphide but no cells could be detected by microscopy. It is, therefore, unlikely that the Mid LOT samples contained viable SRB and AA. The low concentration of sulphides in three Mid LOT samples is likely due to sulphides present naturally in the bentonite.

Enumeration results for the LOT samples obtained by Sweden are shown in Table 16. Aerobes were present in all Top LOT samples and in three of the 10 Mid LOT samples. SRB could be enumerated from four of the 10 Top LOT samples but not from any of the Mid LOT samples. AA could be enumerated from seven of the 10 Top LOT samples but not from any of the Mid LOT samples. The ATP analysis showed levels below the detection limit of the method (which translates to less than 10^4 to 10^6 cells/g) except in one Mid LOT sample for which the ATP

analysis suggested the presence of about 5 x 10^6 viable cells/g. These results reflect the difference in dry density, water content and temperature in the Top LOT and Mid LOT samples.

A comparison between the culture results from Sweden and AECL for the LOT samples (Table 16) shows in general a reasonably good agreement for the aerobes in the Top LOT sample, considering that different media and methods were used. SRB values are up to an order of magnitude higher in the Swedish analyses compared to the AECL result. This could perhaps be due to the fact that Sweden used a 12 to 16 h old enrichment culture as inoculum for the enumerations. The PLFA cell equivalent data obtained by AECL appear to be of the same order of magnitude as the ATP cell equivalent data obtained by Sweden, although the latter show a "less than" result in all but one case. Sweden was able to culture some aerobes in the Mid LOT samples, but the errors on these analyses were generally large. AECL's analyses for aerobes in the Mid LOT sample were below the detection limit. No SRB could be cultured from the Mid LOT samples by either laboratory. The AA results obtained by Sweden were also negative in all locations of the Mid LOT sample.

Table 18 gives the enumeration results for the CRT samples obtained by Sweden. The aerobes results generally show ranges from 10^1 to 10^4 cells at the rock-bentonite interface and from 10^1 to 10^3 cells in the bentonite bulk and at the Cu-bentonite interface. This order of magnitude difference likely reflects the slightly more advantageous conditions at the rock-bentonite interface (i.e., generally a higher water content and a lower dry density). The errors on the enumerations are generally large, and in some cases the error is larger than the actual result. SRB could not be cultured in the R10 and R6 samples and only at the rock-bentonite interface samples in the R9 layer, whereas AA occurred in most samples except in the R10 layer. The ATP analysis showed numbers below detection limit of the method (i.e., $<10^4$ to 10^6 cells/g) for all but one sample. The Cu-bentonite sample in the R6 layer contained a surprisingly high ATP content, which is possibly due to contamination, although culture results were negative.

The comparison between Swedish and AECL results in Table 18 suggests that in most cases the enumeration results obtained by Sweden were somewhat higher (i.e., up to about one order of magnitude) than those obtained by AECL. This could be due to a number of reasons, because different media and enumeration methods were used. Also, the samples enumerated by AECL were stirred for 30 minutes in a sterile saline solution prior to analysis whereas the samples enumerated by Sweden consisted of 12 to 16 h old enrichment culture suspensions. It is possible that during this time some revival and/or growth occurred in the liquid media, which would be reflected in the generally higher enumeration results obtained by Sweden. A third factor could have been the shipping time to AECL; Sweden enumerated fresh samples in comparison.

Both laboratories found high numbers of cells in samples from the CRT R6 layer, as indicated by ATP and PLFA results. However, Sweden found high ATP-derived populations at the Cu-bentonite sample in this layer whereas the AECL results indicated both high aerobic culture results and high PLFA results at the rock-bentonite interface in this layer. It is not obvious why layer R6 should have contained a higher number of viable and culturable organisms, because this layer likely experienced higher temperatures than layer R9. The possibility of sample contamination during sample retrieval was suggested by both AECL and Swedens. However, correspondingly high culture results would also be expected in that case, which was the case in the AECL sample but not in the Micans sample.

6. SUMMARY AND CONCLUSIONS

Two LOT bentonite samples and nine CRT bentonite samples were shipped from the Äspö HRL, Sweden to AECL, Canada, for microbial analysis to establish the culturability and viability of naturally present bacteria in these compacted 100% bentonite samples, in order to assess the potential for MIC of used fuel containers in a future repository. Culturability and viability were assessed as a function of sample temperature, water activity (a_w) and dry density. One of the two LOT samples was subjected to very high temperature (~100^{o}C) which was clearly reflected in the absence of any culturability in that sample and a reduction in PLFA-based viability by a factor of three. The other LOT sample was subjected to much lower temperature (19^{o}C) and had a low dry density and high a_w, which resulted in considerable culturability of aerobes and anaerobes while the presence of culturable SRB was also indicated.

The CRT culturability showed no distinct trend with temperature although the samples from the blocks around the Cu canister generally contained less culturability at or near the bentonite canister interface (~87^{o}C) than in the samples near the rock interface (~73^{o}C). Samples from blocks located above the canister appeared to have slightly less culturability than samples from around the container near the rock interface, possibly because the average a_w was slightly lower and the dry density slightly higher in these upper blocks compared to the blocks located around the canister. A lower a_w generally resulted in lower culturability and viability and a higher dry density generally reduced both culturability and viability as well. These results are in agreement with laboratory studies with the same type of bentonite (Stroes-Gascoyne et al., 2006). PLFA-derived viability was always orders of magnitude higher than culturability, suggesting that the majority of cells naturally present in the bentonite are in the VBNC state and are, therefore, likely metabolically not very active in situ. Culture results obtained from samples right at the bentonite-rock interfaces and bentonite-canister interfaces were generally higher than in bulk samples nearby, despite harsher conditions right at these interfaces with respect to a_w and temperature in the case of the bentonite-canister interface. This suggests that interfaces are more conducive to microbial activity, possibly related to the availability of more space (lower dry density) right at these interfaces. It is not entirely clear what implications this could have for the development of MIC at canister-bentonite interface locations. However, it is unlikely that the effects would be significant because culturabilty found at the canister-bentonite interface was very low and possibly spurious (i.e., contamination was a possibility).

The community structure of the CRT samples showed similar profiles in samples from the same CRT block, but no other patterns were obvious. The ratios for “slowed growth” and “decreased membrane permeability” increased with increasing depth into the CRT experiment. Only three CRT samples gave sufficient PCR product to attempt DGGE separation and band dentification. Two organisms were identified, *Tepidomonas* spp., an aerobic chemolithotrophic organism, which possibly has thermophilic characteristics and *Clostridium* spp., a common spore-forming Gram-positive fermenter, also indicated by the PLFA profiles in these samples.

Overall the only significant culturability was found right at the rock-bentonite interfaces in the CRT samples and in the one, low temperature, low dry density, high a_w, LOT sample. This suggests that microbial activity in the bulk of compacted bentonite will be severely suppressed as long as dry density remains high and a_w relatively low. Temperature also has an (be it less clear) effect but over time in a repository, temperature will decrease while a high dry density would develop at the canister interface upon saturation and swelling of the bentonite. Overall, these results suggest that it is unlikely that significant MIC will occur at the Cu-bentonite interface.

There is reasonable agreement between the Swedish and AECL analyses for most sample types. Viable and culturable bacteria (aerobes, SRB and AA) were present in the Top LOT samples, which, given the opportunity, have the potential to produce sulphide and acetate in situ and, therefore, could contribute to MIC. However, dry density and temperature were low in the Top LOT samples, whereas in a repository both these parameters will be affecting viability negatively. The Mid LOT sample is in fact more representative of a repository situation, with its higher dry density and temperature and lower water content compared to the Top LOT sample. Very few culturable and viable organisms were found in the Mid LOT layer and, therefore, although a potential for MIC still exists, because there were still some viable cells present in the Swedish analyses, the chance that this potential is realized is very low. Similar conclusions can be drawn from the CRT results. There is a potential for MIC because of the presence of viable cells in many samples, but it is unlikely that this potential will be realized as long as high dry density and low water activity conditions are maintained in a future repository. Such conditions will limit in situ microbial activity to insignificant levels.

Wersin (2013) also concludes that microbially induced sulphate reduction is inhibited or at least strongly limited in compacted bentonitic clay and considers the process of MIC not relevant for the conditions in LOT test parcel A2. Wersin (2013) noted that some Cu-rich sulphide had formed at the interface between the hot Cu tube and the bentonite and explained this by non-microbial sulphide sources such as pyrite in the bentonite and possibly MoS_2 in the lubricant used in the LOT test.

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