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Microbial analysis of the buffer/ container experiment at AECL´s Underground Research Laboratory

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MICROBIAL ANALYSIS OF THE BUFFER/CONTAINER EXPERIMENT AT AECL'S UNDERGROUND RESEARCH LABORATORY

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by

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PREFACE

This report summarizes the results of the microbial analysis of the Buffer/Container Experiment (BCE). The BCE was carried out by Atomic Energy of Canada Limited (AECL) in its Underground Research Laboratory (URL) with support from Ontario Hydro under the auspices of the CANDU Owners Group. However, the microbial sampling and analysis during decommissioning of the BCE (in May 1994) was a joint effort between AECL, Swedish Nuclear Fuel and Waste Management Company (SKB) and Agence Nationale pour la Gestion des Déchets Radioactifs, France (ANDRA).

The report documents all methodologies used during the microbial sampling and analysis of the BCE and all results obtained. The methodologies are presented in appendices, written by participants from AECL, SKB and ANDRA and no attempt has been made to harmonize the format and style of the Appendices.

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ABSTRACT

The Buffer/Container Experiment (BCE) was carried out at AECL's Underground Research Laboratory (URL) for 2.5 years to examine the in situ performance of compacted buffer material in a single emplacement borehole under vault-relevant conditions. During decommissioning of this experiment, numerous samples were taken for microbial analysis to determine if the naturally present microbial population in buffer material survived the conditions (i.e., compaction, heat and desiccation) in the BCE and to determine which group(s) of microorganisms would be dominant in such a simulated vault environment. Such knowledge will be very useful in assessing the potential effects of microbial activity on the concept for deep disposal of Canada's nuclear fuel waste, proposed by AECL.

Microbial analyses were initiated at WL within 24 hours of sampling for all types of samples taken. Representative samples were shipped to a laboratory in France experienced in the microbial analysis of clays. Microbial analysis of the BCE samples included analyses for total viable aerobic and anaerobic heterotrophs at 25 and 50°C, viable specialized organisms, such as sulphate-reducing bacteria (SRB), methanogens, fermenters, fungi, sulphur-oxidizing bacteria (SOB), iron-related bacteria and slimeformers. Microbial activity was measured by determining the assimilation of ^{3}H-Leucine and the mineralization of ^{14}C-labelled glucose and a ^{14}C-labelled amino acids mixture. Bacteria in selected samples were identified from biochemical reactions in API® strips and DNA sequencing of the 16S rRNA genes. Phospholipid fatty acid (PLFA) analysis was used to determine potential viability, nutritional status and community structure without the need for culturing. Electron microscopy (both ESEM and TEM) allowed direct observation of any bacteria present. All culture results (almost 400) were evaluated with a statistical method (General Linear Model) to determine which variables affected the number of colony-forming units (CFU) in each sample analyzed.

The culture results showed an almost universal disappearance of viable microorganisms (both heterotrophic and specialized bacteria) in the samples taken from near the heater surface, where the moisture content was low (<15%) and the temperature high (as high as 60°C). The microbial activity measurements confirmed the lack of viable organisms with very weak or no activity measured in most of these samples. Generally, aerobic heterotrophic culture conditions gave the highest mean CFU values at both 25 and 50°C. Under anaerobic conditions, and especially at 50°C, lower mean CFU values were obtained. In all samples analyzed, numbers of SRB were <1000 CFU/g dry material. Methanogens were either not present or were found in very low numbers (<200 CFU/g dry material). Anaerobic SOB were found in higher numbers in most sample types with sufficient moisture.

More than 67% of the aerobically isolated strains were identified with the API method as belonging to either the species *Pseudomonas stutzeri* or the genus *Bacillus*. Two of the three dominating species found using 16S rRNA gene sequencing had a high identity with the typical groundwater bacteria *Pseudomonas flavescens* and

Acinetobacter calcoaceticus. A third organism identified was an actinomycete related to *Streptomyces*. The PLFA analysis suggested the presence of potentially viable populations, but these showed characteristics of severe starvation, implying that the bacteria were not actively growing in situ. The electron microscopy examinations revealed an absence of easily observable microorganisms.

The statistical evaluation of all culture data demonstrated clearly that the water content was the variable limiting the viability of the bacteria present, and not the temperature. The water content below which viable bacteria could not be detected on culture media was about 15%. Calculations have shown that a_w for buffer material (used in the BCE) with a 15% moisture content is ~0.96. This is at the boundary where most Gram negative bacteria cease to grow although some groups of bacteria such as *Pseudomonas*, SRB and *Vibrio* are still able to grow. Virtually no viable bacteria were found in the samples of buffer material from the BCE that contained 15% water or less.

The present attention at AECL with regard to microbially influenced corrosion (MIC) effects on waste containers is focused on the effects of SRB, because of the expected reducing conditions in a vault and the presence of sulphate in buffer materials (as a gypsum impurity). The harsh conditions (radiation, high temperature, desiccation) in the zone adjacent to the container would likely result in the destruction of microbes. If repopulation of this zone is limited or prevented, SRB activity would be limited to regions outside this depleted zone. In this case the only microbial impact on the container would result from the diffusion of microbially reduced sulphur species to the container surface. But the results from the BCE have also shown that in buffer samples with sufficient moisture for microbial activity, the numbers of viable SRB were not large, most likely because conditions during the BCE were not sufficiently reducing, but maybe also because of a lack of suitable organics. The latter would limit SRB activity outside the affected zone around the container, which would imply that the indirect MIC effects (i.e., sulphide diffusion) could also be limited, as long as low redox conditions have not developed. The present attention in Sweden is on experiments to determine at which water activity SRB can no longer survive in buffer materials.

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SAMMANFATTNING

Det så kallade buffer/container experimentet (BCE) utfördes under en period av 2.5 år vid det underjordiska forskningslaboratoriet URL i Kanada av Atomic Energy Canada Limited (AECL) i syfte att studera egenskaperna hos buffertmaterial i full skala under relevanta förvarsförhållanden. Man placerade en värmare med samma storlek som en förvarsbehållare i ett depositionshål vid URL (240 m under jordytan) och packade in den med en sand/bentonit blandning av den typ man planerar att använda i det kanadensiska konceptet för slutförvar av högaktivt kärnbränsle från deras reaktorer. Samtidigt med att man avslutade experimentet så togs ett stort antal prover för mikrobiologiska analyser i syfte att studera om de mikrober som förekom naturligt i buffertmaterialet vid start av experimentet hade överlevt under 2.5 år vid höga temperaturer i kompakterad bentonit. Man avsåg också analysera vilka grupper av mikroorganismer som kunde överleva och dominera under simulerade förvarsförhållanden. Resultaten är värdefulla för den kommande utvärderingen av hur mikrobiell aktivitet potentiellt kan inverka på det kanadensiska förvarskonceptet. Eftersom det finns vissa likheter mellan det kanadensiska och svenska koncepten så bedömdes experimentet tillräckligt intressant för en svensk medverkan i de planerade mikrobiologiska undersökningarna. Svenska forskare besökte vid ett par planeringstillfällen URL och deltog sedan under tre intensiva veckor när BCE avslutades och provtogs. Från Sverige bidrog vi med hjälp vid provtagning och odlingar samt med aktivitetsmätningar och DNA analyser.

De mikrobiologiska analyserna gjordes på Whiteshell Laboratories (AECL) inom 24 timmar efter det att provtagningen utförts. Dessutom skeppades representativa prover till forskare i Frankrike med god erfarenhet av mikrob-odling på prover av lera. Analyserna omfattade totalantal viabla aeroba och anaeroba heterotrofer vid 25 och 50°C, samt antal viabla specialiserade mikrober såsom sulfatreducerande bakterier (SRB), metanogener, fermenterande bakterier, svamp, svaveloxiderande bakterier (SOB), järnrelaterade bakterier och slembildande bakterier. Mikrobiell *in situ* aktivitet bestämdes genom mätning av ^{3}H-leucine upptag och mineralisering av ^{14}C-inmärkt glukos och en ^{14}C-inmärkt aminosyrablandning. Förekommande bakterier i utvalda prover analyserades med API® remsor och genom sekvensering av deras 16S rRNA gener. Fettsyraanalyser (PFLA) utnyttjade för bestämning av bakteriernas metaboliska aktivitetsnivåer, näringsmässiga status och artsammansättning, vilket kan göras med denna metod utan att man behöver odla bakterierna. Elektronmikroskopi (EM) utnyttjades (Scanning EM och Transmission EM) för direkta studier av funna bakterier. Alla odlingsresultat analyserades statistiskt med multivariat variansanalys för bestämning av vilka variabler som hade inverkan på antalet odlingsbara bakterier i respektive prov.

Odlingsresultaten visade på näst intill total frånvaro av odlingsbara mikroorganismer i prover tagna nära värmarens yta, där fuktigheten var lägre än 15% och temperaturen var hög (upp till 60°C). Mätningarna av mikrobiell aktivitet konfirmerade detta resultat, ingen aktivitet registrerades i de torra proverna. Överlag gav aeroba, heterotrofa odlingsförhållande högst antal odlingsbara bakterier vid de båda studerade

temperaturerna, 25 och 50°C. Vid anaeroba odlingsförhållanden, särskilt vid 50°C, erhölls betydligt lägre värden. Antalet SRB var mindre än 1000 st per g buffertmaterial i samtliga analyserade prover. Metanogener påträffades bara i mycket låga tal, mindre än 200 st per g buffertmaterial, eller inte alls. Däremot påträffades aeroba SOB i större mängd i samtliga prover med tillräckligt hög fuktighet (>15%).

Med hjälp av API metoden befanns 67% av de aeroba framodlade isolaten tillhöra antingen *Pseudomonas stuzerii* eller släktet *Bacillus*. Två av de tre dominerande arterna som bestämts med 16S rRNA analys hade en hög likhet med de typiska grundvattenbakterierna *Pseudomonas flavescens* och *Acinetobacter calcoaceticus*. En tredje dominerande organism kunde identifieras såsom släkt med aktinomycetsläktet *Streptomyces*. PFLA analyserna indikerade närvaro av potentiellt viabla mikrob-populationer, men med tydliga tecken på låg näringsstatus vilket tyder på att de inte var i aktiv tillväxt i buffertmaterialet. De elektronmikroskopiska undersökningarna gav endast ett begränsat utbyte, få prover visade på förekomst av bakterier.

Den statistiska utvärderingen av alla odlingsdata visade entydigt och med en hög signifikansnivå att fuktigheten, den så kallade vattenaktiviten (a_w), var begränsande för viabiliteten hos de förekommande bakterierna. Temperaturen hade däremot ingen signifikat effekt. Fuktighetsvärdet under vilket viabla bakterier inte kunde observeras låg på ungefär 15%. Beräkningar har visat att a_w för det buffertmaterial man använde i BCE är cirka 0.96 vid en fukthalt av cirka 15%. Denna a_w motsvarar ungefär fukthalten i vanligt bröd och utgör en ungefärlig gräns där många bakterier inte längre kan växa. I BCE kunde överhuvudtaget inga bakterier påvisas om fukthalten understeg 15 % i buffertmaterialet.

I förvarssammanhang har man riktat uppmärksamhet mot att mikrobiell korrosion av förvarsbehållarna är möjlig; framförallt oroas man av att SRB skall bilda sulfid vilket är korrosivt för kopparkapslar i syrefri miljö. Resultaten från BCE visade att överlevnaden av mikrober i det experimentet begränsades av vattenaktiviteten. Det svenska förvarskonceptet förutsätter 100 % bentonitlera med en vattenaktivitet på ca 0.96. En antal laboratorieexperiment som bekräftar BCE resultaten har nyligen genomförts och publicerats (SKB TR 95-27). Mycket höga halter av viabla SRB (100 miljoner SRB per cm^3 bentonit), var inte längre viabla efter ett dygn i bentonit vid en vattenaktivitet på 0.96. De svenska laboratorie-experimenten kommer inom kort att följas upp med fältexperiment vid Äspö-laboratoriet under realistiska förvarsförhållanden.

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1 INTRODUCTION

AECL has developed a concept for the permanent geological disposal of used nuclear fuel waste, in plutonic rock of the Canadian Shield (AECL 1994). The concept is based on a multi-barrier system and involves the disposal of nuclear fuel waste (from CANDU (CANada Deuterium Uranium) reactors) in an engineered excavation (vault), at a depth of 500 to 1000 m in granitic rock (Johnson et al. 1994). The disposal vault would consist of arrays of rooms, connected by access tunnels for transportation of the excavated rock, waste containers and backfill materials. Fuel wastes would be isolated in corrosion-resistant metal containers (ASTM Grade-2 titanium is the reference material; copper is another option in the concept). The reference scenario is emplacement in boreholes drilled in the floor of the disposal rooms. A minimum container-design lifetime of 500 years has been specified in the reference disposal system to ensure isolation of the fuel waste during the period of high fission-product activity. The containers would be surrounded by a compacted buffer material (50 wt.% sodium bentonite and 50 wt.% silica sand) that would swell on saturation with groundwater and ensure that transport of contaminants from the containers was controlled by diffusion. A 5-cm thick compacted pure sand layer would separate the containers from the compacted buffer material. After the waste emplacement, the rooms would be backfilled with a mixture of 75 wt.% crushed and graded host rock and 25 wt.% glacial lake clay. Figure 1 shows a schematic of such a waste vault. On completion of the vault operations (~ 70 a) the remaining volume, including all the shafts and exploratory boreholes, would be backfilled and sealed, using cementitious and clay-based sealants. Once the facility is sealed, no further actions would be required to ensure adequate isolation of the waste (Johnson et al. 1994).

Such a vault would not be a sterile environment and the presence and activity of a microbial population would be expected. Therefore, the potential effects of microbial action in a vault on the performance of the multi-barrier system have been assessed (Stroes-Gascoyne and West 1994). One of the issues identified in this assessment is that the environment immediately surrounding the containers with the used fuel waste would be very adverse to the survival of microbes because of the high radiation fields, temperature and desiccation expected here. This is further discussed by Stroes-Gascoyne and West (1995, 1996). The work described in this report has been performed to investigate how the effects of heat and desiccation may affect the microbial population naturally present in buffer materials in situ in a full scale simulation test. Radiation effects have been investigated separately in laboratory experiments (Stroes-Gascoyne et al. 1995). Results from this work will contribute to further defining the potential impact of microbial activity on the multibarrier system.

2 THE BUFFER/CONTAINER EXPERIMENT

The Buffer/Container Experiment (BCE) was carried out at AECL's Underground Research Laboratory (URL) for 2.5 years to examine the in situ performance of compacted buffer material in a single emplacement borehole under vault-relevant conditions. The test was run in a full-size borehole, located at the 240 m level in the URL. An electric heater, with the same dimensions of a waste container, was used in the test to provide the heating. A detailed description of the experiment and its emplacement has been given by Kjartanson et al. (1993, 1995), and a schematic representation is shown in Figure 2. The BCE was essentially an engineering test with a number of non-microbial general objectives to:

- Evaluate and document the full-scale, in situ performance of the reference buffer material within a realistic geologic setting, under the effects of heat and lower and upper bound moisture availability boundary conditions;

- Provide for full-scale experimental qualification and further development of numerical and conceptual models for the processes of heat and moisture transfer, development of swelling pressure and contact stresses and volume changes in the buffer. The interaction of the buffer with the heater, the backfill and the surrounding rock was also evaluated;

- Provide experience with experimental methods, geotechnical instrumentation, underground materials handling, in-situ buffer compaction and large diameter borehole drilling in granitic rock.

Specific non-microbial objectives were also formulated to:

- Assess the heat transfer properties of the buffer and rock under natural rock boundary conditions, with the potential for thermal drying of the buffer adjacent to the heater;

- Assess the degree of thermal drying and allow any associated drying shrinkage effects, particularly cracking, to be observed and mapped on completion and disassembly of the test; document the moisture distribution within the buffer for the boundary conditions prevailing;

- Assess the rate of water uptake of the buffer under the natural boundary conditions, through moisture balance calculations;

- Assess and document the mechanical interaction of the buffer with the heater, the emplacement borehole wall and the backfill and vertical restraint under the test conditions.

2.1 MICROBIAL OBJECTIVES OF THE BCE

Microbial analyses were not performed at the onset of the experiment. However, during the 2.5 years of test conditions it became apparent that the BCE could provide very important data on the survival of microorganisms naturally present in buffer materials under vault-realistic temperature and moisture content conditions. Therefore, microbial objectives for the BCE were formulated, to be realized during decommissioning of the experiment to:

- Assess the survival of the naturally present microbial population in buffer material that has been subjected to compaction upon emplacement and to the subsequent heat and drying cycle during the experimental phase (2.5 year) of the test;

- Determine, if a surviving microbial population was found, which group(s) of microorganisms dominate in this simulated vault environment.

3 MICROBIAL ANALYSIS OF ARCHIVED BUFFER MATERIAL USED IN THE BCE

3.1 BACKGROUND LEVELS

Microbial analysis of the buffer material used in the BCE was not carried out during the emplacement of the experiment. However, excess material prepared for the experiment was archived at the URL and this material was sampled for microbial analysis. The analysis was carried out at AECL's Whiteshell Laboratories (WL) and involved total microscopic counts, and viable counts on three growth media. Details of these analyses are described by Haveman et al. (1995a). The total microscopic counts gave results that seemed very high, likely because the stains used to colour the bacteria also stained the clay which made counting very difficult (Haveman et al. 1995a). Table 1 gives the results of the total aerobic heterotrophic viable bacteria (cultured at 30°C) in the various batches of buffer prepared for a number of experiments at the URL (Avonlea buffer mix (458 BM) was used in the BCE). This Table also gives the viable heterotrophic counts in the sand fractions, the bentonites, and in the water used to prepare the buffer material. The actual preparation and mixing methods of the buffer material are described by Dixon et al. (1992).

Anaerobic heterotrophic viable counts were only carried out for Avonlea buffer mix (458BM) on R2A medium at 30°C and were in the range of $5.2 \pm 2.1 \times 10^1$ CFU/g (Haveman, unpublished results 1995).

The results reported in Table 1 and in Haveman et al. (1995a) are the benchmark against which the results from the BCE can be compared.

4 INVOLVEMENT OF OTHER ORGANIZATIONS

The Buffer/Container Experiment (BCE) has created a unique opportunity to obtain microbial data from an in-situ environment that has relevance with respect to deep geological disposal of nuclear waste. Several other countries are considering clay-based buffer materials, or clay as a host medium, for a waste vault. The Swedish Nuclear Fuel and Waste Management Corporation (Svensk Kärnbränslehantering AB (SKB)) contracts microbial work related to nuclear waste disposal out to the University of Göteborg (UoG) (Sweden) and expertise and personnel from UoG was made available during the BCE decommissioning. Participants from UoG performed a number of microbial analyses at WL on the samples as they became available during decommissioning. In addition, a number of samples were shipped to Sweden for further specific analysis. The Agence National pour la Gestion des Déchets Radioactifs (ANDRA) (France) contracts microbial analysis of clay deposits out to Guigues Recherche Appliquée en Microbiologie (GRAM) in Aix-en-Provence (France) and offered this expertise in microbial analysis of clay for the BCE decommissioning. Samples were shipped to the GRAM laboratory in France for a number of independent analyses to be compared with the analyses carried out at WL during decommissioning.

5 MICROBIAL SAMPLING OF THE BCE MATERIAL AT DECOMMISSIONING

Figure 3 shows a more detailed schematic of the BCE and the various zones for engineering samples to be taken (i.e., for moisture content determination). The temperature of the heater was kept steady at 85°C for the duration of the experiment. Figure 4 shows the vertical temperature profile in the buffer through the centre of the emplacement borehole, obtained from thermocouples distributed throughout the buffer. This Figure shows that after 8 weeks the temperature profile was well established and did not change much thereafter (i.e., to 124 weeks, at which time the experiment was terminated). Figure 5 shows the temperature profile in the experiment as a function of the distance from the centre of the emplacement hole at heater mid-height. There is a considerable temperature drop over the 5-cm-thick sandlayer surrounding the heater (~ 20°C) and a further drop of another 20°C over the 25-cm-thick buffer layer.

Sampling this experiment upon decommissioning for microbial characterization was done to provide information on:

- The survival of microorganisms naturally present in the buffer materials under the high temperature and probably dry conditions close to the heater.
- The survival of microorganisms naturally present in the buffer materials under the lower temperature and probably wet conditions close to the granite-buffer interface.
- Biofilm formation at the granite-buffer interface.
- Biofilm formation at the buffer-heater interface.

The metabolic activity of surviving bacteria.

To obtain the maximum amount of microbial data from this experiment, samples were taken from eight different 'environments' in the experiment as indicated in Figure 6, resulting in eight different types of samples. These are:

- The backfill above the actual experiment,
- The backfill-buffer interface,
- The buffer in every layer (G through Q) (using metal tubes to obtain 15-cm long cores),
- The buffer across one entire layer (or several partial layers), using a hollow-stem auger to obtain a 40-cm long core),
- The sand around the heater in several places,
- The buffer-granite interface above, around and below the heater,
- The heater surface (i.e., the Teflon™ cloth in which the heater was wrapped before emplacement), and
- Pore water inflow from the rock, after completion of decommissioning.

The sterile sampling Procedures for the eight different types of samples described above are detailed in Appendix 1, which also contains a complete list of all samples taken per layer and if and where these samples were sent for analysis. There were a total of 88 metal tube samples taken, three backfill samples, two backfill/buffer interface samples, four sand samples, five buffer-granite interface samples, six hollow-stem auger samples and five heater samples (i.e., Teflon heater cloth and adhering material). Pore water inflow samples could not be taken because no water inflow into the borehole was observed for almost a year after removal of the experiment. Table 2 summarizes all types of samples taken and a short description is given here.

5.1 SAMPLE TYPE 1: BACKFILL

After the restraining columns (Figure 2) were removed at the start of the BCE decommissioning (May 2, 1994), the 1-m-thick backfill layer at the top of the experiment was excavated. The backfill sample for microbial analysis was taken as described in Appendix 1, Procedure 1 (Sample F-001-B). During the excavation of the backfill, several additional samples were taken. Some rust was detected on the surface of the backfill underneath the concrete constraint cap, after it was lifted. This was sampled with a sterile scalpel, and the scraped material was deposited in a sterile glass vial (sample F-003-B). Moisture drops were detected underneath an earth pressure cell at the backfill-buffer interface and this area was therefore also sampled by scraping with a sterile scalpel (sample F-007-B). Table 2 describes the locations (and some other details) of sample type 1.

5.2 SAMPLE TYPE 2: BACKFILL-BUFFER INTERFACE

The backfill-buffer interface was sampled in two places (samples F-002-IB and F-002-IB-F) and one of these samples (sample F-002-IB-F) was shipped to the GRAM laboratory for analysis. The sampling procedure is described in Appendix 1, Procedure 2. Table 2 describes the locations (and some other details) of sample type 2.

5.3 SAMPLE TYPE 3: BUFFER IN EVERY LAYER (METAL-TUBE SAMPLES)

The BCE was divided into 11 layers (or lifts) during emplacement of the experiment. These layers were all targeted for extensive moisture content sampling during disassembly of the experiment. These moisture content samples (109 per layer) were taken with metal tubes (i.e., 24 cm long with a diameter of 2.5 cm). The sampling was carried out in a reproducible manner by using a template that fitted on top of the buffer (Figure 3). The template was placed in exactly the same location for each layer to be sampled by using a laser survey technique (Chandler et al. 1995, Roach et al. 1995). The template contained 109 numbered holes through which the metal-tube samples were taken, such that their

exact location could be determined for reconstruction of the three-dimensional moisture profile, developed during the 2.5 years for which the experiment continued. The metal tubes were pushed though the holes in the template into the buffer layer using a pneumatic hammer. The metal tubes were pulled out of the buffer by using an overhead crane (Chandler et al. 1995, Roach et al. 1995).

Eight microbial samples (~ 15 cm long) were taken in each of the 11 layers, in conjunction with the moisture content samples, but using 45-cm-long metal tubes. The locations of these samples are shown in Figures 7 (above and below heater) and 8 (around the annulus of heater). Immediately following the taking of a moisture content sample (with an alcohol-sterilized 24-cm metal tube) in a location chosen for microbiology, the microbiology sample was taken (from exactly the same location, but going deeper into the buffer) with an oven-sterilized (24 h at 550°C) 45-cm-long metal tube. As soon as each sample was retrieved from the borehole, it was capped with sterile caps at both ends and either transported immediately to an underground glovebox, at 20 m distance of the test hole, in the Temporary Microbial Laboratory (TML) for rapid analysis, or carefully sealed. The samples to be shipped or archived were sealed on both ends with hot sterile paraffin and a sterile cap, and wrapped in double plastic bags containing some iron filings, to ensure no air exposure. The samples were kept at 4°C until shipment to France or Sweden, or indefinitely in the case of archived samples. Details of the metal-tube sampling procedure can be found in Appendix 1, Procedure 3. Tables 2 and A-1-1 (Appendix 1) and Figures 7 and 8 give the identification numbers, location and other details of all the metal-tube samples taken.

5.4 SAMPLE TYPE 4: HOLLOW-STEM AUGER BUFFER SAMPLES

Six hollow-stem auger samples (H-001-AB, K-001-AB, L-001-AB, M-001-AB, N-001-AB-F and P-001-AB) were taken according to the procedure described in Appendix 1, Procedure 4. These samples were 40 to 45 cm long cores with a diameter of 5 cm. Table 2 gives the location and identification number of these samples. The hollow-stem auger works according to the principle of a corkscrew and the sample is pushed into a hollow plexiglass tube in the center of the 'screw'. The tube was sterilized with alcohol prior to sampling, because autoclaving would have melted the plexiglass. The hollow-stem auger samples were sealed with hot sterile paraffin to minimize air exposure and, wrapped in double plastic, frozen to -18°C (for subsequent Phospholipid Fatty Acid analysis) or stored at 4°C. The sampling procedure is described in Appendix 1, Procedure 4.

5.5 SAMPLE TYPE 5: SAND SAMPLES

The heater was surrounded by a 5-cm layer of sand. Upon disassembly of the experiment, the sand was removed from around the container with a large vaccuum cleaner. Four sand samples were taken (samples J-001-SB, L-001-SB, L-004-SB-F and N-001-SB) during several breaks in the removal of the sand. The depth of the sand samples was determined by measuring the distance from the top

of the borehole. The samples were scooped from near the heater surface using sterile glass vials tied to a long stake. The procedure is described in Appendix 1, Procedure 5. Tables 2 and A-1-1 give the sample location, identification number and other details of the sand samples taken.

5.6 SAMPLE TYPE 6: BUFFER-GRANITE INTERFACE

The buffer-granite interface was sampled at five locations above, around and below the heater (samples H-001-IB, L-001-IB, L-003-IB-F, N-001-IB and Q-001-IB). The buffer adjacent to the granite wall was removed as a wedge with sterile tools. Both the wall and the buffer surface that had touched the wall were scraped with a sterile spatula in an attempt to sample any biofilm that may have formed at this interface location. The scraped material was transferred into sterile glass vials for analysis. The procedure is described in Appendix 1, Procedure 6. Table 2 describes the sample locations (and some other details) of the buffer-granite interface samples taken.

5.7 SAMPLE TYPE 7: THE HEATER SURFACE

The aluminium heater was wrapped in a thick Teflon cloth before emplacement in the borehole, to protect it against corrosion-failure. During decommissioning of the experiment, the heater was lifted out of the hole after the buffer layers above the heater had been excavated and most of the sand around the heater had been removed by suction. The heater was lifted out by overhead crane and care was taken that nothing touched the surface of the heater before several samples were removed for microbial analysis. Pieces of Teflon cloth (with adhering sand) were cut out from several locations, using a sharp, sterile knife (HE-001-B and HE-003-B-SEM; the latter sample was submitted for analysis of biofilm using an Environmental Scanning Electron Microscope (ESEM)). The procedure is described in Appendix 1, Procedure 7. Sand adhering to the bottom of the heater was also sampled (H-004-B). An unplanned sample from the cloth was taken because it appeared that a small strip of black (electrical) tape remained attached to the cloth upon emplacement of the experiment and this piece of tape was also sampled (sample HE-005-B) and subsampled for ESEM examination (HE-005-B-SEM) because it constituted a different medium for biofilms to form on. Table 2 gives details of sample locations.

5.8 SAMPLE TYPE 8: PORE WATER INFLOW

Before the BCE was emplaced, hydrogeochemical characterization of the water flowing into the borehole from the rock wall was carried out, but no microbial analysis. The buffer was emplaced at 18 wt.% moisture content, whereas buffer saturation is around 23 wt.%. The pore water from the rock may therefore diffuse into the buffer adjacent to the rock and this may introduce additional or different bacteria if the pore water contains any. Therefore, it was planned to sample the rock pore water inflow after decommissioning. However, this has not been possible, because pore water did not reappear up to ten months after removal of the experiment.

5.9 ADDITIONAL SAMPLES

A small segment of a corroded thermocouple (SEM-001-N) was retrieved from near the buffer-granite interface in layer N and submitted for ESEM analysis for the presence of bacterial cells. The buffer in which the piece was embedded (SEM-002-N) was also investigated by ESEM.

5.10 SHIPPING OF THE SAMPLES

The samples destined for analysis at the GRAM laboratory (France) and at UoG (Sweden) were shipped in insulated wooden boxes on ice within 3 to 5 days of sample retrieval. During the lag time between sampling and shipping, the samples were stored airtight at 4°C as described in Section 5.3. The time between leaving the WL laboratory at AECL and arriving in Sweden (UoG) and France (GRAM) varied from 3 to 7 days, depending on the shipping company. The first batch of samples took 7 days to arrive and as a result a different shipping company was chosen for the second batch of shipping, reducing the arrival time to 3 days.

6 MICROBIAL ANALYSES

Microbial analyses of the BCE samples were carried out at AECL's Whiteshell Laboratory (WL), by the French company Guiges Recherche Appliquée en Microbiologie (GRAM) and at the department of Marine and General Microbiology of the University of Göteborg (UoG). Appendix 1 (Table A-1-1) contains a complete list of all samples taken and where they were analyzed or archived.

6.1 CHOICE OF METHODS

Prior to the decommissioning of the BCE extensive consultation took place between the three laboratories on how analysis and culturing should proceed, taking into account the conditions in the BCE. Culture protocols, temperatures and media for microbial analysis had to be chosen such as to resemble the actual conditions in the BCE closely while keeping the amount of cultures down to a manageable number.

6.1.1 Culture Temperatures

Figures 4 and 5 show that the in situ temperatures of the buffer- and other samples varied anywhere from 65 to 45°C (the heater cloth samples were an exception at 85°C). The buffer material was mixed, stored and emplaced at ambient URL temperatures at the beginning of the BCE. Any bacteria surviving after the 2.5 year experiment may still favour this temperature. Ambient URL TML temperature (i.e., 17 to 25°C) was therefore chosen as a suitable culturing temperature for mesophiles. However, some adaptation to higher temperatures may also have occurred and, therefore, a second culture temperature of 50°C was chosen for thermophiles.

6.1.2 Growth Media

Microbial analysis of the buffer material used in the BCE was not carried out during the emplacement of the experiment. However, excess material prepared for the test was archived at the URL and this material was sampled for microbial analysis. The analysis involved viable heterotrophic counts on three growth media, PTYG (Balkwill and Ghiorse 1985), PTYG (1:100 dilution) and R2A (Reasoner and Geldreich 1985). Highest viable counts (Table 1) were obtained on R2A, a low-nutrient medium, originally formulated for the enumeration and isolation of bacteria from water and wastewater which has also proven very effective for groundwater samples (Haveman et al. 1995b). This medium was, therefore, chosen as a culture medium for heterotrophs at WL. Its composition is given in Appendix 2. The medium used for the same purpose at the GRAM laboratory was somewhat different and is given in Appendix 4. Analyses for specific organisms (see below) were carried out on media suitable for these organisms and the composition and preparation of these media are described in Appendices 2 and 4.

6.1.3 Redox Conditions

The buffer was mixed and emplaced in the BCE under aerobic conditions which could have prevailed throughout the experiment. However, anaerobic conditions could possibly have developed during the 2.5 year experiment as a result of chemical reactions or microbial activity. It was therefore decided to carry out the incubations for total heterotrophs both under aerobic and anaerobic conditions. Anaerobic conditions were either obtained by using a glovebox with N_2 atmosphere or anaerobic bags (GasPac^{TM}).

6.1.4 Specific Organisms

Stroes-Gascoyne and West (1994) have argued that sulphate-reducing bacteria (SRB) and methanogens may be of particular importance with regard to container corrosion, cement degradation and gas production in backfill materials, in a nuclear fuel waste vault. It was, therefore, decided that specific methods should be used to analyze the samples for the occurrence of SRB and methanogens. Analyses for the occurrence of fermenting organisms, fungi and sulphur-oxidizers were also carried out. In addition, a rapid field method, the Biological Activity and Reaction Test (or BART), was employed to investigate the samples semi-quantitatively for the presence of iron-related bacteria (IRB), sulphate-reducing bacteria (SRB) and slime-forming bacteria (SLYM) (Appendix 2). This method has been described by Cullimore (1993) and more detail is given in Appendix 2. BARTs are not designed to give quantitative results, but rather an indication of ball park numbers of bacteria that could be present in a sample. The BART test for IRB was tested in the UofG laboratory with strains of *Shewanella putrefaciens* (iron-reducer) and *Gallionella ferruginea* (iron-oxidizer) in concentrations of 10^7 bacteria/mL. Results (Pedersen, unpublished results 1994) indicated an IRB population of <10 bacteria/mL for each of the strains. Used in combination, for a total concentration of 10^{-7} bacteria/mL, the test indicated a population of 2.8×10^3 bacteria/mL. The IRB BART medium contains ferric-citrate, and some of the reactions observed when using this test may be due to bacterial utilization of citrate followed by chemical precipitation of Fe^{3+}. However, when using this test for the analysis of URL groundwaters, a large IRB population with mostly anaerobic activity was indicated. The results obtained at the U of G Laboratory suggest that the interpretation of the IRB-BARTs results could be in error and that IRB-BART results should be used with caution.

6.1.5 Metabolic Activity

It is now well-known that many bacteria in stressful environments (in this case highly compacted buffer material with low water content, high temperatures and probably very limited nutrient availability) may not be very active metabolically. Therefore, metabolic activity measurements, using several different substrates, were chosen as an essential part of the microbiological characterization of the BCE samples. Phospholipid fatty acid (PLFA) analysis was chosen to determine potential viability, nutritional status and community structure without the need for culturing.

6.1.6 Identification of Organisms

Identification of culturable organisms was undertaken, using standard identification techniques (i.e., the Analytical Profile Index (API) method, Appendix 4) (Analytab Products, 1987) to determine the kinds of organisms that can survive in this very specific environment. However, it is well-known that culturing may actually only detect between 1 and 10% of the viable organisms actually present. It was therefore decided that a limited number of samples (limited, because of the extremely labour-intensive and costly method) should be identified by sequencing amplified DNA using the 16S rRNA method (Appendix 3) which does not require culturing. PLFA analysis will also give information about the community structure without the need for culturing.

6.1.7 Total Microscopic Counts

Total microscopic counts are useful to get an indication of the total population of bacterial cells (both live and dead) present in a sample. Total microscopic counts using either AO or DAPI stains (Haveman et al. 1995a) were planned for all BCE samples. However, counting proved very difficult for these samples because of interference from the clay particles present. Counts obtained on the archived pre-BCE buffer material yielded numbers that may be too high (Haveman et al. 1995a). Subsamples for microscopic counting were prepared from all samples taken during BCE decommissioning, but counting was not completed for most of these samples due to difficulty in interpreting stained cells.

6.2 ANALYSES AT WL (IN COOPERATION WITH UNIVERSITY OF GÖTEBORG STAFF)

A number of recent publications stress the importance of rapid, (i.e., within 24 hrs) sample analysis initiation upon sampling of both groundwater and core samples (Frederickson et al. 1995; Brockman et al. 1992; Hirsch and Rades-Rohkohl 1988; West et al. 1986a). Therefore, all analyses of the BCE samples at WL were carried out within 2 to 24 h of sample taking. All sample preparations were carried out in an anaerobic glovebox with N_2 atmosphere. All analyses carried out for each sample type are listed below, whereas detailed protocols and recipes for growth media used are given in Appendix 2. These protocols were designed before decommissioning and were adhered to strictly during sample analysis.

6.2.1 Backfill Samples

Three backfill samples were analyzed at WL (Table 2) for:

- Aerobe heterotrophs at laboratory temperature (17°C) and 50°C on R2A medium (Appendix 2, Procedure 1).
- Anaerobic heterotrophs at laboratory temperature (17°C) and 50°C on R2A medium (Appendix 2, Procedure 1).
- BART tests for sulphate-reducing bacteria (SRB), iron-related bacteria (IRB) and slime- forming bacteria (SLYM) (Appendix 2).

6.2.2 Backfill-Buffer Interface Samples

One sample was analyzed at WL (Table 2) for:

- Aerobe heterotrophs at laboratory temperature (17°C) and 50°C on R2A medium (Appendix 2, Procedure 2).
- Anaerobic heterotrophs at laboratory temperature (17°C) and 50°C on R2A medium (Appendix 2, Procedure 2).
- BART tests for sulphate-reducing bacteria (SRB), iron-related bacteria (IRB) and slime-forming bacteria (SLYM) (Appendix 2).

6.2.3 Metal Tube Samples

Two samples per layer were analyzed at WL (Table 2 and Figures 7 and 8) for:

Aerobe heterotrophs at laboratory temperature (17oC) and 50oC on R2A medium (Appendix 2, Procedure 3).

- Anaerobic heterotrophs at laboratory temperature (17°C) and 50°C on R2A medium (Appendix 2, Procedure 3).
- BART tests for sulphate-reducing bacteria (SRB), iron-related bacteria (IRB) and slime-forming bacteria (SLYM) (Appendix 2).
- Bacterial activity tests using ^{3}H Leucine (Pedersen and Ekendahl 1992a,b) (Appendix 2).
- Analysis for fermenters and for SRB and methanogens using the Hungate method (Appendix 2).

6.2.4 Hollow-Stem Auger Samples

Three samples (H-001-AB, M-001-AB and P-001-AB) were analyzed for:

- Phospholipid fatty acid analysis, carried out by Microbial Insights, Knoxville, Tennessee (Appendix 6) (White et al. 1983).

These samples were frozen to -20°C within a few hours after sample retrieval and were shipped overnight on ice in frozen state to Microbial Insights.

6.2.5 Sand Samples

Three samples were analyzed (Table 2) at WL for:

- Aerobe heterotrophs at laboratory temperature (17°C) and 50°C on R2A medium (Appendix 2, Procedure 4).
- Anaerobic heterotrophs at laboratory temperature (17°C) and 50°C on R2A medium (Appendix 2, Procedure 4).
- BART tests for sulphate-reducing bacteria (SRB), iron-related bacteria (IRB) and slime-forming bacteria (SLYM) (Appendix 2).

6.2.6 Buffer-Granite Interface Samples

Four samples were analyzed (Table 2) at WL for:

- Aerobe heterotrophs at laboratory temperature (17°C) and 50°C on R2A medium (Appendix 2, Procedure 2).
- Anaerobic heterotrophs at laboratory temperature (17°C) and 50°C on R2A medium (Appendix 2, Procedure 2).
- BART tests for sulphate-reducing bacteria (SRB), iron-related bacteria (IRB) and slime-forming bacteria (SLYM) (Appendix 2).

6.2.7 Teflon Cloth- and Related Samples

Three samples were analyzed (Table 2) at WL for:

Aerobe heterotrophs at laboratory temperature (17oC) and 50oC on R2A medium (Appendix 2, Procedure 5).

- Anaerobic heterotrophs at laboratory temperature (17°C) and 50°C on R2A medium (Appendix 2, Procedure 5).
- BART tests for sulphate-reducing bacteria (SRB), iron-related bacteria (IRB) and slime-forming bacteria (SLYM) (Appendix 2).

Two samples, one a piece of Teflon cloth with adhering sand (HE-003-B-SEM), the other a small piece of black tape that was found on the Teflon covering the heater (HE-005-B-SEM) (Section 5.7) were analyzed for:

- Evidence of microbial cells by ESEM (Appendix 6).

These samples were preserved in cacodylic buffer solution 0.1 M $(CH_3)_2As(O)ONa{\cdot}XH_2O$ and shipped overnight on ice to the USA.

6.2.8 Pore Water Sample

No samples were obtained because the rock remained dry for 10 months after completion of the decommissioning of the BCE.

6.2.9 Additional Analyses

A piece of corroded thermocouple (SEM-001-N) retrieved from near the buffer/granite interface in layer N was sent for ESEM analysis for the presence of bacterial cells. This sample was preserved in cacodylic buffer and shipped overnight on ice. The buffer material (SEM-002-N) in which the piece of thermocouple was embedded was also investigated by ESEM. This sample was not preserved but shipped on ice overnight.
Four samples were submitted to the Department of Microbiology, University of Guelph, for transmission electron microscopy examination (TEM). These samples were subsamples of the metal tube sample K-072-B, L-072-B, M-072-B and N-024-B that were analyzed at WL. The subsamples were stored and shipped at 4oC. TEM analysis took place several months after sample retrieval in this case.

6.3 ANALYSES AT THE GRAM LABORATORY

A total of 20 samples were sent to the GRAM laboratory for analysis. Because of the geographical constraints and shipping problems these samples were not analyzed until approximately 10 to 14 days after sample retrieval. However, the samples were stored at 4°C and shipped on ice. All protocols used at GRAM are described in Appendix 4.

6.3.1 Backfill Sample

No backfill sample was submitted for analysis.

6.3.2 Backfill/Buffer Interface Sample

One sample (F-002-IB-F) was submitted and was cultured at 25°C (Table 2) for:

- Heterotrophic strict aerobic and facultative anaerobic bacteria.
- Heterotrophic strict and facultative anaerobic bacteria.
- Fungi (molds).
- Sulphate-reducing bacteria.
- Methanogenic bacteria.
- Aerobic sulphur-oxidizing bacteria.
- Anaerobic sulphur-oxidizing bacteria.
- Identification with conventional tests and the API method.

6.3.3 Metal Tube Samples

Sixteen samples (Figures 7 and 8 and Tables 2 and A-1-1) were analyzed at 25°C (all samples) and 50°C (samples from around the heater annulus only) for:

- Heterotrophic strict aerobic and facultative anaerobic bacteria.
- Heterotrophic strict and facultative anaerobic bacteria.
- Fungi (molds).
- Sulphate-reducing bacteria.
- Methanogenic bacteria.
- Aerobic sulphur-oxidizing bacteria.
- Anaerobic sulphur-oxidizing bacteria.
- Microbial activity as measured by ^{14}C-glucose and ^{14}C-amino acid mixture mineralization on 5 metal tube samples (samples G-008-B, Q-092-B, K-054-B, K-006-B and N-006-B, (Figures 7 and 8 and Table A-1-1).
- Identification with conventional tests and the API method.

6.3.4 Hollow-Stem Auger Sample

One sample (N-001-AB-F) (Table 2) was submitted for analysis by GRAM for:

- Heterotrophic strict aerobic and facultative anaerobic bacteria.
- Heterotrophic strict and facultative anaerobic bacteria.
- Fungi (molds).
- Sulphate-reducing bacteria.
- Methanogenic bacteria.
- Aerobic sulphur-oxidizing bacteria.
- Anaerobic sulphur-oxidizing bacteria.
- Identification with conventional tests and the API method.

6.3.5 Sand Sample

One sand sample (L-004-IB-F) (Table 2) was submitted for analysis for:

- Heterotrophic strict aerobic and facultative anaerobic bacteria.
- Heterotrophic strict and facultative anaerobic bacteria.
- Fungi (molds).
- Sulphate-reducing bacteria.
- Methanogenic bacteria.
- Aerobic sulphur-oxidizing bacteria.
- Anaerobic sulphur-oxidizing bacteria.
- Identification with conventional tests and the API method.

6.3.6 Buffer-Granite Interface Sample

One buffer-granite interface sample was sent to GRAM and analyzed (Table 2) for:

Heterotrophic strict aerobic and facultative anaerobic bacteria.

- Heterotrophic strict and facultative anaerobic bacteria.
- Fungi (molds).
- Sulphate-reducing bacteria.
- Methanogenic bacteria.
- Aerobic sulphur-oxidizing bacteria.
- Anaerobic sulphur-oxidizing bacteria.
- Identification with conventional tests and the API method.

6.3.7 Teflon Cloth Sample

No Teflon cloth or related samples were submitted to GRAM for analysis.

6.3.8 Pore Water Sample

Pore water samples were not obtained.

6.4 ANALYSES AT THE UNIVERSITY OF GÖTEBORG LABORATORY

Most of the analyses performed by UoG staff (i.e., SRB, methanogens, fermenters and microbial activity measured by ^{3}H- Leucine uptake) were done at WL during the decommissioning of the BCE. Additional metal tube samples (Figures 7 and 8) were sent to UoG for 16S rRNA analysis.

6.4.1 16S rRNA Analysis

Because of the large amount of work involved, only three metal tube samples were investigated with this method (samples H-097-B, M-018-B and P-066-B, Figures 7 and 8). The protocols used are described in detail in Appendix 3.

6.4.2 Statistical Evaluation of the CFU Counts (Canada, France and Sweden)

A large amount of data (close to 400) on viable counts (colony forming units or bacterial cells) was obtained from the buffer-container experiment at the different laboratories and it was decided to analyze these data with a statistical method (the General Linear Model, Appendix 5) to determine the variables that may have had an effect on the number of viable cells in each sample. Appendix 5 gives details of the assumptions in this model.

7 RESULTS

A large amount of data has resulted from the microbial sampling and analysis of the BCE. In order to facilitate the discussion of all these results, they have been grouped in a number of categories:

1. Temperature and moisture content data.
2. Total viable heterotrophs.
3. Specific organisms..
4. Activity measurements.
5. Bacterial identification.
6. Other analyses (PLFA, ESEM, TEM).
7. Statistical analysis of all culture data.

7.1 TEMPERATURE AND MOISTURE CONTENT DATA FOR THE METAL TUBE SAMPLES

Table 3 gives the temperatures at the approximate locations in the BCE where the metal tube samples for microbial analysis were taken. These temperatures were measured by thermocouples located throughout the experiment, and the data were recorded by a central computer system. Table 3 also gives the values for the moisture contents of the metal tube microbiology samples measured at WL or at the GRAM laboratory, as well as the moisture contents measured by URL personnel in the engineering samples taken at the locations directly above the microbiology samples (Column 'BCE results' in Table 3). The latter values are likely more correct, since these moisture content measurements were measured using the entire sample, whereas the moisture content measured in the microbial samples was done on a much smaller subsample. Also, the microbiology samples were handled in a glovebox and no special precautions were taken to avoid moisture loss or gain while subsampling for moisture content. The engineering moisture content samples were treated with the utmost care to avoid any loss or gain of moisture prior to moisture content measurements, since those measurements were one of the main objectives of the experiment. Figure 9 compares the moisture content data for the microbiology samples analysed at WL and the moisture contents measured in the equivalent engineering samples ('BCE results').

7.2 TOTAL VIABLE HETEROTROPHS

Total viable heterotrophs were measured in the metal tube samples at WL and at the GRAM laboratory, as well as in the backfill, the buffer-granite interface, the buffer-backfill interface, the sand and the heater cloth and related samples.

7.2.1 Total Viable Heterotrophs in Metal Tube Samples

Tables 4 and 5 give the results of total viable heterotrophs for the analyses carried out at WL and GRAM, under aerobic and anaerobic conditions at two temperatures on R2A medium (WL protocols, Appendix 2) and on Plate Count Agar (PCA) (GRAM protocols, Appendix 4). These results are also shown in Figures 10, 11, 12 and 13 (WL data) and Figures 14 and 15 (GRAM data). At the GRAM laboratory no growth was obtained for the 50°C incubations and therefore no figures were produced for these results.

7.2.2 Total Viable Heterotrophs in Other Samples

Tables 6, 7, 8 and 9 give the total viable heterotrophic results obtained at WL on R2A medium for the backfill samples, the buffer-granite interface samples, the sand samples and the heater cloth and associated samples, respectively. Figure 16 shows the buffer-granite interface samples results obtained at WL. Tables 10, 11, 12 and 13 give the viable heterotrophic results obtained at the GRAM laboratory on PCA medium for the backfill sample, the buffer-granite interface sample, the sand sample and the hollow-stem auger sample that were sent to France.

7.3 ANALYSES FOR SPECIFIC ORGANISMS

The metal tube samples and the other type of samples were analyzed for specific physiological groups: sulphate-reducing bacteria (SRB)(at both GRAM and WL laboratories), methanogens (GRAM and WL), fermenters (WL), aerobe and anaerobe sulphur oxidizers (GRAM), fungi (GRAM), iron-related bacteria (IRB) (WL) and slimeforming bacteria (SLYM) (WL).

7.3.1 Analysis for Specific Organisms in Metal Tube Samples

SRB

SRB analysis was done on the metal tube samples at WL at an incubation temperature of 50°C (procedure given in Appendix 2) and the results are given in Table 14 and shown in Figure 17. SRB analysis was also done using BART tests at 25°C (Appendix 2) and these results are given in Table 15 and Figure 18. SRB analysis was carried out at both 25°C (Table 16) and 50°C (Table 17) at the GRAM laboratory (Appendix 4), but no results were obtained at the higher temperature. Figure 19 shows the GRAM results for 25°C.

Methanogens

Analysis for methanogens was done at WL at an incubation temperature of 50°C (Appendix 2) and results are shown in Table 14 and Figure 17. Analysis for methanogens was also carried out at the GRAM laboratory (Appendix 4) on the samples sent to France, and results are shown in Table 16 (25°C) and 17 (50°C). No methanogens could be cultured at 50°C; results for 25°C are shown in Figure 19.

Fermenters

Table 14 and Figure 17 show the results of the analysis for fermenters in the metal tube samples, carried out at WL (Appendix 2) at an incubation temperature of 50°C.

Sulphur-oxidizing bacteria

The samples sent to the GRAM laboratory were analyzed for sulphur-oxidizing bacteria at 25°C and 50°C under aerobic and anaerobic conditions (procedure given in Appendix 4). Tables 16 (25°C) and 17 (50°C) show the results. The 25°C results are also shown in Figure 19. Again, nothing grew at an incubation temperature of 50oC.

Fungi

The samples sent to the GRAM laboratory were also analyzed for fungi (Appendix 4) and results are given in Tables 16 (25°C) and 17 (50°C). No fungi could be grown from these metal tube samples.

Iron-related bacteria

BART tests (Appendix 2) were used at room temperature at WL to examine the metal tube samples for the presence of iron-related bacteria (IRB) and results are given in Table 15 and Figure 18.

Slime-forming bacteria

BART tests (Appendix 2) were also used at WL to examine the metal tube samples for the presence of slime-forming bacteria and results are given in Table 15 and Figure 18.

7.3.2 Analysis for Specific Organisms in Other Samples

Tables 10, 11, 12 and 13 give the results obtained at the GRAM laboratory for specific organisms (SRB, methanogens, fungi, sulphur-oxidizers) in the backfill sample, the buffer-granite interface sample, the sand sample and the hollow-stem auger sample, respectively, that were sent to France.

Table 18 gives the BART results for iron-related bacteria, SRB and slimeformers in the buffer-granite interface samples, the sand samples, the backfill samples and the heater cloth and associated samples analyzed at WL.

7.4 ACTIVITY MEASUREMENTS

7.4.1 ^{3}H-Leucine

Activity measurements were carried out on the metal tube samples analyzed at WL, using ^{3}H-leucine uptake measurements. The procedure is described in Appendix 2. Table 19 and Figure 20 give the results.

7.4.2 D(-U-^{14}C)-Glucose and ^{14}C-Labelled Amino Acid Mixture

Mineralization of D(-U-^{14}C)-glucose and a ^{14}C-labelled amino acids mixture was determined under aerobic and anaerobic conditions at 20, 40 and 60°C on a number of the samples sent to France. These results are shown in Tables 20 (D(-U-^{14}C)-glucose and 21 (^{14}C amino acid mixture). Figures 21 (glucose) and 22 (amino acids) show the results graphically.

7.5 IDENTIFICATION OF ORGANISMS

7.5.1 Identification of Culturable Organisms

Organisms cultured from all the samples analyzed at the GRAM laboratory were identified using conventional tests and the API method (Appendix 4). Table 22 gives the results from these identifications

7.5.2 Identification Using the 16S rRNA Method

This identification method was applied to 3 metal tube samples sent to the UoG laboratory in Sweden (samples H-097-B, M-018-B and P-016-B, Figures 7 and 8 for location). The method used is described in Appendix 3. The results are given in Table 23 and Figures 23 and 24.

7.6 OTHER ANALYSES

7.6.1 Phospholipid Fatty Acid (PLFA) Analysis

Three hollow-stem auger samples were sent to Microbial Insights Inc., Knoxville, Tennessee for PLFA analysis. The method is described in Appendix 6 . The complete results for the samples, including a blank, are given in Table A-6-1 (Appendix 6). A summary of the PLFA data is given in Table 24. Figures 25, 26 and 27 show the results for PLFA-biomass, PLFA-community structure and PLFA growth indicators, respectively.

7.6.2 Environmental Scanning Electron Microscope Examination of the Heater Cloth and Associated Samples

Several samples were analyzed by Environmental SEM for the presence of biofilms. EDS profiles were also obtained for these samples. The method is described in Appendix 6.

ESEM of corroded thermocouple

A piece of corroded thermocouple (sample SEM-001-N) taken from near the buffer-granite interface in layer N was examined for the presence of microbes. Figures A-6-1, A-6-2 and A-6-3 (Appendix 6) give the EDS profiles for the uncorroded, the corroded and the pitted areas on the thermocouple segment, respectively. Figure 28 (main text) shows a pit in the thermocouple. Figure A-6-4 (Appendix 6) gives the EDS spectrum for the fragment of buffer (SEM-002-N) in which the thermocouple segment had been embedded. No bacteria were observed on the buffer fragment by ESEM and therefore no photo is shown.

ESEM of Teflon cloth and black tape segment

Two samples, a piece of Teflon cloth with adhering sand (HE-003-B-SEM), and a small piece of black electrical tape adhering to the Teflon cloth (HE-005-B-SEM) were analyzed by ESEM for evidence of microbial cells and biofilms. No bacteria were found on the Teflon cloth and, therefore, no photo's are shown. Figure 29 (a and b) shows the black tape segment and a 2000x micrograph indicating strings of bacteria.

7.6.3 Transmission Electron Microscopy (TEM) Analysis of Metal Tube Samples

Several samples of buffer material from the metal tube samples analyzed at WL were sent for Transmission Electron Microscopy (TEM) analysis. The method is described in Appendix 6. Results of the successively more intrusive treatments of the samples are given in Figures 30 (sample N-024-B, washed four times with 0.05M HEPES buffer), 31 (sample K-072-B, washed eight times with 0.15M HEPES buffer), 32 (sample K-072-B, washed eight times with 0.15M HEPES buffer, embedded with Nanoplast resin and stained with Uranyl) and 33 (sample K-072-B, grown in M-9 salts broth and NB medium).

7.7 STATISTICAL ANALYSIS OF ALL CULTURE DATA

All input data for the statistical analysis of all the culture results are given in Table A-5-1 (Appendix 5). The results of the statistical analysis are given in Tables 25 to 29 and Figures 34 (temperature) and 35 (moisture content).

8 DISCUSSION OF THE RESULTS

8.1 TEMPERATURE AND MOISTURE CONTENT DATA

The temperature data in Table 3 were obtained from thermocouples located throughout the experiment, by extrapolating the temperature of the thermocouple nearest to the sample location. No in situ temperature readings were taken during decommissioning and sampling.

The moisture content data in Table 3 were measured on the actual microbiology samples both at WL and at the GRAM laboratory. However, these moisture content measurements were obtained from small subsamples (5 to 10 g) taken from the large metal tube samples when they were extruded from the tubes in a glovebox environment. No special precautions were taken during subsampling to avoid moisture loss (wet samples) or gain (dry samples).

Therefore, Table 3 also contains the moisture content data obtained by the engineering staff during the BCE commissioning from the samples taken specifically for moisture content measurements. These samples were located directly above the microbiology samples. Since determining the moisture distribution in the buffer material was one of the main engineering objectives of the experiment, great care was taken to avoid moisture loss or gain in these samples: Upon retrieval of each sample (109 per layer), it was immediately plugged, capped tightly, sealed with tape and transported to an on-site laboratory for immediate analysis. The analysis was done on the whole sample under strict precautions (Chandler et al. 1995). It is therefore likely that these moisture content data (BCE moisture data in Table 3) are more accurate than those measured in the microbiology samples.

Figure 9 compares the moisture content data measured in the metal tube microbiology samples at WL and the BCE moisture data measured on the corresponding moisture content samples. Although there is some difference in the data, the trends (peaks) are very similar for both sets of data. Therefore, since the BCE moisture values are likely more correct, these values are used in all other figures in this report that show moisture content data.

Table 3 shows quite a discrepancy between the moisture content data measured in the metal tube samples analyzed at WL, and the equivalent samples analyzed at the GRAM laboratory. This is due to the fact that moisture contents at the GRAM laboratory were measured by drying the sample to constant weight at 55°C (Appendix 4) rather than drying to constant weight at 110°C as was done at WL and for the BCE moisture samples (Appendix 2). Therefore, all results obtained at the GRAM laboratory were plotted against the BCE moisture data, since they are believed to be more correct.

A discussion on how moisture content (in wt.%) can be converted to water potential and water activity for this material is given in Section 8.8.

8.2 TOTAL VIABLE HETEROTROPHS

Total viable heterotrophs were measured both at the WL and GRAM laboratories in the metal tube samples, the backfill and backfill-buffer interface samples, the buffer-granite interface samples, the sand samples and the heater cloth and associated samples.

8.2.1 Total Viable Heterotrophs in Metal Tube Samples

Results are given in Tables 4 (WL) and 5 (GRAM) and in Figures 10 through 15.

Aerobes at 17 to 25°C

Figures 10 (WL data) and 14 (GRAM laboratory results) can be compared directly because they both represent aerobic heterotrophs cultured at about room temperature (17 to 25°C) from equivalent metal tube samples (Figures 7 and 8). The comparison shows a very similar pattern in culturable organisms, with the overall numbers up to about one order of magnitude higher in the samples analyzed at the GRAM laboratory. It appears that these culture results are reproducible for equivalent samples, despite the fact that culture media and temperatures were slightly different (R2A versus PCA medium and 17°C versus 25°C). Also, the samples at WL were worked up within 24 hours of sampling whereas it took from about 10 to 14 days before the samples reached the GRAM laboratory.

It has been shown recently (Frederickson et al. 1995) that storage of solid subsurface samples appears to increase the number of culturable aerobic heterotrophic bacteria, possibly by a thus far poorly understood process of 'reactivation'. Frederickson et al. (1995) subjected subsurface samples to various storage times and found that, generally, the number of culturable aerobic heterotrophic organisms increased (by up to 6 orders of magnitude in some samples) with sample storage time (up to 77 d). They posed the question whether this was due to the growth of a few starved or dormant cells, or to a more general resuscitation of many inactive cells. Evidence in their work pointed towards a complex interplay between different lag times for resuscitation, growth and succession of various bacteria and even the heterogeneous distribution of microorganisms in subsurface samples. They also found that storage effects were more pronounced in samples that had been homogenized prior to storage than samples left intact. The samples shipped to the GRAM laboratory in France were left completely intact, as they were retrieved and the distribution of microbes in these samples was probably fairly homogeneous, because the buffer underwent a thorough mixing before emplacement in the BCE. It is therefore uncertain whether the differences in culture techniques or the longer sample storage time caused the generally slightly higher number of culturable aerobic heterotrophs in the samples analyzed at the GRAM laboratory.

Table 3 shows that the moisture content in the samples from near the granite-buffer interface (the 'outside' samples) varied from about 19.5 to 23% throughout all layers. The temperature in these samples varied from 20 to 45°C (Table 3) with the higher temperatures (i.e., 40 to 45°C) occurring in the samples taken from the

layers around the heater (i.e., layers K, L, M and N). The number of culturable aerobic heterotrophs (17 to 25°C) in these samples (Figures 10 and 14) is considerably lower than the number of culturables in the 'outside' samples from the layers above and below the heater. This is most likely due to the prolonged exposure to 40 to 45°C of the microbes in these samples, such that the microbes present are no longer growing optimally at 17 to 25°C. This is confirmed by the results in Figure 11, showing a considerable increase in culturable heterotrophs at 50°C, compared to 17 to 25°C (Figure 10).

For the samples taken near the centre of the borehole (and therefore near the heater in those layers surrounding the heater) the moisture content drops from about 19% above the heater to about 13% in the layers around the heater and then increases again to about 20% in the layers below the heater. The temperature, responsible for this redistribution of moisture, varied from about 20°C in the top layers to up to 60°C in the layers around the heater (Table 3). This combination of environmental factors has had a pronounced effect on the number of culturable aerobic heterotrophs at 17 to 25°C. Both Figures 10 and 14 show that bacteria could no longer be cultured in the layers around the heater. However, at the higher moisture levels in the samples from above and below the heater, aerobic heterotrophs could be cultured.

The sample taken in layer J close to the top of the heater (J-072-B, analyzed at WL, Table 4) had a moisture content of about 20%, a temperature of 55°C and a culturable aerobic heterotrophic population of 3 x 10^3 CFU/g dry weight. The sample taken just below, in layer K (K-072-B) had about the same temperature, but a moisture content of only 13%. No culturable organisms could be found in this sample and in the equivalent sample analyzed at the GRAM laboratory (K-054-B). This suggests that a low moisture content and not a temperature as high as 55°C may be the cause of the 'sterilization' of the buffer layers around the heater. This is further explored with statistical techniques in Section 8.7.

Aerobes at 50°C

Figure 11 represents total viable aerobic heterotrophs cultured at 50°C on R2A medium at WL. No viable bacteria could be cultured at the GRAM laboratory at 50°C (Table 5) but culturing was only carried out on a limited number of samples that had been exposed to higher temperatures during the BCE (i.e., samples from layers K, L, M, N and O). At WL, all samples were cultured aerobically at 50oC. Figure 11 shows that in the samples taken close to the granite-buffer interface ('outside' samples) in the layers with increased temperature and a sufficiently high moisture content (21 to 23%) (layers J though N), more viable heterotrophs could be cultured at 50oC than in the layers with lower temperatures above and below the heater. This could suggest a selection towards thermophilic bacteria. It is not clear why such results were not found at the GRAM laboratory. The fairly prolonged storage time (10 to 14 d) on ice of the GRAM samples may possibly have reduced the viability of any thermophilic bacteria present. Figure 11 shows that no aerobic heterotrophic organisms could be cultured at 50°C from the samples taken close to the heater, similar to what was observed for the culturable organisms at 25°C. This is presumably due to the low moisture content in these samples, as discussed in Section 8.7.

Figure 11 shows that in the sample from layer J (55°C, 20% moisture) a peak amount of viable heterotrophs could be cultured at WL. No J samples were sent to the GRAM laboratory because it was assumed at the beginning of the decommissioning that all material from layer J was needed for engineering analysis. Fortunately, J samples for microbiology were possible, but none were shipped out for analysis. The peak in viable organisms in layer J in Figure 11 is also visible in Figure 10. By tracing back the history of the BCE it appeared that layer J was exposed to the atmosphere for several months during a break in the construction of the experiment. To protect this layer, wood boards were at one point placed on this layer, but many people may also have walked on this layer. It is therefore possible that layer J was more contaminated than any other layer in the BCE and this could possibly explain the higher viable cell numbers in this layer. The moisture content in layer J is also higher than expected considering the temperature and this may be due to the very complex redistribution of moisture content that took place in the BCE.

Anaerobes at 17 to 25°C

Figures 12 (WL) and 15 (GRAM) can be compared for the results of anaerobic heterotrophs cultured at 17 to 25°C. The patterns in these two graphs are again fairly similar, and also similar to the results for the viable aerobes cultured at 25°C. The GRAM results appear up to two orders of magnitude higher than the WL results, and this could possibly be caused by either small differences in culture techniques or storage times, as discussed above.Anaerobes at 50°C

Figure 13 shows results for viable heterotrophic anaerobes cultured at 50°C at WL. This Figure shows that in neither the 'outside' samples (close to the buffer-granite interface) nor the 'inside' samples (close to the heater) any anaerobic heterotrophs could be cultured at 50°C in the layers around the heater. These results coincide with the results obtained on the equivalent samples analyzed at the GRAM laboratory (Table 5). This suggests that no selection for anaerobic thermophiles has occurred in the buffer during the 2.5 years of the BCE.

8.2.2 Total Viable Heterotrophs in Other Samples

Backfill samples

The results given in Table 6 (WL) and Table 10 (GRAM) show that a large number of viable heterotrophic aerobes and anaerobes could be cultured from the backfill-buffer interface samples (F-002-IB and F-002-IB-F). The aerobes results obtained at the GRAM laboratory are an order of magnitude lower than the results obtained at WL, whereas the anaerobes results are similar. The moisture content of the interface sample was 17.5%, compared with only 6.7% for the bulk of the backfill (sample F-001-B). This may account for the larger number of viable organisms found at the interface compared to the bulk sample. It should be noted that the backfill consisted of 75% crushed granite and only 25% of a glacial lake clay, compared to the buffer which contained 50% sodium bentonite. The availability of water to microbes in backfill material is, therefore, likely considerably larger at a lower moisture content, because the 'suction' in backfill would be much lower. This may explain why large numbers of aerobic bacteria could be cultured at a moisture content of only 6.7% in the backfill, whereas no culturable microbes were found at moisture contents of below 15% in the buffer (Section 8.8).

Aerobic incubation at 50°C was not carried out at the GRAM laboratory, but the results obtained at WL (Table 6) show that aerobe thermophiles could be cultured in all backfill samples analyzed at WL. Anaerobic culture results at 50°C are much lower. The rust sample (F-003-B) taken at the top of the backfill just underneath the concrete cap contained mostly molds. Molds were also found in the interface sample analyzed at the GRAM laboratory. This is further discussed in Section 8.5 (bacterial identification).

Buffer-granite interface samples

Tables 7 (WL) and 11 (GRAM) present the viable heterotrophs results for the buffer-granite interface samples. Figure 16 shows the results for the four samples analyzed at WL. The number of aerobic heterotrophs cultured at 25°C in the sample analyzed at the GRAM laboratory (L-001-IB-F) is two orders of magnitude higher than in the equivalent WL sample (L-001-IB) and possible reasons for this are again the differences in culture techniques or storage time. Counts of anaerobic heterotrophs cultured at 25°C in the GRAM sample were two orders of magnitude lower than the equivalent WL sample. The sample sent to the GRAM laboratory was sealed under N_2 atmosphere. But, since no overpressure of N_2 was applied, it is possible that air may have leaked into the sample during shipping, causing most anaerobic cells to die. However, only one sample was analyzed at GRAM and differences in culture techniques may also account for the difference.

Three of the four buffer-granite interface samples analyzed at WL (Table 7) contained small numbers of aerobic thermophiles. Only one sample contained thermophilic anaerobes but this number may be suspect; anaerobic thermophiles were cultured in GasPac anaerobic bags and these bags did not keep their seals very well during incubation at 50°C. Neither aerobic nor anaerobic thermophiles were found in the sample analyzed at the GRAM laboratory.

Sand samples

Three sand samples were analyzed at WL (Table 8) and one at the GRAM laboratory (Table 12). An accurate temperature of the sand samples was not known. Figure 5 shows how the temperature across the 5-cm wide sand layer dropped 20°C. Therefore, the sand samples taken from layers L and M may have experienced temperatures in the 85 to 65°C range, and a temperature of ~ 75°C was assumed in Tables 8 and 12. However, the samples were only cultured at 25 and 50°C. The sand sample from layer J was taken just above the heater and may have had a temperature of about 55°C. This sample contained a small number (18 ± 15) of mesophilic aerobes which may not be significantly different from zero. It contained a larger number of thermophilic aerobes ($(1.87 \pm 1.47) \times 10^3$). Because this sample contained no moisture it is likely that these thermophilic aerobes grew from surviving spores. As already discussed in the previous section, during emplacement of the experiment, layer J was open to the atmosphere for a considerable time, due to a break in experiment construction that could have caused enhanced contamination. It is therefore possible that this layer was contaminated by bacterial spores that subsequently germinated when cultured.

Samples from layer L were analyzed at both laboratories (L-001-SB at WL and L-004-SB at GRAM). No viable bacteria were detected in these samples at either 25 or 50°C, likely because of the extreme dehydration and high temperature of the sand close to the heater (0% moisture, ~ 75oC, Table 8). However, 1000 molds/g were found in the sample cultured at 25°C at the GRAM laboratory, but not at 50°C. These molds likely subsisted but were not active in situ since they were mesophilic species. Their origin is unknown but they probably survived burial and the subsequent high temperatures and desiccation in the spore state. The sand sample from layer M, analyzed at WL contained very small numbers of heterotroph aerobes. The small number of anaerobes found is not significantly different from zero (Table 8.).

Hollow-stem auger sample

Only one hollow-stem auger sample was analyzed for viable heterotrophs, at the GRAM laboratory. Results are shown in Table 13. This sample was taken in layer N near location 70 (Figure 8), close to the heater. The microorganisms in this sample would, therefore, have experienced a moisture content of ~ 13% and a temperature of ~ 58°C (compare metal tube sample N-072-B in Table 4 and N-054-B in Table 5). No aerobic organisms could be cultured at 25 or 50°C and only a very small amount of anaerobes were found at 25°C in PCA medium in this sample. No anaerobes were cultured at 50°C. These results compare reasonably well with the metal tube data for samples N-072-B (Table 4) and N-054-B (Table 5). Three other hollow-stem auger samples were analyzed for phospholipid fatty acids (PLFA) and the results of that analysis are discussed in Section 8.6.

Heater cloth and associated samples

Three heater cloth and associated samples were analyzed for viable heterotrophs at WL. Results are shown in Table 9. The actual Teflon cloth sample HE-001-B (plus some adhering sand), cut immediately and aseptically from the heater upon retrieval from the borehole, contained no culturable anaerobes, but ~ 25 (±2) cells/cm^2 of aerobic mesophiles and ~ 135 (±31) cells/cm^2 of aerobic thermophiles were found. This could indicate selection towards thermophiles although the numbers are small. It is known that Teflon is not prone to biofilm formation and ESEM examination of a Teflon cloth sample for the presence of bacteria did not detect any cells. This is further discussed in Section 8.6.

Sand scraped from the Teflon cloth underneath the heater (HE-004-B) contained very small numbers of culturable bacteria but these numbers are not significantly different from zero. The sand and black tape sample scraped from the side of the heater (HE-005-B) showed similar results as sample HE-001-B, no anaerobes but a detectable amount of thermophilic aerobes. The samples taken from the surface of the heater were at 85°C for 2.5 years. Great care was taken in sampling these, to prevent contamination. The fact that some thermophilic aerobes could be cultured may indicate some selection towards thermophiles. These organisms survived high temperatures and extreme desiccation and must have survived in an inactive state (possibly as spores).

8.3 SPECIALIZED ORGANISMS

The metal tube and other types of samples were analyzed for specific physiological groups and specialized organisms, such as sulphate-reducing bacteria (SRB), methanogens, fermenters, sulphur-oxidizing bacteria (SOB), fungi, iron-related bacteria (IRB) and slime-forming bacteria. The results are discussed below.

8.3.1 Sulphate-Reducing Bacteria (SRB)

Analysis for SRB was done by three independent methods, on the metal tube samples and the other types of samples analyzed at WL with BART tests (Appendix 2), on the metal tube samples analyzed at WL using a method developed by the UoG (Appendix 2) and on the metal tube and other samples sent to the GRAM laboratory with Labège test kits (manufactured by the GRAM laboratory, Appendix 4)

SRB in metal tube samples

The SRB results obtained for the metal tube samples analyzed at WL are shown in Table 14 and Figure 17 (50°C, UoG method, Appendix 2) and Table 15 and Figure 18 (room temperature, BART tests). Both sets of results indicate that the numbers of SRB that could be cultured are very small, especially in the samples from near the heater surface (<100 CFU/g). The results for the samples from near the buffer-granite interface are all <1000 CFU/g, with most <400 CFU/g. The results obtained at the GRAM laboratory are shown in Tables 16 (25°C) and 17(50°C). The 25°C results indicate the same trend of very low numbers for SRB. The 50°C results indicate that no thermophilic SRB could be grown with the GRAM method. The UoG method was able to detect some thermophilic SRB, but the numbers are very low (Table 14).

SRB in other samples

Table 18 shows the results of the BART test SRB analysis for the other types of samples. It is apparent that only the backfill contains a sizable number of SRB, with a population size of <4000 bacteria/g dry material. The buffer-granite interface samples indicated the presence of SRB but in very small quantities (20 bacteria/g dry material). In the sand and heater cloth and associated samples, no SRB could be indicated with the BART test. These results are in agreement with the results from the GRAM laboratory for the other types of samples, as shown in Tables 10, 11, 12 and 13. In the backfill sample (Table 10) analyzed at the GRAM laboratory, a very small presence of SRB was indicated (2.5 CFU/g dry material) at 25°C. The buffer-granite interface sample (Table 11) and the sand sample (Table 12) did not contain any SRB as detected by the Labège kits used at the GRAM laboratory at both 25 and 50°C. Table 13 contains the results for the hollow-stem auger sample (N-001-AB-F which had a temperature of about 58°C and a moisture content of about 13%). Only a very small presence of SRB (7 CFU/g dry material) was found when culturing at 25°C, but nothing at 50°C. This concurs with the results found for the metal tube samples.

In general, the culture results for SRB suggest that, although the presence of SRB could be indicated in some samples, the population size is very small. The SRB are not limited by sulphate; the bentonite used in the buffer mixture was quite rich in gypsum ($CaSO_4$) (Oscarson and Dixon 1989) and the groundwater used for mixing the buffer materials was also sulphate-rich (sulphate concentrations in Fracture Zone 2 water at the URL range typically from 30 to 300 mg/L (Gascoyne and Kamineni 1994). The occurrence of SRB is, therefore, dictated by other factors: The buffer material used in the BCE was mixed and emplaced aerobically. Since there is not much reducing capacity in the buffer *per se*, oxidizing or at least microaerophilic conditions may have prevailed during the 2.5 year experiment. SRB (and methanogens, Section 8.3.2) would not develop under such conditions. SRB require short-chain organic acids such as lactate and acetate, which are produced by other (hydrolyzing and fermenting) bacteria. Therefore, the occurrence of SRB also depends on the presence of other bacteria that produce the required nutrients for SRB from more complex organic material. The possible lack of a suitable source of organic material for fermenting and hydrolyzing bacteria will, therefore, also limit SRB growth.

It should be noted that upon opening the BCE at the beginning of the decommissioning procedure, no smell of H_2S could be detected to emanate from the emplacement borehole. During decommissioning, no black precipitates or smells were observed in any layer of the experiment. It should also be mentioned here that in laboratory experiments at WL with columns consisting of compacted buffer material, the presence of SRB was indicated, as evident by smell and BART test results, especially at the buffer-water interfaces in these experiments. However, to keep the buffer from extruding from the experimental cell, cellulose filters were used and it appeared that the presence of cellulose supported the growth of SRB. Although cellulose is not a suitable nutrient for SRB, it can indirectly enhance SRB growth because cellulose can be used by other bacteria to produce suitable short-chain organic acids for SRB, as mentioned above. Once the use of cellulose filters was terminated, the presence of large quantities of SRB was no longer evident in laboratory experiments (Dixon 1995). These observations suggest that the growth of SRB and other bacteria in a buffer environment may be limited by the absence of a suitable or sufficient source of organic material. The organic material naturally associated with the bentonite component of the buffer does not appear to be suitable to support substantial SRB growth. As already discussed in the previous paragraph, another factor could be that the buffer environment was not sufficiently anaerobic for SRB growth.

8.3.2 Methanogens

Analysis for methanogens was carried out on the metal tube samples analyzed at WL, using a method developed by UoG (Appendix 2), and on the metal tube and other types of samples sent to the GRAM Laboratory, using a modified Widdel and Pfennig medium (Appendix 4).

Methanogens in metal tube samples

Table 14 and Figure 17 give the results of the analysis for methanogenic bacteria in the metal tube samples analyzed at WL. The results show a small presence of methanogens in most of the samples analyzed but the population size was always <200 CFU/g dry material. Also, the small numbers of viable methanogens detected had large errors associated with them. Methanogens could not be found in the metal tubes analyzed at the GRAM laboratory either at a culture temperature of 25°C (Figure 19 and Table 16) or 50°C (Table 17) with the exception of one sample (G-092-B) in which a very small number of methanogens were detected (7 CFU/g dry material).

Methanogens in other samples

The presence of methanogens in other samples was only investigated at the GRAM laboratory, and no methanogens were detected in any of these samples with the modified Widdel and Pfennig medium (Tables 10 to 13).

In general it can be concluded from these results that methanogens are either not present in most samples or are present in very low numbers. This suggests that the buffer environment is not a very suitable environment for the growth and activity of methanogenic organisms, because the environment was likely oxidizing or microaerophilic. The buffer material was emplaced at 80% saturation, with about 20% of the porosity taken up by air. Aerobic metabolism and chemical reactions that use O_2 may not have proceeded enough during the 2.5 years of the test to create a suitable environment for methanogens to thrive. It is also possible that the buffer environment lacked suitable nutrients, electron donors and acceptors (CO_2, H_2,, one-carbon organic compounds and acetate) needed for methanogens. In this context it should be mentioned that experiments are currently being carried out at WL, in which backfill materials and groundwater are incubated in an $N_2/CO_2/H_2$ atmosphere (in the presence of powdered elemental iron to induce reducing conditions), to study the kinetics of methane production from the clay component of backfill materials. Initial results suggest that the presence of clay almost completely suppresses the production of methane in these experiments (Sheppard et al. 1996). This could indicate that the environment was not sufficiently anaerobic, despite the presence of elemental iron and the $N_2/CO_2/H_2$ atmosphere, which could imply that a buffer environment contains large amounts of residual oxygen that are reduced very slowly. The absence of methane production may also be due to the presence of sulphate and to the fact that sulphate reduction is thermodynamically more favourable than the reduction of CO_2 to CH_4. It is known from other studies that the formation of methane is insignificant in environments with abundant sulphate. This is further discussed in Section 8.5.1.

8.3.3 Fermenters

Analysis for thermophillic fermenting organisms was carried out on the metal tube samples analyzed at WL using a method developed by UoG (Appendix 2). Only selected samples were incubated anaerobically, at a culture temperature of 50°C. The results are given in Table 14 and Figure 17 and show that the number of fermenters found was <2000CFU/g dry material in all samples analyzed. No growth was observed in samples that had a combination of high temperature and low moisture content.

These fermenters can be considered a special group of anaerobe heterotrophs, and therefore the fermenter results in Table 14 can be compared with the results for anaerobe heterotrophs cultured at 50°C, in Tables 4 (WL results) and 5 (GRAM results). The WL results for anaerobe heterotrophs at 50°C are <1200 CFU/g, somewhat lower than the results obtained for fermenters in the same samples. This suggests that the plating method used to culture anaerobe heterotrophs was less suited for enumerating fermenters than the liquid medium method used specifically to culture fermenters. The analysis for anaerobic heterotrophs at 50°C at the GRAM laboratory did not give any results. However, the GRAM AnHB medium has higher nutrient concentrations than R2A or the fermenter medium (at least 10x) and this may have inhibited oligotrophs. In addition, the pH of the liquid growth medium in the UoG method was adjusted to 8.5 to 9.0, whereas the pH in the GRAM liquid medium was 7.2 to 7.4 and the pH of the semi-solid R2A medium was 7.2 $\pm$ 0.2. Previous studies carried out at WL, in which pore-solution compositions were measured for Avonlea bentonite at various solution/clay ratios, determined that the pore water pH would be around 7.6 (Oscarson and Dixon 1989). However, the enumeration results for fermenters are generally higher than the results for anaerobe heterotrophs at 50°C and this could indicate that the pH value of the culture medium was a critical parameter (with the higher value being more suitable).

8.3.4 Fungi

Analyses for fungi were carried out on the metal tube and other types of samples sent to the GRAM laboratory in France, using Sabouraud Chloramphenicol standard solid medium (Appendix 4). The results are given in Tables 16 (25°C) and 17(50°C) for the metal tube samples and in Tables 10 to 13 for the other types of samples.

No fungi were found in any of the buffer samples (i.e., the metal tube samples and the hollow-stem auger sample). Fungi were found in the backfill-buffer interface sample, as shown in Table 10. The fungi may have come from the backfill rather than from the buffer; Table 6 shows that in one of the backfill samples (F-003-B, rust from the top of the BCE) analyzed at WL, the presence of considerable quantities of molds were noted. Table A-2-1 (BART results) indicates that sample F-003-B (rust) showed the presence of molds in the IRB BART tube and sample F-007-B (from underneath an earth pressure cell, where drops of condensation were visible) also contained molds, as indicated by the SRB BART tube for this sample.

Molds could potentially be important in a buffer environment, because they are filamentous in nature and as such may be able to penetrate into very narrow pores. Similar to some bacteria, they also form spores that are very resistant to drying. The only other sample containing fungi (25°C) was the sand sample sent to the GRAM laboratory for analysis (Table 12). These molds likely subsisted at the high temperatures in the sand but were not active in situ because these were mesophilic species (i.e., could only be cultured at 25°C and not at 50°C). They probably were originally present in the sand and survived in their spore state.

8.3.5 Sulphur-Oxidizing Bacteria (SOB)

Enumeration of aerobic and anaerobic sulphur oxidizing bacteria (SOB) was carried out on the metal tube and other types of samples analyzed at the GRAM laboratory (Appendix 4).

Aerobic sulphur-oxidizing bacteria

Aerobic SOB were cultured on the Starkey medium (Appendix 4) and results are shown in Tables 16 (25°C) and 17 (50°C) for the metal tube samples and in Tables 10 to 13 for the other types of samples. Aerobic SOB are detected in only one sample, P-008-B, cultured at 25°C, in a very low amount (7.2 bacteria/g dry weight).The difference between this result and the "nil" results found in all the other samples is not significant since the detection level is 5/g. Therefore, aerobic SOB are basically not present in any of the samples analyzed at the GRAM laboratory.

Anaerobic sulphur-oxidizing bacteria

Anaerobic SOB were enumerated on the Taylor, Hoare and Hoare medium (Appendix 4). Results are shown in Table 16 and Figure 19 (25°C results) and in Table 17 (50°C results) for the metal tube samples and in Tables 10 to 13 for the other types of samples analyzed at the GRAM laboratory. In contrast to the aerobic SOB, anaerobic SOB are numerous in all samples analyzed, except in the sand sample, hollow-stem auger sample and samples K-054-B, M-054-B and N-054-B, the metal tube samples taken close to the heater (L-054-B was not analyzed for SOB). In these samples, anaerobic SOB likely did not survive because of the observed dehydration.

The difference in the enumeration results between aerobic and anaerobic SOB cannot be explained by a lack of oxygen, since numerous aerobic heterotrophic bacteria are found. In fact, the difference may be due to the initial presence of nitrates in the clay, that would have favoured the growth of anaerobic SOB, which are composed of the species *Thiobacillus denitrificans* (Section 8.5.1 and Table 22). This species is in fact capable of reducing nitrates into nitrogen anaerobically. It is capable of growing aerobically too, using oxygen instead of nitrates.

8.3.6 Iron-Related Bacteria (IRB)

BART tests (Appendix 2) were used to analyze the metal tube and other samples analyzed at WL for IRB. The results are shown in Table 15 and Figure 18 (metal tube samples) and in Table 18 (other types of samples). Detailed results from the BART tests are given in Table A-2-1 (Appendix 2). The results in Table 15 show considerable IRB activity in many of the samples analyzed. This activity, if present, is mostly very aggressive and dominated by anaerobic species (Table A-2-1). The BART software indicates genera potentially present on the basis of the reactions observed over time in the BART tubes. Possible genera responsible for the aggressive anaerobic activity observed in many of the samples include methanogenic bacteria, enteric bacteria and vibrioids. Often, the presence of pseudomonads is indicated. The enumeration numbers given in Tables 15, 18 (and A-2-1) are possible population sizes and should not be taken as absolute numbers. In Section 6.1.4, it was discussed that the reactions in the IRB BART test could be due to citrate metabolizing bacteria, rather than iron-related bacteria. The IRB results should therefore be regarded as dubious. However, generally the trends observed from the viable heterotrophs are confirmed by the BART IRB results, i.e., no activity in samples with a low moisture content and high temperature.

8.3.7 Slime-Forming Bacteria (SLYM)

BART tests were used to enumerate the metal tube and other types of samples analyzed at WL for slime-forming bacteria. The results are shown in Table 15 and Figure 18 (metal tube samples) and Table 18 (other types of samples). Detailed results for the BART tests are given in Table A-2-1 (Appendix 2). The results show considerable slime-producing activity in many of the samples analyzed. The BART software indicates that many species may potentially be present in these samples, most often *Pseudomonas*, *Micrococcus*, *Bacillus* and *Zoogloea*. The trends observed from the viable heterotrophs are generally confirmed by the BART SLYM results, i.e., no activity in samples with a low moisture content and a high temperature.

8.4 RESULTS FROM THE ACTIVITY MEASUREMENTS

Activity measurements were carried out on selected metal tube samples analyzed at WL using a method developed by UoG and on selected metal tube samples analyzed at the GRAM laboratory. The results of these measurements are discussed below.

8.4.1 Activity of Bacteria as Measured by the Assimilation of ^{3}H-Leucine

Activity measurements were carried out on the metal tube samples analyzed at WL, using ^{3}H-leucine uptake measurements. The procedure is described in Appendix 2; Table 19 and Figure 20 show the results. The measured assimilation of ^{3}H-leucine in selected metal tubes from the layers above and around the top of the heater was significant, indicating viable bacteria. This activity was greatest in

layers I and J, for both the 'outside' and 'inside' samples (Figure 20). Activity dropped off to almost nil in the samples taken from close to the heater except for in sample M-072-B (Table 19) where considerable activity was observed, indicating viable bacteria, although nothing could be cultured in this sample. Unfortunately, no samples deeper into the experiment were assessed for ^{3}H-Leucine uptake due to a lack of time and manpower (these analyses were done within 24 h of sample retrieval). It is possible to calculate the assimilation per individual bacterium by making the bacteria visible using microautoradiography as explained in Appendix 2. These calculated activities can then be compared with data from groundwater and surfaces exposed to flowing ground water in Swedish granitic rock at the Stripa research mine (Pedersen and Ekendahl 1992a) and the Äspö hard rock laboratory environments (Pedersen and Ekendahl 1992b) (Table 31). The percentage bacteria active in ^{3}H- Leucine uptake in Stripa and Äspö ranged from 9 to 99%. However, the samples analyzed at WL yielded only negative results for microautoradiography, and therefore, calculations of activity per individual bacterium could not be done for the BCE experiment samples. In general, however, the ^{3}H-Leucine assimilation activity in the BCE samples analyzed was similar to what has been registered for the deep ground water of the Stripa research mine, which contained between 10^{4} to 10^{5} bacterial cells per ml of groundwater.

8.4.2 Mineralization of D(-U-^{14}C)-Glucose and a ^{14}C-Labelled Amino Acids

Microbial activity was studied at the GRAM laboratory in five representative metal tube samples: G-008-B above the heater, Q-092-B below the heater and three samples from around the annulus of the heater (K-054-B close to the heater, K-006-B and N-006-B closer to the buffer-granite interface). Mineralization of D(-U-^{14}C)-glucose and a ^{14}C-labelled amino acids mixture was determined under aerobic and anaerobic conditions at 20, 40 and 60°C on the samples sent to France. These results are shown in Tables 20 (D(-U-^{14}C)-glucose and 21 (^{14}C amino acid mixture). Figures 21 (glucose) and 22 (amino acids) show the results graphically.

The results indicate that the bacterial metabolism is mainly oriented towards a carbohydrate utilization pathway, rather than towards an amino-acids utilization. The CO_2 production obtained in the glucose experiments was 5 to 200 times larger than in the experiments with the amino acids mixture. In sample K-054-B from near the heater the microbial activity was very weak both under aerobic and anaerobic conditions and at the three temperatures used. This is in agreement with the culture results for this sample, i.e., no evidence of any culturable bacteria either at 25°C or at 50°C, but not in agreement with the ^{3}H-Leucine assimilation results for sample M-072-B as discussed in Section 8.4.1, which also came from near the heater.

For samples G-008-B and K-006-B, CO_2 production is somewhat higher under aerobic conditions. In these two samples there are more aerobic heterotrophic bacteria than anaerobic heterotrophs (Table 5). At an incubation temperature of 20°C, sample G-008-B, which among the five samples tested for mineralization activity contained the largest number of bacteria (Table 5), showed the highest

activity. In samples G-008-B, N-006-B and K-006-B, the bacterial activity seemed mainly mesophilic, i.e., the CO_2 production decreased from 20°C to 60°C, regardless of the substrate or redox conditions. Only mesophilic bacteria were found in these samples. In contrast, the larger activity in sample Q-092-B occurs at 40°C. In this sample, the active bacteria are mesophilic with an optimum temperature of about 40°C.

These results are compatible with the enumeration results shown in Table 5. A very weak activity is detected in the samples located at 5 cm from the heater surface, whereas in the samples at 20 cm from the heater surface a mesophilic activity is detected but no thermophilic activity. This activity increases with the number of bacteria.

8.5 BACTERIAL IDENTIFICATION

8.5.1 Identification of Culturable Organisms Using the API Method

Organisms cultured from all the samples analyzed at the GRAM laboratory were identified using conventional tests and the API method (Appendix 4). Identifications were done for ten randomly chosen heterotrophic aerobic or facultative anaerobic isolates per sample enumerated at the GRAM laboratory on PCA medium (Appendix 4). Some anaerobic isolates were also identified, i.e., some non-specialized isolates grown in Widdel and Pfennig medium and some SRB grown in Labège test kits (Appendix 4). Some sulphur-oxidizing bacteria (autotrophic) have also been identified. Table 22 gives the results from these identifications.

Only culturable bacteria have been enumerated and identified (some non-cultured bacteria were identified in a limited number of metal tube samples with the 16S rRNA method discussed in Section 8.5.2). Some other bacteria may require particular growth conditions or take longer to grow on standard nutrient agar (e.g., some kinds of actinomycetes need 60 days incubation) and, therefore, would not be studied with the methods used here.

Only the dominant colonies have been studied, e.g., since the detection limit on PCA medium is 50 CFU/g wet material, there must be at least 50 bacteria in the sample belonging to a particular genus to be detected. Moreover, only ten colonies were chosen at random per sample for further identification. This implies that only bacteria present in large numbers were identified with this method.

Certain bacterial groups cannot be identified with the API system. In this case, non-classical tests may be necessary or useful to confirm identification, e.g., some Gram positive bacteria are easier identified when the cell-wall composition is known. This type of non-classical testing is very time consuming and labour intensive. It is, therefore, not always possible to distinguish between isolates that possess very similar characteristics. Also, bacterial taxonomy is in constant evolution, new genera or species appear, some species are reclassified in another genus and difficulties may be encountered with genera that are not well-defined.

The main characteristics of the identified genera and species are discussed in Appendix 4.

More than 67% of the aerobic isolated strains were identified as either the species *Pseudomonas stutzeri* or belonging to the genus *Bacillus*. These are motile rod-shaped bacteria that can grow both in the presence and absence of O_2 and therefore are completely adaptable to the changing conditions in the buffer material of the BCE. Generally, both are ubiquitous in soil, but it is interesting that in the samples from the BCE they do not have the same distribution. *P. stutzeri* is almost exclusively found in samples with enough moisture to allow its growth (i.e., in the interface samples and in O-006-B and P-008-B). Contrary, *Bacillus* is found in samples with lower moisture contents. *P. stutzeri* may be predominant in the buffer material, but when desiccation prevents it from growing, the genus *Bacillus* may become predominant because of its ability to form endospores. The genera suggested as possibly present in the BCE samples by the BART analysis (Table A-2-1) also show the ubiquitous presence of *Pseudomonas* and *Bacillus*.

Another interesting result is the large number of denitrifying organisms (*P. stutzeri*, some *Bacillus*, *Thiobacillus denitrificans*) that were found in all samples except the sand samples and the metal tube samples from close to the heater where no viable bacteria were found. Denitrification is a series of anaerobic respiration processes in which nitrate is used as an electron acceptor instead of O_2. In the buffer material from the BCE, nitrate concentrations are low or absent. However, it has recently been found that conventional respiring denitrifiers have the capacity for long-term survival at high population densities without O_2 or nitrate and appear to be capable of providing for their maintenance by carrying on a low level of fermentation (Jorgenson and Tiedje 1993). Therefore, denitrifying bacteria may have used the nitrates originally present in as much as 400 ppm in bentonite (West et al. 1986b). Since total N in the Avonlea bentonite is <0.01 mg/kg (Stroes-Gascoyne 1989), N could be the limiting factor for bacterial growth in the buffer materials, contributing to the observed low bacterial activity. However, some of the species identified in the samples from the BCE are N_2 fixing organisms that could provide for their own and others' N needs. In natural environments in an ecological balance, N limitation is not usually a problem. Since N_2 fixation is a process requiring large amounts of energy (16 mol ATP are needed per mol N_2 fixed, Paul and Clark 1989) it is more likely that energy rather than N is the limiting factor in many environments.

Thiobacilli are known for their involvement in concrete and metal corrosion (Rogers et al. 1993). Sulphur is oxidized to sulphates which form sulphuric acid, decreasing the pH and creating favourable corrosion conditions. Chemical analyses of the buffer material have shown that this material is sulphate rich. This sulphate can be used by SRB to produce H_2S which is implicated in the corrosion of copper materials. Since in the buffer samples both groups of bacteria are present, one could speculate that it is possible that *Thiobacilli* provide SRB with an electron acceptor (sulphate) and that SRB provide *Thiobacilli* with an energy source, i.e., the sulphide produced by the SRB can be used by the sulphur-oxidizing bacteria to produce sulphate which can then be used by SRB to produce sulphides. Such a cycle would allow these corrosion bacteria to develop continuously, even if organic carbon sources are not available, since all *Thiobacilli* and some SRB are capable of autotrophic growth with CO_2 as the sole carbon source. However, no evidence was obtained from the BCE samples for the occurrence of such a cycle.

Almost no methanogenic bacteria are found, most likely because the buffer environment was not sufficiently reducing. In addition, sulphate reduction is thermodynamically more favourable than the reduction of CO_2 to CH_4 (Claypol and Kaplan 1974) and it is known from other studies that the formation of methane is insignificant in environments with abundant sulphate (Winfrey and Zeikus 1977, Abram and Nedwell 1978a,b; Oremland and Policin 1982; Winfrey and Ward 1983). And the buffer material and groundwater used in the BCE are both rich in sulphates. The results reported here, therefore, may indicate that sulphate-reducing bacteria compete successfully with methanogenic bacteria for the available electron donors (Pedersen and Ekendahl 1990). However, methanogens have been found in groundwaters containing 100 to 150 ppm sulphate (Pedersen et al. unpublished results 1995).

8.5.2 Distribution and Diversity of Bacteria in the Buffer Analysed by 16S rRNA Gene Analysis

This identification method was applied to 3 metal tube samples sent to the UoG laboratory in Sweden (samples H-097-B, M-018-B and P-016-B, Figures 7 and 8 for location). The method used is described in Appendix 3. The results are given in Table 23 and Figures 23 and 24.

Distribution

A total of 21 unique clone groups could be identified. The three samples (from three different layers in the BCE) investigated indicated a fairly homogenous system with three identical and dominating bacteria occurring in each sample (Figure 23). Each sample also shared about 50% of its clones identities (Table 23) with each of the other two samples. This result was expected as the buffer mass was mixed from common sources of groundwater, clay and sand. The temperature differences did not correlate with any drastic changes in the distribution of the dominating species, although the frequencies of single clones differed between the sampled layers. One such minor difference noticed was the presence of eukaryotic DNA. The closest species in the database was the yeast *Saccharomyces cerevisiae*. This sequence, clone K20, appeared only in the two lower layers analysed, in samples M-018-B and P-016-B. This observation coincides with the PLFA analysis indicating eukaryotic fungi in these layers but not at the top layer H-097-B (Section 8.6.1). Table A-2-1 (BART results, Appendix 2) also indicated the possible presence of yeasts in the lower layers O (sample O-024-B) and Q (Q-001-B). The results from the analyses performed at the GRAM laboratory do not indicate the presence of fungi (Tables 16 and 17) in these layers.

Diversity

Three distinct phylogenetic groups of bacteria were found, proteobacteria alpha and gamma groups and Gram-positive bacteria belonging to the actinomycetes (Figure 24). Most clones belonged to the gamma group of the proteobacteria and this is a group were many bacteria living close to man can be found. Clones indicating anaerobic sulphate reducing bacteria (SRB) (proteobacteria delta group) were not found and methanogenic sequences (archaeobacteria) could not be found either. This result supports the negative or low viable counts obtained for both SRB and methanogens (Sections 8.3.1 and 8.3.2) in the metal tube samples.

Two of the three dominating species found had high identity with the typical groundwater bacteria *Pseudomonas flavescens* (95.6%) (Hildebrand et al. 1994) and *Acinetobacter calcoaceticus* (98.3%). The third one was an actinomycete related to *Streptomyces*. Filamentous actinomycetes are adapted to life in soils and their mycelial morphology makes them well adapted to desiccation. The finding of these groups of bacteria in the buffer samples is, therefore, not surprising. Pseudomonads are also indicated by the BART results for almost all of the metal tube and other samples with viable bacteria analyzed at WL and *Acinetobacter* was indicated in a few of the metal tube samples by the BART tests (Table A-2-1, Appendix 2). *Pseudomonas stutzeri* was indicated frequently in the identifications carried out at the GRAM laboratory and the presence of an actinomycete (possibly Nocardia) was also observed.

The nucleotide sequence data determined for the clones identified will appear in the EMBL, GenBank and DDBJ Nucleotide Sequence Databases under the accession numbers X91516 (K1, Figure 24) to X91536 (K20, Figure 24).

The results of the 16S rRNA analyses, although only performed on three samples, are very important, because this method does not require that the organisms to be identified are culturable. The fact that the results from the 16S rRNA analysis agree quite well with the other methods of identification (based on culturing) provides further confidence that the dominating species in the buffer samples have been identified correctly.

8.6 OTHER ANALYSES

Some of the samples retrieved during decommissioning of the BCE were sent out for special analysis, such as analysis for phospholipid-ester-linked fatty acids (these are part of intact cell membranes and are therefore a measure of total biomass presence; because phospholipid fatty acids are also unique for certain groups of bacteria, identification can also be pursued with this kind of analysis) (White et al. 1983). In addition, electron microscopy (both Scanning and Transmission) was also carried out on a number of samples. The results of these additional analyses are discussed below.

8.6.1 Phospholipid Fatty Acid Analysis (PLFA)

Three hollow-stem auger samples were sent to Microbial Insights Inc. (Knoxville, Tennessee, USA), H-001-AB, M-001-AB and P-001-AB (Table 2 for locations) for PLFA analysis. Results are given in Tables 24 (summary) and A-6-1 (Appendix 6, complete results) and Figures 25 (biomass), 26 (community structure) and 27 (old age or slowed growth). Table 24 shows that the laboratory blank contains 35 pmoles of PLFA which is normal when analyzing with single ion detection with the GC/Mass spectrometer. The PLFA pattern in the blank contains PLFA characteristic of eukaryotic (human dandruff type) contamination without the classical bacterial biomarkers. This did not compromise the analysis of the buffer samples because the samples contained 400 to 2200 pmoles of PLFA, at least ten times the biomass that could be present in the laboratory blank.

Viable biomass

The viable biomass (Figure 25) indicated by the PLFA concentrations in the samples varied from 3.7 x 10^5 to 1.9 x 10^6 bacterial cell equivalents per gram of dry buffer (data reference sheet in Appendix 6 for conversion factors). There are significant numbers of bacteria with intact membranes in the samples tested, and these are, therefore, potentially viable. Sample M-001-AB (from near location 56, Figure 8, with a temperature of ~ 60°C and a moisture content of ~ 13%) contained the most cell equivalents, and sample H-001-AB (from near location 74, Figure 7, with a temperature of ~ 20°C and a moisture content of ~ 19.5%) the least amount of cell equivalents. Sample P-001-AB (from near location 76, Figure 7, with a temperature of ~ 45°C and a moisture content of ~ 18.5%) contained almost as much biomass as sample M-001-AB.

Community structure

The community structure is illustrated in Figure 26. The proportion of Gram-positive bacteria based on the proportion of terminally-branched saturated PLFA varied between 7 and 13% in these samples. The predominant PLFA were i16:0 and a17:0 with *iso/antiiso* ratio in the seventeen carbon series of 0.36 and 0.45 for samples P-001-AB and M-001-AB respectively, and a high ratio for sample H-001-AB. A low ratio suggests the presence of *Arthrobacter/ Micrococci* type Gram-positive aerobes rather than anaerobic Gram-negative *Desulfovibrio* which have a significantly greater ratio. *Desulfovibrio* are obligate anaerobes and characteristically have a higher ratio of *iso/antiiso* terminally-branched saturated PLFA (5.6 to 7.5 for *Desulfovibrio*) compared to common Gram positive-bacteria (i.e., 0.1 for *Arthrobacter* and 0.2 for *Micrococcus*). The microbial communities in all three samples were predominantly Gram-negative, based on the sum of monoenoic and normal PLFA. The proportion of monoenoic PLFA characteristic of Gram-negative heterotrophs was higher in sample P-001-AB and H-001-AB than in sample M-001-AB.

The microeukayotes represented a small proportion of the community based on the polyenoic PLFA. The major polyenoic PLFA was 18:2w6 which is especially prominent in fungi and was found in samples P-001-AB and M-001-AB.

Mid-chain branched saturated PLFA, particularly 10Me16:0, are indicative of the anaerobic sulphate-reducer *Desulfotobacter.* The presence of i17:1w7c in the PLFA often indicates the presence of another sulphate- (or iron-) reducer, *Desulfovibrio*, suggesting there are anaerobes present. The low levels of 10Me18:0 suggest the aerobic actinomycetes characteristic of surface soils are absent. The actinomycetes also contain a mixture of 10Me branched saturated PLFA. Classically they contain more 10Me18:0 than 10Me16:0.

Nutritional/physiological status

The degree of nutritional stress (Figure 27) is indicated by the ratio of cyclopropane PLFA/monounsaturated precursor for the 16 carbon PLFA and the 18 carbon PLFA (Table 24, data summary sheet). Vigorously-growing systems usually have ratios of <0.1. In the samples analyzed here, the rations are >0.4, and this ratio is particularly high in sample P-001-AB. High levels of

cyclopropanization indicate prolonged stationary phase (slowed growth) and/or low oxygen or high carbon dioxide. Toxicity as indicated by the *trans/cis* PLFA ratio is indicated when the ratio is >0.1 and in sample P-001-AB this ratio was 0.2 for the 18 carbon PLFA.

In summary, the PLFA analysis shows that there are clearly bacterial communities in the three samples with intact cytoplasmic membranes and thus there is the potential for growth. The communities are complex with evidence of Gram-positive, Gram-negative, aerobic and anaerobic bacteria. The Gram-negative bacteria show strong signs of nutritional stress in the high cyclopropane levels and there is some evidence of toxicity, particularly in sample P-001-AB.

These results do not agree with the results obtained from culturing in corresponding metal tube samples. The potentially viable biomass (Figure 25) found in sample H-001-AB, a cool sample with ample moisture present, was lower than in the other two samples, which came from hot and dry (M-001-AB) and warm and moist (P-001-AB) locations. Tables 4 and 5 show considerable growth in layer H, in contrast with no growth in the samples from layer M (in the hot locations adjacent to the heater) and less growth in layer P. Also, sample M-001-AB had a much lower ratio of cyclopropane PLFA/monounsaturated precursor than in H-001-AB and P-001-AB in Figure 27. This ratio increases as bacteria move from a logarithmic (log) to stationary growth phase and usually falls within the range of 0.5 (log phase) to 2.5 (stationary phase). Figure 27 shows that only the ratio for sample P-001-AB indicates a definite stationary phase, whereas especially for sample M-001-AB a log phase is indicated. However, this ratio may also indicate starvation or toxicity and the range for this is 0.05 (healthy) to 0.3 (starved). Figure 27 shows that all samples indicate starved bacteria, and this is especially pronounced for sample P-001-AB. Unbalanced growth, as indicated by the ratio PHA/PLFA (Appendix 6, data reference sheet) is indicated for sample M-001-AB (Table 24), but could not be calculated for the other two samples because no PHA was measured (PHA is a storage lipid; PLFA is a membrane lipid).

These results suggest that initially, shortly after commissioning of the BCE (i.e., starting the heater), the temperature in the layers around the heater increased rapidly (Figure 4), possibly stimulating growth more in these layers than anywhere else. The moisture content would likely change considerably less quickly than the temperature in these layers. Growth would be stimulated but not all necessary nutrients may have been present and this may explain the presence of PHA in layer M. When conditions became more harsh and dry, growth slowed and starvation and toxicity may have been the result. The fact that less biomass was indicated for the coolest sample H-001-AB may indicate no growth stimulation by higher than normal temperatures in this layer (~ 20°C). The higher biomass in sample P-001-AB may also reflect stimulation by temperature, but the change in temperature is shown in Figure 4 to be considerably slower in layer P.

The species indicated as being present from the PLFA analysis agree fairly well with the 16S rRNA and IPA identifications, except that the PLFA analysis indicates the absence of actinimycetes, which were indicated in some samples with both API and 16S rRNA identification.

8.6.2 Environmental Scanning Electron Microscopy (ESEM)

Four samples (Table 2) were examined with ESEM for the presence of individual microorganisms and the formation of biofilms. These samples were: the Teflon cloth with adhering sand grains (HE-003-B(SEM)), adhesive black tape with adhering sand (HE-005-B(SEM)), a portion of a corroded stainless steel thermocouple (SEM-001-N) and a fragment of buffer that had been in contact with the thermocouple segment (SEM-002-N). Sample treatment is described in Appendix 6. Figure 28 shows a corrosion pit on the thermocouple segment and Figure 29 shows the piece of black tape (Figure 29a) and the bacteria on this tape (Figure 29b). Figure A-6-1 to A-6-4 can be found in Appendix 6. They show EDS spectra of, respectively, the uncorroded thermocouple tip, the corroded zone on the thermocouple, the corrosion pit and the buffer fragment.

The thermocouple section showed signs of localized corrosion along the mid-section. The tip of the thermocouple was free of any visible indication of corrosion. That area was used to determine the composition of the base metal. The EDS spectrum of the uncorroded thermocouple tip indicated stainless steel with the constituents: nickel (26.17%), molybdenum (3.24%), chromium (19.85%), iron (49.33%) and copper (1.41%). A corrosion pit is shown in Figure 28. The corrosion scale (Figure A-6-2) contained magnesium, silicon, phosphorus, sulphur, calcium and titanium in addition to alloying elements. The scale was enriched in chromium and nickel relative to the uncorroded alloy. Areas under the scale were similar in composition to the base metal. Pitted regions were predominantly chromium and nickel (Figure A-6-3) with inorganic crystals. There were no indications of a biofilm anywhere on the surface of the thermocouple. Similarly, individual microorganisms were not found on the surface or in the corrosion pits. The clay fragment that had been next to the thermocouple was predominantly silicon with aluminum, calcium and iron with lesser amounts of sodium, magnesium, sulphur, potassium, titanium, chromium and nickel (Figure A-6-4). No bacteria were observed in the clay fragment.

Adherent sand grains on the Teflon cloth were clean silicon, a few individual sand grains were bright-red due to the presence of iron. There were no indications of individual microorganisms or a biofilm either associated with the sand grains or on the Teflon cloth. Sand grains were found on the adhesive side of the piece of black tape (Figure 29a) and long chains of cocci-shaped bacteria were found between the sand grains (Figure 29b). The side of the tape that had not been in contact with the heater (i.e., Teflon cloth) was free of sand and bacteria.

These results indicate that no bacteria were involved in corroding the stainless steel thermocouple; the corrosion characteristics are indicative of an oxidative chemical process (D. Shoesmith, pers. comm., 1995). No bacteria were found on the heater cloth surface. This may be due to the fact that Teflon is a poor substrate for the adhesion of biofilms. However, it is likely that this cloth was handled substantially when it was wrapped around the heater and lowered into the borehole, and therefore it would have been contaminated with (human) bacteria. The fact that none were indicated under the SEM may suggest that the contamination likely present was either not severe or did not survive the extreme

conditions. This agrees with the culture results for sample HE-001-B (Table 9) in which a maximum of only 135 CFU/cm^2 of Teflon cloth were found at a culture temperature of 50°C, or 1.35 x 10^{-8} cells per μm^2 which would make them difficult to find under the SEM. The sand sample containing a piece of the black tape contained a maximum of 172 CFU/g, cultured at 50°C, considerably more than in the sand samples from layers L and M analyzed at WL (Table 8) and from layer L analyzed at the GRAM laboratory (Table 12) (in which only molds were found). This agrees with the SEM results for this sample, which showed long strings of cocci-shaped bacteria (Figure 29b) that appear to have resulted from contamination during emplacement, survived exposure and may have proliferated. The fact that culture results obtained at 50°C were larger than those obtained at 25°C may indicate that surviving bacteria were thermophilic.

8.6.3 Transmission Electron Microscopy (TEM)

Four samples were submitted for TEM analysis. These samples were subsamples of metal tube samples K-072-B, L-072-B, M-072-B and N-024-B, analyzed at WL (Table 4). The sample treatment is described in Appendix 6. Three of these four samples came from the extreme environment around the heater, and did not give culturable results (Table 4), except sample K-072-B in which 1.6 x 10^2 CFU/g dry material was found at 50°C (aerobic results). Sample N-024-B came from the periphery and contained culturable aerobic organisms in the range of 1.7 x 10^2 to 4.2 x 10^3 CFU/g dry material.

The samples examined contained microorganisms but in very small numbers, that were very difficult to see under the TEM. A number of subsequent sample manipulations were carried out (Appendix 6) to attempt to increase the quality of the photographs. Figure 30 shows sample N-024-B, washed four times with 0.05M HEPES buffer. The bacteria that could be seen in this sample were adhered to larger particles and very difficult to see clearly. The other samples that underwent the same treatment showed badly lysed bacteria and were not photographed.

Figure 31 shows sample K-072-B, washed eight times with 0.15 M HEPES buffer. This treatment improved the quality of vision somewhat but definite cells could still not be seen. All samples were taken though this treatment and subsequently embedded in two types of resin, Nanoplast and LRwhite (Appendix 6). Extremely thin sections of the embedded material were examined by TEM and proved again that the bacteria were indeed present in very low numbers and were very difficult to preserve. The Nanoplast embeddings did contain a few cells. Figure 32 shows sample K-072-B, washed eight times with 0.15M HEPES buffer, embedded in Nanoplast and stained with Uranyl. This photo suggests that the cells prefer to reside in groups rather than singly. They also show a great deal of associated material, indicating a reactive surface component, probably a capsule or slime. No photos of the other samples were provided. It should be pointed out here that the photographed sample K-072-B contained some culturable organisms, as discussed above (Table 4).

The embedding and sectioning procedures are very time consuming and it was attempted, therefore, to multiply the cell numbers in the samples to increase the chances of viewing greater numbers and types of organisms with TEM. A subsample of each of the samples was therefore suspended in M-9 salts broth (Appendix 6), but although cell numbers increased, they were still too low for successful TEM. A medium of equal parts of Nutrient Broth (NB) and M-9 salts (Appendix 6) was inoculated from the M-9 growth experiment. Cell numbers increased and could be viewed successfully in sample K-072-B as shown in Figure 33.

These results confirmed the results of most of the other analyses, i.e., that very few, if any, viable cells were present in the samples taken from near the heater.

8.7 STATISTICAL ANALYSIS

All culture results were included in a statistical evaluation of which the details are given in Appendix 5. Table A-5-1 contains all data included in the analysis. Tables 25 to 29 show the various results of the statistical tests and Figures 34 and 35 illustrate the results.

8.7.1 Statistic Evaluation of the CFU Counts (WL, GRAM Laboratory and UoG)

Figures 34 and 35 show the data used for the statistical analysis as functions of the in situ temperatures and water contents, respectively. The F-test in Table 25 shows significance on the 99% level for the effects from water content, nested within the in situ temperature, and the crossed effect from culturing temperature, oxygen culturing conditions, the medium used and the laboratory doing the analysis, i.e. the culturing conditions. The in situ temperature did not have a significant effect on the results obtained. The variance component analysis in Table 26 shows that the variance component of the in situ temperature effect was small indeed, only 4.9 %. The major variance components were those that were shown to be significant in the analysis of variance, the in situ water content of the buffer mass and the culturing conditions used to produce the CFU numbers.

Table 27 shows the mean values of CFU for the buffer temperature class levels used in the statistical analysis. The mean CFU numbers decrease with increasing temperature. In Table 28 the relation between in situ temperature and water content is depicted. A total of 75 observations at temperatures above 50°C had a water content below 15% and also a much smaller CFU mean value than any other of the class levels. The two other classes at a temperature above 50°C had a water content above 15 and 18% respectively and had among the highest CFU mean values. This is probably the best demonstration that the water content is the variable that limits the viability of the present bacteria, and not the temperature per se. Table 29 shows that aerobic heterotrophic culture conditions generally gave the highest CFU mean values at both 20 and 50°C. At anaerobic culturing conditions, and especially at 50°C, much lower mean CFU values were obtained. This suggests that anaerobic conditions were not widely distributed in the buffer material after 2.5 years.

8.8 WATER POTENTIAL DISCUSSION

Since the statistical analysis of all culture data has clearly indicated that the presence of water is the most important factor in determining whether or not viable bacteria will be present in a sample, a discussion will follow below exploring the reasons for the important role of water availability on the viability of bacteria in this specific buffer environment.

8.8.1 Solutes

Because a selectively permeable plasma membrane separates bacteria from their environment, they can be affected by changes in the osmotic concentration of their surroundings. If a bacterium is placed in a hypotonic solution, water will enter the cell and cause it to burst unless something is done to prevent the influx. Most bacteria have rigid cell walls that maintain the shape and integrity of the cell. Indeed, many bacteria keep the osmotic concentration of the protoplasm above that of the habitat by use of compatible solutes, so that the plasma membrane is always pressed firmly against their cell wall. Compatible solutes are solutes that are compatible with cell metabolism and growth when at high concentrations. It can be synthesis of choline, betanine, proline, glutamic acid and other amino acids; elevated levels of potassium ions are also involved to some extent. A few bacteria (e.g., *Halobacterium salinarium)* raise their osmotic concentration with potassium ions and have enzymes that require high salt concentrations for normal activity.

8.8.2 Water Activity

The amount of water available to bacteria can be reduced by interactions with solute molecules (the osmotic effect) and by adsorption to the surface of solids (the matrice effect). Because the osmotic concentration of a habitat has such profound effects on bacteria, it is useful to be able to express quantitatively the degree of water availability. Microbiologists generally use water activity (a_w) for this purpose which is a thermodynamic parameter. The water activity of a solution is 1/100 the relative humidity of air in equilibrium with the solution (when the latter is expressed as percent). This corresponds to the ratio of the solution's vapour pressure (P_{sol}) to that of pure water (P_{water}) at a fixed temperature.

$$a_w = P_{sol}/P_{water} \quad (1)$$

The water activity of a solution or solid can be determined by sealing it in a chamber and measuring the relative humidity after the system has come to an equilibrium. Water activity is inversely related to osmotic pressure; if a solution has a high osmotic pressure, its a_w is low.

Bacteria differ greatly in their ability to adapt to habitats with low water activity. A bacterium must expend extra efforts to grow in a habitat with a low a_w value because it must maintain a high internal solute concentration to retain water. Some bacteria do this and are osmotolerant; they will grow over wide ranges of water activities or osmotic concentrations. For example, *Staphylococcus aureus* can be cultured in media containing any sodium chloride solution up to about 3 M. Although a few bacteria are truly osmotolerant, most only grow well at water activities around 0.98, (the approximate a_w for sea water) or higher. Extreme halophiles have adapted so completely to saline conditions that they require high levels of sodium chloride to grow, i.e., concentrations between about 2.8 M and saturation, about 6.8 M. Extreme halophilic bacteria have adapted successfully to environmental conditions that would destroy most organisms. In the process, they have become so specialized that they have lost ecological flexibility and can prosper only in a few extreme habitats.

8.8.3 Chemical Potential

The activity of water (a_w) is related to its concentration through an activity coefficient (γ_w) where $a_w = \gamma_w N_w$. N_w is the mole fraction of water in the system (Potts 1994). The chemical potential of water (μ_w) in a system is expressed according to the following equation:

$$\mu_w = \mu_w^* + RT \ln a_w + V_w P + Z_w FE + m_w gh \qquad (2)$$

In equation 2, the term RTln a_w - the activity term (where R is the gas constant) - gives the water activity term in units of energy per mole. V_w is the partial molar volume of water, i.e., in a bacterial cell, in contrast to μ_w which is the partial molar Gibbs free energy ($\delta G/\delta n_w$). V_w is the differential increase or decrease in the volume of a bacterial cell when a differential amount of water is added or removed, respectively, and it is expressed as the volume per mole. Pure water, or a very dilute solution, has a value of V_w equal to 1.8×10^{-5} m^3 mol^{-1}. P is the hydrostatic pressure in excess of the atmospheric pressure, so that the V_wP term in equation 2 reflects the effects of pressure on the chemical potential of water and is expressed, therefore, in units of energy per mole. Z_wFE is the electrochemical potential, and because water is uncharged ($Z_w = 0$), the electrical term Z_wFE can be ignored. The gravitational term, m_wgh, represents the work needed to move a given mass per mole of water, m_w (18.016 g mol^{-1}), to a given height (h) against gravitational acceleration (g). Only under circumstances when cells are distributed at high altitudes throughout a water vapour can this term contribute significantly to the overall μ_w^*. μ_w^* is an additive constant and represents the chemical potential of water in a standard (ideal) reference state where RT ln a = 0,$V_wP = 0$, Z_w FE = 0, and $m_wgh = 0$. For practical purposes, the chemical potential of cells is compared with different intermediate water contents, e.g., those of a dried bacterial cell (μ_w^D) and/or a cell at some stage of rehydration (μ_w^R). During comparison of these chemical potentials, the two μ_w^* terms cancel out.

8.8.4 Water Potential

The water potential of a system (Ψ) is proportional to μ_w-μ_w*, so that the term μ_w-μ_w* has considerable utility when the water relations of bacterial cells are compared. The term represents the work involved in moving 1 mol of water from some point in a system (at constant pressure and temperature) to a pool of pure water at atmospheric pressure and at the same temperature as the system under consideration (the gravitational term is ignored for reasons describe above). A difference between two locations in values of μ_w-μ_w* indicates that water is not in equilibrium, such that there will be a net tendency for water to move towards the region where μ_w-μ_w* is lower.

8.8.5 Water Available for Bacteria in the BCE

When the buffer was emplaced at the start of the experiment, the water content was homogenous and averaged about 18% (Dixon et al. 1992). During the experiment, the heat from the heater caused a mass transport of water outward from the vicinity of the heater to the peripheral parts of the buffer. Gradients of water contents developed. Approximately 50% of the buffer mass is sand and this part will not influence the amount of free water by a matrix adsorption effect more than marginally compared to the bentonite part. The clay will sorb water until it is saturated, and only water in excess of what is sorbed by the clay will be available for the bacteria. The water content at which water becomes available in a 50/50% sand-bentonite mixture is about 15% which correlates well with the values below which viable bacteria could generally not be demonstrated.

8.9 IMPLICATIONS FOR WASTE MANAGEMENT

8.9.1 General Implications

The main result from the statistical analysis of all culture data is the demonstration (Table 27, Figure 35) that the water content is the variable that limits the viability of the bacteria present and not the temperature. As discussed in Section 8.8.5, the water content in the buffer below which viable bacteria could not be demonstrated on culture media is about 15% (Figure 35). This is in agreement with the PLFA analysis which showed the presence of a potentially viable but severely starved and, therefore, inactive microbial population.

Measurements at WL have shown that the total suction of buffer material containing 15% moisture at 25°C is 6.08 MPa, as calculated by the experimentally derived formula (3) for this buffer material (Wan 1995). (Note that extrapolation of this formula to zero suction is invalid, because suction is never zero because of the osmotic component of the suction).

$$\text{moisture content (\%)} = 25.3369 \quad 0.0017 \times \text{suction (kPa)} \qquad (3)$$

The water potential of buffer with 15% moisture is, therefore, -6.08 MPa. Water potential (ψ) can be converted to water activity (a_w), using the following equation (Brown 1990):

$$\psi \text{ (MPa)} = RT \ln a_w / V_w \qquad (4)$$

in which

$R = 8.3143 \times 10^{-6}$ m^3 .MPa / mol.K

$T = 298$ K

$V_w = 1.8 \times 10^{-5}$ m^3/mol

$\psi = -6.08$ MPa

This yields a water activity of ~ 0.96 for buffer with 15% moisture content. Table 30 gives the water activity of several materials with some microbes growing at that water activity in comparison with a number of buffer materials (100% Na-bentonite) from the Swedish nuclear fuel waste disposal program (Pedersen and Karlsson 1995). This table shows that Gram negative bacteria disappear around an a_w value of ~ 0.95, but that *Pseudomonas*, SRB and *Vibrio* are able to grow at an a_w value of 0.96. An a_w value between 0.95 and 0.96, therefore, appears to form the boundary of where microbial life is easily possible and it is not surprising that virtually no viable bacteria were found in the samples of buffer material from the BCE that contained 15% water or less.

8.9.2 Implications for the Canadian Waste Management Concept

These findings imply that, for some time after the disposal of containers with used fuel waste in a vault, the area around these containers (i.e., part of the buffer directly adjacent to the containers) would be virtually devoid of microbial activity because of the redistribution of the initial moisture (18%) as a result of the high temperatures of the waste containers. This effect would be further enhanced by the initially high radiation emanating from the containers (Stroes-Gascoyne et al. 1995, King and Stroes-Gascoyne 1995). It has been argued (Stroes-Gascoyne and West 1994) that microbial repopulation of an initially depleted zone around waste containers may be limited due to the extremely small pore size of the buffer material. Experiments are needed, therefore, to study the possibility of repopulation by microbes of this zone, once conditions of moisture, heat and radiation improve as a result of the decay of radioactivity in the waste. These experiments are being conducted at WL and involve the use of radiation-sterilized compacted clay and buffer material plugs. Viable bacteria are added in a suitable growth medium around these plugs and after the test, the plugs are sectioned and tested for viable bacteria with a variety of methods. These experiments will either confirm the argument that repopulation may not easily occur or will provide a measure of the rate at which repopulation may occur.

The present attention at AECL with regard to microbially influenced corrosion (MIC) effects on waste containers is focused on the effects of SRB on Cu containers because MIC of Ti has never been observed (Stroes-Gascoyne and West 1994). If a zone of depleted microbial activity is created during the period of harsh conditions in a vault, and if repopulation of this zone is limited or prevented because of the small pore size of the compacted buffer material, SRB activity will be limited to regions outside this depleted zone. In this case the only microbial impact on the container will result from the diffusion of microbially reduced sulphur species (sulphide, thiosulphate, etc.) to the container surface. Therefore, MIC as a result of corrosion agents (e.g., sulphide) produced by microbes is being studied at WL by electrochemical methods using a copper/compacted clay electrode and inorganic sulphide as an analog for the products of SRB (King and Stroes-Gascoyne 1995). However, the results from the BCE have also shown that in the buffer samples with sufficient moisture for microbial activity, the occurrence of SRB is not large. This suggests that SRB growth is limited, likely because the environment is not sufficiently anaerobic for SRB growth or possibly because of the lack of a suitable source of organic materials (i.e., the organic material naturally associated with the buffer may not be suitable or available for substantial SRB growth). Therefore, SRB activity outside the affected zone around the container could be limited for some time after disposal, which would imply that the indirect MIC effects (i.e., sulphide diffusion) would also be limited. At longer times, conditions will be more reducing and SRB may become more prominent if their nutritional requirements are met (i.e., short-chain organic acids, sulphate etc.).

8.9.3 Implications for the Swedish High-Level Radioactive Waste Repository

The Swedish buffer will consist of 100% Wyoming bentonite and will have a swelling pressure corresponding to a water activity between 0.92 to 0.96 (Table 30). This is low enough to reduce the viability of many bacteria including sulphur and sulphate reducing bacteria. The production of sulphide by SRB constitutes one of the very few chemical circumstances under which copper will corrode anaerobically. If it can be shown that the density of the bentonite is high enough to kill any viable sulphur and sulphate reducing bacteria through desiccation, then copper corrosion induced by sulphide producing bacteria will totally depend on the production of sulphide in the rock outside the bentonite and the diffusivity of sulphide in bentonite. There are spore forming SRB belonging to the genus *Desulfotomaculum* but spores are inactive and do not produce sulphide. Such production may occur after germination, but then the sporeforming SRB species are as intolerant to desiccation as most other SRB. In other words, sporeforming SRB species will not constitute a larger problem with respect to sulphide production than any other SRB.

As a consequence of the above conclusions, a series of experiments studying the survival of SRB at different swelling pressures to determine the water activity at which SRB no longer survive are presently being launched in Sweden. It is executed as a collaborative project between Clay technology AB, Lund, Sweden, Department of General and Marine Microbiology, Göteborg and SKB AB (Pedersen and Karlsson 1995).

9 SUMMARY AND CONCLUSIONS

The Buffer/Container Experiment (BCE) was carried out at AECL's Underground Research Laboratory (URL) for 2.5 years to examine the in situ performance of compacted buffer material in a single emplacement borehole under vault-relevant conditions. The test was run in a full-size borehole at the 240 m level in the URL. An electric heater, with the same dimensions of a waste container, was used in the test to provide the heating (constant output of 85°C).

During the decommissioning of the BCE, numerous samples were taken for microbial analysis to satisfy two objectives, to (i) assess the survival of the naturally present microbial population in buffer material that has been subjected to compaction upon emplacement and to the subsequent heat and drying cycle during the experimental phase (2.5 year) of the experiment, and (ii) to determine, if a surviving microbial population was found, which group(s) of microorganisms are dominant in this simulated vault environment.

Great care was taken to obtain non-compromised samples during decommissioning of the BCE, by using stringent sampling procedures to ensure sterility and anaerobic conditions. Eight different types of samples were taken as well as some incidental ones. Microbial analyses were initiated at WL within 24 hours of sampling for all types of samples. Staff from the Department of General and Marine Microbiology at the University of Göteborg (UofG) were present during decommissioning and performed a number of the analyses. Representatives of most sample types were shipped to the GRAM laboratory in Aix-en-Provence, France where considerable expertise has been developed in the microbial analysis of clays.

The microbial analysis of the BCE samples involved analyses for total viable aerobic and anaerobic heterotrophs at 25 and 50°C, viable specialized organisms, such as sulphate-reducing bacteria (SRB), methanogens, fermenters, fungi, sulphur-oxidizing bacteria (SOB), iron-related bacteria (IRB) and slimeformers. Microbial activity was measured in a number of the samples, by determining the assimilation of ^{3}H-Leucine and the mineralization of ^{14}C-labelled glucose and a ^{14}C-labelled amino acids mixture. Identification was attempted by using conventional tests and the API method on isolates from culture plates and 16S rRNA gene sequencing on selected samples. Phospholipid fatty acid (PLFA) analysis was performed on three samples to determine potential viability, nutritional status and community structure without the need for culturing. Electron Microscopy (both ESEM and TEM) was done on a few samples, to observe any bacteria present directly. All culture results (almost 400) were evaluated with a statistical method to determine which variables affected the number of colony-forming units (CFU) in each sample analyzed.

As a result of a redistribution of moisture in the buffer material around the heater, the moisture content in samples taken from near the heater surface dropped considerably, from the initial 18% at which the material was emplaced at the beginning of the BCE, to as low as 12%. The culture results showed an almost universal disappearance of viable microorganisms (both heterotrophic non-specialized and specialized bacteria) in the samples taken from near the heater surface, where the moisture content was low (<15%) and the temperature high (as high as 60°C). The microbial activity measured was either insignificant or zero in most of these samples, reflecting the lack of culturables. The activity measured in samples from more moderate regions of the BCE correlated with the numbers of viable aerobic heterotrophs found. The culture results at 50°C gave some indication of selection towards aerobic heterotrophic thermophiles in samples with a high temperature and sufficient moisture (>15%). Generally, aerobic heterotrophic culture conditions gave the highest CFU mean values at both 25 and 50°C. At anaerobic culturing conditions, and especially at 50°C, much lower mean CFU values were obtained. This suggests that anaerobic conditions were not widely distributed throughout the buffer material, after 2.5 years.

Few to very few SRB (<1000 CFU/g dry material) were observed in all samples analyzed, despite high concentrations of sulphate (as gypsum) in the buffer material. It is likely that the environment in the BCE was not sufficiently anaerobic for SRB growth. The buffer material was emplaced at 80% saturation, with about 20% of the porosity taken up by air. Aerobic metabolism and chemical reactions that use O_2 may not have proceeded enough during the 2.5 years of the test to create a low enough redox potential for SRB to thrive. Lack of organic material corresponding to the very restricted nutritional spectrum of SRB may also have been a factor.

Methanogens were either not present in most samples or were present in very low quantities, suggesting that the buffer environment was not a very suitable environment for the growth and activity of methanogenic bacteria. One likely factor is that the redox potential in the buffer environment may not have been low enough for methanogens to develop. Another factor is that sulphate reduction is thermodynamically more favourable than the reduction of CO_2 to CH_4 and it has been reported that the formation of methane is insignificant in environments with abundant sulphate (i.e., gypsum in the clay).

Numerous anaerobic sulphur-oxidizers were found in most sample types. Almost all were identified as *Thiobacillus denitrificans*, a facultative denitrifying species, that can grow anaerobically using nitrate as the terminal electron acceptor. Both SRB and *Thiobacilli* were present in the buffer samples and one could speculate that *Thiobacilli* could provide SRB with an electron acceptor (sulphate) and that SRB provide *Thiobacilli* with an energy source. This cycle could allow these bacteria to develop continuously, even if organic carbon sources are not available, since all *Thiobacilli* and some SRB are capable of autotrophic growth with CO_2 as the sole carbon source. However, an abundant presence of SRB was not found.

A large number of denitrifying organisms (*P. stutzeri*, some *Bacillus*, *Thiobacillus denitrificans*) were found in most samples with viable bacteria. These denitrifying bacteria may have used any nitrates originally present in buffer materials and groundwater, and are now only subsisting in an inactive state. Total N in the buffer materials is low and N could, therefore, be the limiting factor for bacterial growth in the buffer materials, contributing to the observed low bacterial activity. However, some of the species identified in the samples from the BCE are N_2 fixing organisms. Since N_2 fixation is a process requiring large amounts of energy it is likely that energy rather than N was a factor in limiting bacterial activity in the BCE environment.

More than 67% of the aerobically isolated strains were identified with conventional tests and the API method as belonging to either the species *Pseudomonas stutzeri* or the genus *Bacillus*. These bacteria can develop both in the presence and absence of O_2 and therefore are completely adaptable to the changing conditions in the buffer material of the BCE. *P. stutzeri* was almost exclusively found in samples with enough moisture to allow its growth, and *Bacillus* was found in the samples with lower moisture contents where they probably survived in spore form. Two of the three dominating species found with 16S rRNA gene sequencing had a high identity with the typical groundwater bacteria *Pseudomonas flavescens* and *Acinetobacter calcoaceticus*. A third species was an actinomycete related to *Streptomyces*. Many actinomycetes are adapted to life in soils and their mycelia-like morphology makes them well adapted to desiccation. The finding of these groups of bacteria in the buffer samples is, therefore, not surprising.

The PLFA analysis suggested the presence of potentially viable populations, but they showed many characteristics of starvation and toxicity, implying that the present population was not actively growing in situ. About 7 to 13% of the bacteria present were Gram-positive. The presence of SRB was indicated by the PLFA results, but no actinomycetes, contrary to what was indicated by 16S rRNA gene sequencing.

The electron microscopy examinations revealed either low numbers or no microorganisms in the buffer samples, in agreement with the culture results and activity measurements. The Teflon cloth from around the heater did not show any presence of a biofilm or even individual cells. However, a piece of black tape taken from the Teflon cloth appeared to have strings of cocci present, despite the high temperature of this sample (85°C). The statistical evaluation of all culture data demonstrated clearly that the water content was the variable that limited the viability of the present bacteria, and not the temperature per se. The water content below which viable bacteria could not be demonstrated on culture media was about 15%. Calculations have shown that the water activity for buffer with 15% moisture content is ~ 0.96. This is precisely at the border where most Gram negative bacteria are unable to grow, although some species such as *Pseudomonas*, SRB and *Vibrio* are still able to survive. Virtually no viable bacteria were found in the samples of buffer material from the BCE that contained 15% water or less.

The results from this study imply that, for some time after the disposal of containers with used fuel waste, the area around these containers (i.e., the buffer) would be virtually devoid of microbial activity because of the redistribution of the initial moisture (18%) as a result of the high temperatures of the waste containers. This effect would be further enhanced by the radiation emanating from the containers. Experiments are needed, therefore, to study the possibility of repopulation by microbes of this zone from non-affected areas, once conditions of moisture, temperature and radiation improve in the area surrounding the containers.

The present attention at AECL with regard to microbially influenced corrosion (MIC) effects on waste containers is focused on the effects of SRB, because of the expected reducing conditions that should develop after emplacement and the presence of sulphate in granitic groundwaters and in the clay used for buffer materials (as a gypsum impurity). If a zone of depleted microbial activity is created during the period of harsh conditions, and if repopulation of this zone is limited or prevented, SRB activity would be limited to regions outside this depleted zone. In this case the only microbial impact on the container would result from the diffusion of microbially reduced sulphur species (sulphide, thiosulphate, etc.) to the container surface. But the results from the BCE have also shown that in the buffer samples with sufficient moisture for microbial activity, the occurrence of SRB is very limited, most likely because of conditions that are not sufficiently reducing, or because of a lack of suitable organics. Therefore, SRB activity outside the affected zone around the container may be limited, which would imply that the indirect MIC effects (i.e., sulphide diffusion) would also be limited, as long as low redox conditions have not developed. The focus in the Swedish program is on a series of experiments in which the survival of SRB is studied at different swelling pressures, to determine the water activity at which SRB can no longer survive in buffer materials. Reducing the water activity by increasing the bentonite content of buffer material may be a potential approach to limiting microbial activity in the vicinity of waste containers.

In conclusion, the extensive microbial analysis of the BCE has shown that the water activity in buffer material is the dominant crucial factor for the survival and activity of a viable microbial population.

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* Internal report, available from SDDO, AECL, Chalk River Laboratories, Chalk River, Ontario K0J 1J0.

12 TABLES

Table 1 Total heterotrophic viable counts (aerobes, 30°C) in buffer mixes and buffer components.

Material	Aerobe Viable Counts (in CFU#/g)		
	PTYG Medium	PTYG(1:100) Medium	R2A Medium
Sand mesh size 70 - 140	1.8×10^1	$<1.8 \times 10^1$	$(4.4 \pm 2.4) \times 10^1$
Sand mesh size 40 - 70	$<1.9 \times 10^1$	$<1.9 \times 10^1$	$(6.9 \pm 3.5) \times 10^1$
Sand mesh size 20 - 40	$<2.1 \times 10^1$	$<2.1 \times 10^1$	$<2.0 \times 10^1$
Sand mesh size 16 - 30	$<2.1 \times 10^1$	$<2.1 \times 10^1$	$<2.1 \times 10^1$
Sand mesh size 12 - 20	$<1.9 \times 10^1$	$<1.9 \times 10^1$	$<1.9 \times 10^1$
Sand mesh size 8 - 12	7.4×10^1	$<2.0 \times 10^1$	$(1.9 \pm 0.5) \times 10^2$
Avonlea bentonite	$(6.4 \pm 0.29) \times 10^3$	$(9.1 \pm 1.1) \times 10^3$	$(3.5 \pm 0.6) \times 10^4$
Wyoming bentonite	$<1.6 \times 10^2$	$<1.1 \times 10^2$	$<1.1 \times 10^2$
URL fracture zone 2 water		$10^1 - 10^3$*	$10^1 - 10^3$*
URL tap water	$(6.7 \pm 0.6) \times 10^1$	$(3.6 \pm 0.5) \times 10^3$	$(6.7 \pm 0.2) \times 10^3$
Avonlea buffer mix (457 ISO)**	$(3.0 \pm 0.3) \times 10^4$	$(1.4 \pm 0.2) \times 10^5$	$(7.9 \pm 0.5) \times 10^6$
Avonlea buffer mix (458 BM)**	$(2.3 \pm 0.1) \times 10^4$	$(6.8 \pm 0.3) \times 10^4$	$(2.0 \pm 0.1) \times 10^6$
Wyoming buffer mix (459TR)***	$(7.8 \pm 1.5) \times 10^3$	$(3.6 \pm 0.2) \times 10^4$	$(2.2 \pm 0.2) \times 10^6$

* From Haveman et al. (1995b)

** Mixed with URL fracture zone 2 water

*** Mixed with URL tap water

CFU = Colony-forming units (average of 3 plates; detection limit 20 CFU/g) 457 ISO, 458BM and 459 TR indicate the engineering purpose for which the buffers were mixed, buffer mix (458 BM) was used in the BCE

Table 2 Location of all sample types taken

Sample Type	Identification Number	BCE Layer	Depth (m)	Position (°)	Specifics
1. back- fill	F-001-B	F	0.43	343.5	radius 0.305m
	F-003-B	F	~0	n.r.	rust
	F-007-B	F	~1.0	n.r.	underneath cell*
2. buffer/ backfill interface	F-002-IB	F	~1.0	n.r.	
	F-002-IB-F	F	~1.0	n.r.	GRAM Lab
3. buffer (metal tubes)	G-xxx-B	G	1.25	see Fig.7	8 samples
	H-xxx-B	H	1.86	see Fig.7	8 samples
	I-xxx-B	I	2.47	see Fig.7	8 samples
	J-xxx-B	J	2.63	see Fig.8	8 samples
	K-xxx-B	K	3.18	see Fig.8	8 samples
	L-xxx-B	L	3.64	see Fig.8	8 samples
	M-xxx-B	M	4.23	see Fig.8	8 samples
	N-xxx-B	N	4.79	see Fig.8	8 samples
	O-xxx-B	O	5.22	see Fig.8	8 samples
	P-xxx-B	P	5.34	see Fig.7	8 samples
	Q-xxx-B	Q	5.74	see Fig.7	8 samples
4. buffer (hollow stem auger)	H-001-AB	G+H	1.3-1.7 (74)	see Fig.7	PLFA
	K-001-AB	K	3.0-3.4 (56)	see Fig.8	
	L-001-AB	L	3.5-3.9 (70)	see Fig.8	
	M-001-AB	M	4.0-4.4 (56)	see Fig.8	PLFA
	N-001-AB-F	N	4.6-5.0 (70)	see Fig.8	GRAM Lab
	P-001-AB	P	5.2-5.6 (76)	see Fig.7	PLFA
5. sand	J-001-SB	J	2.50	0°	
	L-001-SB	L	3.15	0°	
	L-004-SB-F	L	3.15	0°	GRAM Lab
	N-001-SB	N	4.25	0°	
6. buffer/ granite interface	H-001-IB	H	1.60	n.r.	
	L-001-IB	L	~3.5	n.r.	
	L-003-IB-F	L	~3.5	n.r.	GRAM Lab
	N-001-IB	N	~4.8	n.r.	
	Q-001-IB	Q	~5.6	n.r.	
7. heater surface	HE-001-B	N	~4.8	n.r.	teflon
	HE-003-B(SEM)	N	~4.8	n.r.	teflon
	HE-004-B	N/O	~5.0	n.r.	sand**
	HE-005-B	N	~4.8	n.r.	tape***
	HE-005-B(SEM)	N	~4.8	n.r.	tape***
8. pore water inflow, sample not taken, rock dry for 10 months					
9. extra samples	SEM-001-N	N	~4.8-5.0	n.r.	thermo-couple+
	SEM-002-N	N	~4.8-5.0	n.r.	buffer++

* Underneath earth pressure cell, ** Sand scraped from bottom of heater, *** Black electrical tape attached to teflon cloth, + Corroded thermocouple segment from near buffer/granite interface, ++ Buffer fragment in which the corroded thermocouple segment was embedded , GRAM Lab:Shipped to the GRAM Laboratory in France for analysis , PLFA: Shipped to Microbial Insights Laboratory in the U.S.A. for phospholipid fatty acid analysis , n.r. Not recorded

Table 3 Moisture content and temperature results for metal tube samples.

Sample Location (+ Country)	Position (°)	T (°C)	Water Content (% of Sample) Based on Wet Weight		Based on Dry Weight		
			WL	GRAM	WL	GRAM	BCE*
G-024 (C)	0	20.64	14.90		17.62		19.81
G-100 (C)	300	20.67	15.82		18.79		19.05
G-016 (S)	240	19.91					20.17
G-097 (S)	270	19.91					18.76
G-008 (F)	120	19.84		14.3		16.7	19.85
G-092 (F)	120	19.91		16.5		19.8	18.88
H-024 (C)	0	24.31	17.03		20.52		19.46
H-100 (C)	300	24.68	14.79		17.36		18.35
H-016 (S)	240	23.38					20.09
H-097 (S)	270	24.36					18.41
H-008 (F)	120	23.41		14.5		17.0	19.56
H-092 (F)	120	24.22		13.7		15.9	18.52
I-024 (C)	0	28.71	16.95		20.41		20.93
I-100 (C)	300	32.92	13.34		15.40		17.12
I-016 (S)	240	28.91					20.69
I-097 (S)	270	33.84					16.70
I-008 (F)	120	28.99		14.7		17.2	20.65
I-092 (F)	120	33.46		11.4		12.9	16.75
J-024 (C)	0	38.9	18.37		22.51		21.17
J-072 (C)	0	54.64	15.51		18.36		20.41
J-018(Archived)	270	39.44					
J-066(Archived)	270	55.07					
J-006(Archived)	90	39.67					
J-054(Archived)	90	55.74					
K-024	0	38.9	17.63		21.41		22.24
K-072 (C)	0	54.6	11.56		13.08		13.06
K-018 (S)	270	39.44					20.47
K-066 (S)	270	55.07					12.72
K-006 (F)	90	39.67		15.5		18.4	20.55
K-054 (F)	90	55.74		8.3		9.1	12.74
L-024 (C)	0	42.62	18.72		23.04		22.55
L-072 (C)	0	59.17	0.24		11.40		13.49
L-018 (S)	270	45.18					21.45
L-066 (S)	270	56.34					12.47
L-006 (F)	90	45		15.7		18.6	21.14
L-054 (F)	90	58					12.73
M-024 (C)	0	43.60	19.11		23.62		21.94
M-072 (C)	0	60.36	11.83		13.41		14.12
M-018 (S)	270	44.13					20.61
M-066 (S)	270	61.18					12.33
M-006 (F)	90	44.40					22.19
M-054 (F)	90	60.87		10.9		12.2	13.37
N-024 (C)	0	42.68	18.31		22.41		23.00
N-072 (C)	0	58.84	12.21		13.91		13.32
N-018 (S)	270	45.49					21.48

Sample Location (+ Country)	Position (°)	T (°C)	Water Content (% of Sample) Based on Wet Weight		Based on Dry Weight		
			WL	GRAM	WL	GRAM	BCE*
N-066 (S)	270	55.34					12.61
N-006 (F)	90	45		16.2		19.3	22.21
N-054 (F)	90	58		11.0		12.4	13.80
O-024 (C)	0	38.17	18.32		22.43		21.80
O-072 (C)	0	48.55	17.96		21.89		19.16
O-018 (S)	270	37.86					20.40
O-066 (S)	270	49.07					17.53
O-006 (F)	90	37.86		17.3		20.9	22.87
O-054 (F)	90	49.07					20.64
P-024 (C)	0	37.86	16.69		20.04		21.89
P-100 (C)	0	53.96	14.36		16.77		16.89
P-016 (S)	240	37.86					20.79
P-097 (S)	270	53.96					16.44
P-008 (F)	120	37.86		16.9		20.3	21.45
P-092 (F)	120	53.96		14.2		16.6	17.49
Q-001 (C)	0	28.54	15.13		17.82		21.73
Q-100 (C)	0	32.92	16.55		19.83		19.61
Q-016 (S)	240	29.03					20.56
Q-097 (S)	270	32.89					19.06
Q-008 (F)	120	31.38					22.18
Q-092 (F)	120	32.01		11.6		13.1	19.86

* BCE results were obtained from moisture content samples taken directly above microbial tube samples in the same location. The BCE numbers are likely the most accurate because the measurements were done on a large sample size with very strict precautions. GRAM results obtained on microbial samples at GRAM Laboratory. WL results obtained on microbial samples at WL.

Table 4 Total viable heterotrophs results for metal tube samples (R2A medium, WL)

Sample Location	Sample Depth (m)	Sample Temp (°C)	Moisture Content (wt. %)	Lag Time* (h)	CFU/g Dry Material			
					Aerobes		Anaerobes	
					17°C	50°C	17°C	50°C
Samples Near Buffer/Granite Interface								
G -024-B	1.25	20.6	19.81	26.5	$(4.82\pm0.24) \times 10^5$	$(4.00\pm1.38) \times 10^2$	$(4.07\pm1.24) \times 10^3$	ND
H -024-B	1.86	24.7	19.46	22	$(8.55\pm1.12) \times 10^3$	$(4.33\pm1.48) \times 10^2$	$(3.45\pm5.92) \times 10^2$	86±149
I -024-B	2.47	28.7	20.93	21	$(1.39\pm0.82) \times 10^4$	$(2.22\pm3.48) \times 10^2$	$(2.95\pm5.11 \times 10^2$	148±129
J -024-B	2.63	38.9	21.17	12	$(5.12\pm1.28) \times 10^3$	$(2.20\pm0.97) \times 10^4$	$2.56\pm2.56) \times 10^2$	169±149
K -024-B	3.18	38.9	22.24	6.5	$(1.36\pm0.29) \times 10^3$	$(4.25\pm1.18) \times 10^4$	ND	ND
L -024-B	3.64	42.6	22.55	11.5	$(5.71\pm3.73) \times 10^2$	ND	ND	ND
M -024-B	4.23	43.6	21.94	5	$(2.42\pm0.48) \times 10^3$	$(6.80\pm4.26) \times 10^3$	ND	ND
N -024-B	4.79	42.7	23.00	< 12	$(4.15\pm0.64) \times 10^3$	$(1.69\pm1.49) \times 10^2$	ND	ND
O -024-B	5.22	38.2	21.80	6	$(7.05\pm0.65) \times 10^3$	ND	$(4.06\pm2.80) \times 10^2$	ND
P -024-B	5.34	37.9	21.89	6.5	$(4.66\pm0.53) \times 10^3$	$(2.09\pm2.08) \times 10^2$	$(1.39\pm1.21) \times 10^2$	ND
Q -001-B	5.74	28.5	21.73	7	$(3.20\pm1.10) \times 10^3$	$(8.20\pm2.55) \times 10^2$	$(2.24\pm2.24) \times 10^2$	ND
Samples Near Heater Surface								
G -100-B	1.25	20.7	19.05	6	$(7.70\pm0.38) \times 10^3$	$(7.26\pm12.9) \times 10^1$	$(1.32\pm0.22) \times 10^3$	ND
H -100-B	1.86	24.7	18.35	19	$(5.23\pm0.99) \times 10^3$	$(7.29\pm4.16) \times 10^2$	$(1.06\pm0.83) \times 10^3$	66±116
I -100-B	2.47	32.9	17.12	21	$(2.88\pm0.97) \times 10^3$	$(3.90\pm1.35) \times 10^2$	$(1.56\pm1.35) \times 10^2$	ND
J -072-B	2.63	54.6	20.41	12	$(2.95\pm0.60) \times 10^3$	$(5.78\pm0.81) \times 10^4$	$(4.54\pm3.93) \times 10^2$	ND
K-072-B	3.18	54.6	13.06	6	ND	$(1.60\pm1.41) \times 10^2$	ND	ND
L -072-B	3.64	59.2	13.49	11.5	ND	ND	ND	ND
M -072-B	4.23	60.4	14.12	5	ND	ND	ND	ND
N -072-B	4.79	58.8	13.32	< 12	ND	ND	ND	ND
O -072-B	5.22	48.6	19.16	6	$(3.07\pm0.36) \times 10^3$	$(2.61\pm0.59) \times 10^3$	ND	230±328
P -100-B	5.34	54.0	16.29	6.5	$(3.29\pm0.61) \times 10^3$	$(2.15\pm2.15) \times 10^2$	ND	72
Q -100-B	5.74	32.9	19.61	7	$(4.14\pm0.36) \times 10^3$	$(1.75\pm0.50) \times 10^3$	$(2.39\pm4.12) \times 10^2$	$(1.12\pm0.90)\times10^3$

* Time between sample retrieval and incubation

ND = Not Detected; detection limit 200 CFU/g

Table 5 Total viable heterotrophs results for metal tube samples (PCA medium, GRAM LAB)

Sample Location	Depth (m)	Temp. (°C)	Moisture Content (wt. %)	Lag * Time (d)	CFU/g Dry Material		Bacteria/g Dry Material	
					Aerobes**		Anaerobes**	
					25°C	50°C	25°C	50°C
Samples Near Buffer/Granite Interface								
G -008-B	1.25	19.84	19.85	~ 14	1.3×10^6	n.m.	2.9×10^3	n.m.
H -008-B	1.86	23.41	19.56	~ 14	3.3×10^5	n.m.	7.0×10^4	n.m.
I -008-B	2.47	28.99	20.65	~ 14	2.1×10^5	n.m.	2.9×10^4	n.m.
J	NO	SAMPLE	PROVIDED					
K -006-B	3.18	39.67	20.55	~ 14	3.8×10^3	0	3.0×10^2	0
L -006-B	3.64	45	21.14	~ 14	9.5×10^2	0	7.1×10^2	0
M -006-B	4.23	44.40	22.19	n.m.	n.m.	n.m.	n.m.	n.m.
N -006-B	4.79	45	22.21	~ 10	4.8×10^2	0	3.0×10^3	0
O -006-B	5.22	37.86	22.87	~ 10	8.0×10^4	0	3.0×10^4	0
P -008-B	5.34	37.86	21.45	~ 10	8.2×10^4	n.m.	7.2×10^3	n.m.
Q -008-B	5.74	31.38	19.86	n.m.	n.m.	n.m.	n.m.	n.m.
Samples Near Heater Surface								
G -092-B	1.25	19.91	18.88	~ 14	4.3×10^5	n.m.	3.0×10^4	n.m.
H -092-B	1.86	24.22	18.52	~ 14	7.5×10^4	n.m.	1.5×10^5	n.m.
I -092-B	8.47	33.46	16.75	~ 14	4.0×10^3	n.m.	2.8×10^2	n.m.
J	NO	SAMPLE	PROVIDED					
K -054-B	3.18	55.74	12.74	~ 14	0	0	0	0
L -054-B	3.64	58	12.73	n.m.	n.m.	n.m.	n.m.	n.m.
M -054-B	4.23	60.87	13.37	~ 10	0	0	0	0
N -054-B	4.79	58	13.80	~ 10	0	0	1.5×10^3	0
O -054-B	5.22	49.07	20.64	n.m.	n.m.	n.m.	n.m.	n.m.
P -092-B	5.34	53.96	17.49	~ 10	2.9×10^3	n.m.	2.9×10^3	n.m.
Q -092-B	5.74	32.01	19.86	~ 10	3.7×10^3	n.m.	6.8×10^3	n.m.

* Estimated time between retrieval and analysis in France at the GRAM Laboratory

** Total heterotrophic aerobic bacteria; detection limit aerobes 50/g wet wt.Results are averages of two plates containing between 30 and 300 colonies.

*** Total heterotrophic anaerobic bacteria; detection limit 5/g wet wt. Results from MPN method with 2 tubes/dilution

Table 6 Total viable heterotrophs results for backfill samples (R2A medium, WL)

Sample* Location	Sample Depth (m)	Sample Temp. (^{o}C)	Moisture Content (wt. %)	Lag Time** (h)	CFU/g Dry Material			
					Aerobes		Anaerobes	
					17^{o}C	50^{o}C	17^{o}C	50^{o}C
F -001-B	0.43	n.m.	6.74	3	(3.77±2.31) x10^{5}	(1.28± 0.28) x10^{4}	(1.83± 0.30) x 10^{3}	(4.56± 4.07) x 10^{2}
F -003-B	~ 0	n.m.	3.19	81	(2.38± 0.31) x10^{5}***	(2.07± 0.21)x10^{3}***	(6.91± 12.1) x10^{1}	ND
F -007-B	~ 1.0	n.m.	17.29	62	(5.41± 0.49) x10^{5}	(5.01± 2.28) x10^{2}	((5.0± 8.7) x10^{1})	((1.0± 0.9) x 10^{2})
F -002-IB	~ 1.0	n.m.	17.55	10	(1.07± 0.07) x10^{7}	(1.08± 0.16) x10^{4}	(7.33± 1.87) x10^{3}	((1.08± 0.27) x10^{4})

* F -001-B backfill block sample depth 0.43 m radius 0.305 m angle 343.5^{o}

F -002-1B backfill-buffer interface(scraped interface on both sides, more buffer, softer)

F -003-B rust sample, top of backfill, around edge

F -007-B backfill-buffer interface underneath earth pressure cell (visible condensation on cell)

** Lag time = time between sample retrieval and incubation

*** Molds mostly

( ) Numbers in brackets are suspect, because anaerobic incubation bags leaked

ND = Not detected; detection limit 200 CFU/g

Table 7 Total viable heterotrophs results for buffer granite interface samples (R2A medium, WL)

Sample* Location	Sample Depth (m)	Sample** Temp. (^{o}C)	Moisture Content (wt. %)	Lag Time *** (h)	CFU/g Dry Material			
					Aerobes		Anaerobes	
					17^{o}C	50^{o}C	17^{o}C	50^{o}C
H -001-IB	1.60	~ 30 - 35	18.98	8	(4.9±0.17) x 10^{5}	(1.2±0.51) x 10^{3}	(1.6±1.13) x 10^{3}	ND
L -001-IB	~ 3.5	~ 40 - 45	22.78	14	(2.15±0.56) x 10^{4}	ND	(1.59±0.37) x10^{4}	((2.58±2.23) x10^{3})
N -001-IB	~ 4.8	~ 40 - 45	21.94	5.5	(7.55±1.40) x 10^{3}	(3.97±0) x 10^{2}	(4.63±4.16) x 10^{2}	ND
Q -001-IB	~ 5.6	~ 25 - 30	19.56	4	(5.28±1.51) x 10^{3}	(1.55±1.38) x 10^{2}	(6.21±2.74) x 10^{2}	ND

* nterface samples, scraped from clay + granite where they touched

** stimated from corresponding metal tube samples taken nearby

*** ime between sample retrieval and incubation

( ) Number in brackets is suspect; anaerobic incubation bag leaked

ND = Not Detected; detection limit 200 CFU/g

Table 8 Total viable heterotrophs results for sand samples (R2A medium, WL)

Sample Location	Sample Depth (m)	Sample Temp.* (^{o}C)	Moisture Content (wt. %)	Lag Time** (h)	CFU/g Dry Material			
					Aerobes		Anaerobes	
					17^{o}C	50^{o}C	17^{o}C	50^{o}C
J -001-SB	2.50	n.m. (~ 55^{o})	0	13	((1.78±1.54) x10^{1})	(1.87±1.47) x10^{3}	ND	ND
L -001-SB	3.15 (0^{o})	n.m. (~ 75^{o})	0	15	ND	ND	ND	ND
M -001-SB	4.25 (0^{o})	n.m. (~ 75^{o})	0	~ 12	(5.59±0) x10^{1}	(4.35±2.8) x10^{1}	((1.86±3.20) x10^{1})	ND

* Not measured; estimated from temperature at heater surface and at the start of the buffer (Figure 5)

** Time between sample retrieval and incubation

( ) Numbers in brackets are not accurate; close to detection limit (20 CFU/g dry material)

ND = Not Detected; detection limit 20 CFU/g

Table 9 Total viable heterotrophs results for heater cloth - and associated samples (R2A medium, WL)

Sample Location*	Sample Depth (m)	Sample Temp. (°C)	Moisture Content (wt. %)	Lag Time** (h)	CFU/g Dry Material			
					Aerobes		Anaerobes	
					17°C	50°C	17°C	50°C
HE -001-B	~ 4.8	85	n/a	4.5	24.6***±1.9	135***±31	ND***	ND***
HE -004-B	~ 5.0	85	n.m. (est. 0)	8.5	(43±43)	(14±25)	(14±25)	ND
HE -005-B	~ 4.8	85	0	7.5	(18.5±0)	172±121	(6±11)	ND

* HE -001-B teflon from heater

HE -004-B sand from underneath heater

HE -005-B black tape + sand scraped from teflon

** Time between sample retrieval and incubation

*** CFU/cm^2 of teflon cloth; detection limit 4 CFU/cm^2

( ) Numbers in brackets are not accurate; close to detection limit (40 CFU/g)

ND = Not Detected; detection limits ~ 40 CFU/g and 4CFU/cm^2 heater cloth

Table 10 Backfill sample results (Gram Lab).

Sample Location	**F -002-IB-F**	**(buffer/backfill interface)**
Sample Depth (m)	1.0	
Sample Temp (oC)	n.m. (~ 20 - 25)	
Moisture Content (wt. %)	~ 17.6	
Microbial Analysis		CFU/g Dry Material+
	25°C	50°C
Heterotrophic Aerobes*	1.3×10^6	n.m.
Fungi (Molds)**	6.5×10^2	n.m.
		Bacteria/g Dry Material++
	25°C	50°C
Heterotrophic Anaerobes*	2.5×10^4	n.m.
SRB**	2.5	n.m.
Methanogens**	0	n.m.
Aerobic Sulphur Oxidizers**	0	n.m.
Anaerobic Sulphur Oxidizers**	1.3×10^3	

* On or in PCA medium (Appendix 4)

** For media, (Appendix 4)

\+ Average of 2 plates; detection limit 50 CFU/g wet weight

++ MPN method with 2 tubes/dilution; detection limit 5 bacteria/g wet weight

Table 11 Buffer-Granite interface sample -results (Gram Lab).

Sample Location	**L -003-IB-F**	
Sample Depth (m)	~ 3.5	
Sample Temp (oC)	~ 40 - 45	
Moisture Content (wt. %)	~ 22.8	
Microbial Analysis	CFU/g Dry Material+	
	25°C	50°C
Heterotrophic Aerobes*	1.3×10^6	0
Fungi (Molds)**	0	0
	Bacteria/g Dry Material++	
	25°C	50°C
Heterotrophic Anaerobes*	6×10^1	0
SRB**	0	0
Methanogens**	0	0
Aerobic Sulphur Oxidizers**	0	0
Anaerobic Sulphur Oxidizers	7.0×10^3	0

* PCA medium (Appendix 4)

** For media, (Appendix 4)

\+ Average of 2 plates; detection limit 50 CFU/g wet weight

++ MPN method with 2 tubes/dilution; detection limit 5 bacteria/g wet weight

Table 12 Sand sample results (Gram Lab).

Sample Location	**L -004-SB**	
Sample Depth (m)	3.15 (0o)	
Sample Temp (oC)	n.m. ~ 75 est.	
Moisture Content (wt. %)	0	
Microbial Analysis	CFU/g Dry Material+	
	25°C	50°C
Heterotrophic Aerobes*	0	0
Fungi (Molds)**	10^3***	0
	Bacteria/g Dry Material++	
	25°C	50°C
Heterotrophic Anaerobes*	0	0
SRB**	0	0
Methanogens**	0	0
Aerobic Sulphur Oxidizers**	0	0
Anaerobic Sulphur Oxidizers**	0	0

* On or in PCA medium (Appendix 4)

** For media, (Appendix 4)

*** On standard plate count agar (PCA) - (Appendix 4)

\+ Average of 2 plates; detection limit 50 CFU/g wet weight

++ MPN method with 2 tubes/dilution; detection limit 5 bacteria/g wet weight

Table 13 Hollow stem auger sample results (Gram Lab).

Sample Location	**N -001-AB**	**(layer N, near 70)**
Sample Depth (m)	~ 4.6 - 5.0	
Sample Temp (°C)	~ 58	
Moisture Content (wt. %)	~ 13	
Microbial Analysis		
	CFU/g Dry Material[+]	
	25°C	50°C
Heterotrophic Aerobes*	0	0
Fungi (Molds)**	0	0
	Bacteria/g Dry Material[++]	
	25°C	50°C
Heterotrophic Anaerobes*	6.9×10^1	0
SRB**	6.9	0
Methanogens**	0	0
Aerobic Sulphur Oxidizers**	0	0
Anaerobic Sulphur Oxidizers**	0	0

* PCA medium (Appendix 4)

** For media (Appendix 4)

[+] Average of 2 plates; detection limit 50 CFU/g wet weight

[++] MPN method with 2 tubes/dilution; detection limit 5 bacteria/g wet weight

Table 14 Results for sulphate-reducing bacteria (SRB) Methanogens and Fermenters in metal tube samples, Cultured at 50°C at WL in cooperation with U of G

Sample Location	Sample Depth (m)	Sample Temp. (°C)	Moisture Content (wt.%)	Lag Time* (h)	CFU/g Dry Material		
					SRB	Methanogens	Fermenters
Samples Near Buffer / Granite Interface							
G-024-B	1.25	20.6	19.81	26.5	97±95	149±60	<20
H-024-B	1.86	34.7	19.46	22	n.m.	n.m.	n.m.
I-024-B	2.47	28.7	20.93	21	n.m.	n.m.	n.m.
J-024-B	2.63	38.9	21.17	12	n.m.	n.m.	n.m.
K-024-B	3.18	38.9	22.24	6.5	n.m.	n.m.	n.m.
L-024-B	3.64	42.6	22.55	11.5	26±0	<20	181
M-024-B	4.23	43.6	21.94	5	n.m.	n.m.	n.m.
N-024-B	4.79	42.7	23.00	<12	390±80	52±40	207±36
O-024-B	5.22	38.2	21.80	6	n.m.	n.m.	n.m.
P-024-B	5.34	37.9	21.89	6.5	n.m.	n.m.	n.m.
Q-001-B	5.74	28.5	21.73	7	n.m.	n.m.	n.m.
Samples Near Heater Surface							
G-100-B	1.25	20.7	19.05	6	n.m.	n.m.	n.m.
H-100-B	1.86	24.7	18.35	19	98±80	74±65	428±250
I-100-B	2.47	32.9	17.12	21	48±20	<20	241±80
J-072-B	2.63	54.6	20.41	12	<20	63±30	1707±1070
K-072-B	3.18	54.6	13.06	6	<20	<20	<20
L-072-B	3.64	59.2	13.49	11.5	n.m.	n.m.	n.m.
M-072-B	4.23	60.4	14.12	5	93±40	35±30	<20
N-072-B	4.79	58.8	13.32	<12	n.m.	n.m.	n.m.
O-072-B	5.22	48.6	19.16	6	<20	49±20	791±906
P-100-B	5.34	54.0	16.89	6.5	24±0	204±30	242±279
Q-100-B	5.74	32.9	19.61	7	n.m.	n.m.	n.m.

* Time between sample retrieval and incubation; Detection limit for SRB, Methanogens and Fermenters: 20 CFU/g

Table 15 Bart test results for metal tube samples (WL)

Sample Location	Sample Depth (m)	Sample Temp. (°C)	Moisture Content (wt.%)	Lag Time* (h)	Bacteria/g Dry Material**		
					IRB	SRB	SLYM
SamplesNear Buffer / Granite Interface							
G-024-B	1.25	20.6	19.81	26.5	$8.0x10^5$	20	$6.7x10^5$
H-024-B	1.86	34.7	19.46	22	0	0	0
I-024-B	2.47	28.7	20.93	21	$8.0x10^5$	20	$5.7x10^2$
J-024-B	2.63	38.9	21.17	12	0	0	$1.4x10^3$
K-024-B	3.18	38.9	22.24	6.5	0	0	20
L-024-B	3.64	42.6	22.55	11.5	0	0	0
M-024-B	4.23	43.6	21.94	5	0	$1.3x10^4$	$2.2x10^3$
N-024-B	4.79	42.7	23.00	<12	$8.0x10^5$	20	$2.2x10^3$
O-024-B	5.22	38.2	21.80	6	$4.9x10^6$	$6.5x10^2$	0
P-024-B	5.34	37.9	21.89	6.5	$1.3x10^6$	0	$5.7x10^2$
Q-001-B	5.74	28.5	21.73	7	$4.9x10^6$	$2.7x10^2$	$1.4x10^3$
Samples Near Heater Surface							
G-100-B	1.25	20.7	19.05	6	$4.9x10^6$	20	$1.4x10^3$
H-100-B	1.86	24.7	18.35	19	0	0	$6.7x10^5$
I-100-B	2.47	32.9	17.12	21	$2.0x10^3$	0	$2.2x10^2$
J-072-B	2.63	54.6	20.41	12	0	0	$1.4x10^3$
K-072B	3.18	54.6	13.06	6	0	0	0
L-072-B	3.64	59.2	13.49	11.5	0	0	0
M-072-B	4.23	60.4	14.12	5	0	0	0
N-072-B	4.79	58.8	13.32	<12	0	0	0
O-072-B	5.22	48.6	19.16	6	$1.4x10^6$	20	$1.4x10^3$
P-100-B	5.34	54.0	16.89	6.5	0	0	$2.2x10^2$
Q-100-B	5.74	32.9	19.61	7	$8.0x10^5$	20	$3.5x10^2$

* Time between sample retrieval and incubation, ** IRB = Iron Related Bacteria (May in fact be citrate utilizing bacteria) SRB = Sulphate-Reducing Bacteria SLYM = Slime Forming Bacteria

Table 16 Results for fungi, sulphate reducing bacteria methanogenic bacteria aerobic and anaerobic sulphur oxidizing bacteria, cultured at 25°C at the GRAM Laboratory

Sample Location	Sample Depth (m)	Sample Temp. (°C)	Moisture Content (wt.%)	Lag Time* (d)	Fungi**	Bacteria/g Dry Material			
								Sulphur Oxidizing Bacteria	
						SRB***	Methanogens***	Aerobic***	Anaerobic***
SamplesNearBuffer/GraniteInterface									
G-008-B	1.25	19.84	19.85	~14	0	$1.5.X10^1$	0	0	$1.5x10^3$
H-008-B	1.86	23.41	19.56	~14	0	$7.0x10^1$	0	0	$7.0x10^4$
I-008-B	2.47	28.99	20.65	~14	0	0	0	0	$2.8x10^3$
J-	No	Sample	Provided						
K-006-B	3.18	39.67	20.55	~14	0	7.1	0	0	$3.0x10^1$
L-006-B	3.64	45	21.14	~14	0	7.1	0	0	$3.0x10^1$
M-006-B	4.23	44.40	22.19	n.m.	n.m.	n.m.	n.m.	n.m.	n.m.
N-006-B	4.79	45	22.21	~10	0	7.2	0	0	$3.0x10^1$
O-006-B	5.22	37.86	22.87	~10	0	6.0	0	0	$3.0x10^1$
P-008-B	5.34	37.86	21.45	~10	0	7.2	0	7.2	$7.2x10^4$
Q-008-B	5.74	31.38	19.86	n.m.	n.m.	n.m.	n.m.	n.m.	n.m.
SamplesNearHeaterSurface									
G-092-B	1.25	19.91	18.88	~14	0	0	7.2	0	$3.0x10^5$
H-092-B	1.86	24.22	18.52	~14	0	$2.9x10^2$	0	0	$2.9x10^5$
I-092-B	2.47	33.46	16.75	~14	0	0	0	0	$2.8x10^3$
J-	No	Sample	Provided						
K-054-B	3.18	55.74	12.74	~14	0	0	0	0	0
L-054-B	3.64	58	12.73	n.m.	n.m.	n.m.	n.m.	n.m.	n.m.
M-054-B	4.23	60.87	13.37	~10	0	0	0	0	0
N-054-B	4.79	58	13.80	~10	0	0	0	0	0
O-054-B	5.22	49.07	20.64	n.m.	n.m.	n.m.	n.m.	n.m.	n.m.
P-092-B	5.34	53.96	17.49	~10	0	0	0	0	$2.9x10^2$
Q-092-B	5.74	32.01	19.86	~10	0	0	0	0	$6.8x10^2$

*Estimated time between samplere trievaland analysisin France **Detection limit 10/g wet weight ***Detection limit 5/g wet weight (average of 2 plates) (MPN method using 2 tubes / dilution)

Table 17 Results for fungi, sulphate reducing bacteria methanogenic bacteria aerobic and anaerobic sulphur oxidizing bacteria, cultured at 50°C at the GRAM Laboratory

Sample Location	Sample Depth (m)	Sample Temp. (°C)	Moisture Content (wt.%)	Lag Time* (d)	Fungi**	Bacteria /g Dry Material			
								Sulphur Oxidizing Bacteria	
						SRB***	Methanogens***	Aerobic***	Anaerobic***
Samples Near Buffer / Granite Interface									
G-008-B	1.25	19.84	19.85	~14	n.m.	n.m.	n.m.	n.m.	n.m.
H-008-B	1.86	23.41	19.56	~14	n.m.	n.m.	n.m.	n.m.	n.m.
I-008-B	2.47	28.99	20.65	~14	n.m.	n.m.	n.m.	n.m.	n.m.
J-	No	Sample	Provided						
K-006-B	3.18	39.67	20.55	~14	0	0	0	0	0
L-006-B	3.64	45	21.14	~14	0	0	0	0	0
M-006-B	4.23	44.40	22.19	n.m.	n.m.	n.m.	n.m.	n.m.	n.m.
N-006-B	4.79	45	22.21	~10	0	0	0	0	0
O-006-B	5.22	37.86	22.87	~10	0	0	0	0	0
P-008-B	5.34	37.86	21.45	~10	n.m.	n.m.	n.m.	n.m.	n.m.
Q-008-B	5.74	31.38	19.86	n.m.	n.m.	n.m.	n.m.	n.m.	n.m.
Samples Near Heater Surface									
G-092-B	1.25	19.91	18.88	~14	n.m.	n.m.	n.m.	n.m.	n.m.
H-092-B	1.86	24.22	18.52	~14	n.m.	n.m.	n.m.	n.m.	n.m.
I-092-B	2.47	33.46	16.75	~14	n.m.	n.m.	n.m.	n.m.	n.m.
J-	No	Sample	Provided						
K-054-B	3.18	55.74	12.74	~14	0	0	0	0	0
L-054-B	3.64	58	12.73	n.m.	n.m.	n.m.	n.m.	n.m.	n.m.
M-054-B	4.23	60.87	13.37	~10	0	0	0	0	0
N-054-B	4.79	58	13.80	~10	0	0	0	0	0
O-054-B	5.22	49.07	20.64	n.m.	n.m.	n.m.	n.m.	n.m.	n.m.
P-092-B	5.34	53.96	17.49	~10	n.m.	n.m.	n.m.	n.m.	n.m.
Q-092-B	5.74	32.01	19.86	~10	n.m.	n.m.	n.m.	n.m.	n.m.

* Estimated time between sample retrieval and analysis in France, ** Detection limit 50/g wet weight, ** Detection limit 5/g wet weight (average of 2 plates) (MPN method with 2 tubes/dilution)

Table 18 Bart test results fpr buffer/granite interface samples sand samples backfill samples and heater cloth samples analysed at WL

Sample Location	Sample Depth (m)	Sample Temp. (°C)	Moisture Content (wt.%)	Lag Time* (h)	Bacterial /g Dry Material**		
					IRB	SRB	SLYM
Buffer/Granite Interface Samples							
H-001-IB	1.60	~30-35	18.98	8	0	0	$3.5x10^2$
L-001-IB	~3.5	~40-45	22.78	14	$8.0x10^5$	20	$2.2x10^3$
N-001-IB	~4.8	~40-45	21.94	5.5	$4.9x10^6$	20	$5.7x10^2$
Q-001-IB	~5.6	~40-45	19.56	4	$4.9x10^6$	20	$1.5x10^4$
Sand Samples							
J-001-SB	2.50	~55	0	13	0	0	$2.2x10^3$
L-001-SB	3.15	~75	0	15	0	0	0
N-001-SB	4.25	~75	0	~12	0	0	0
Backfill Samples							
F-001-B	0.43	~20	6.74	3	$8.0x10^5$	$3.7x10^3$	$1.9x10^6$
F-003-B	~0	~20	3.19	81	$2.6x10^2$	$3.7x10^3$	$1.9x10^6$
F-007-B	~1.0	~20	17.29	62	$3.3x10^5$	$6.5x10^2$	$1.4x10^3$
F-002-IB	~1.0	~20	17.55	10	$4.9x10^6$	20	$2.2x10^3$
Heater Cloth and Associated Samples							
HE-001-B	~4.8	~85	n/a	4.5	0	0	0
HE-004-B	~5.0	~85	n.m.(est0)	8.5	0	0	0
HE-005-B	~4.8	~85	0	7.5	0	0	0

* Time between sample retrieval and incubation; ** IRB = iron-related bacteria (may in fact be citrate utilizing bacteria); SRB = Sulphate-reducing bacteria; SLYM = Slime forming bacteria

Table 19 Microbial activity measured in metal tube samples as anaerobic ^{3}H leucine assimilation at 50^{O}C at WL in cooperation with University of Göteborg

SampleLocation	Sample Depth (m)	Sample Temp. (^{o}C)	Moisture Content (wt.%)	Lag Time*	10^{14}xMole^{3}H-Leucine Assimilated per g dry weight per h
Samples Near Buffer / Granite Interface					
G-024-B	1.25	20.6	19.81	29.5	0
H-024-B	1.86	34.7	19.46	25	0.24
I-024-B	2.47	28.7	20.93	24	3.39
J-024-B	2.63	38.9	21.17	15	2.46
K-024-B	3.18	38.9	22.24	9.5	1.93
L-024-B	3.64	42.6	22.55	14.5	0.61
M-024-B	4.23	43.6	21.94	8	0
N-024-B	4.79	42.7	23.00	<15	n.m.
O-024-B	5.22	38.2	21.80	9	n.m.
P-024-B	5.34	37.9	21.89	9.5	n.m.
Q-001-B	5.74	28.5	21.73	10	n.m.
Samples Near Heater Surface					
G-100-B	1.25	20.7	19.05	9	0
H-100-B	1.86	24.7	18.35	22	0
I-100-B	2.47	32.9	17.12	24	5.61
J-072-B	2.63	54.6	20.41	15	2.16
K-072-B	3.18	54.6	13.06	9	0.09
L-072-B	3.64	59.2	13.49	14.5	0
M-072B	4.23	60.4	14.12	8	1.33
N-072-B	4.79	58.8	13.32	<15	n.m.
O-072-B	5.22	48.6	19.16	9	n.m.
P-100-B	5.34	54.0	16.89	9.5	n.m.
Q-100-B	5.74	32.9	19.61	10	n.m.

* Estimated time between sample retrieval and incubation

Table 20 Bacterial mineralization of D(U-^{14}C) - Glucose in metal tube samples at different temperatures as indicated by $^{14}CO_2$ production (nmol CO2/L/day)*(GRAM)

Sample Location	Sample Depth (m)	Sample Temp. (^{o}C)	Moisture Content (wt.%)	IncubationTemperature (^{o}C) 20	40	60
Aerobic Incubation: Samples Near Buffer / Granite Interface				$^{14}CO_2$ Production (nmol / CO_2 / L / Day)		
G-008-B	1.25	19.84	19.85	1317	94.5	21
K-006-B	3.18	39.67	20.55	261	128	16
N-006-B	4.79	45	22.21	857	482	100.5
Samples Near Heater Surface						
K-054-B	3.18	55.74	12.74	9	22	22.5
Q-092-B	5.74	32.01	19.86	23	179	18
Anaerobic Incubation: Samples Near Buffer / Granite Interface						
G-008-B	1.25	19.84	19.85	952	106	19
K-006-B	3.18	39.67	20.55	127	92	10
N-006-B	4.79	45	22.21	n.m.	n.m.	n.m.
Samples Near Heater Surface						
K-054-B	3.18	55.74	12.74	7.5	15.5	16.5
Q-092-B	5.74	32.01	19.86	n.m.	n.m.	n.m.

* Measured in 15 mL (1/10 wet clay weight/volume) slurries D-(U-^{14}C) - glucose (specific activity 10.6 GBq / mmol) added to a concentration of 7 mg C/L

n.m. = Not measured

Table 21 Bacterial mineralization of ^{14}C amino acids mixture in metal tube samples at different temperatures as indicated by $^{14}CO_2$ production (nmol CO_2/L/day)* (GRAM)

Sample Location	Sample Depth (m)	Sample Temp. (^{o}C)	Moisture Content (wt.%)	IncubationTemperature (^{o}C)		
				20	40	60
Aerobic Incubation: Samples Near Buffer / Granite Interface				$^{14}CO_2$ Production (nmol / CO_2/ L/ Day)		
G-008-B	1.25	19.84	19.85	53	6.7	0.1
K-006-B	3.18	39.67	20.55	13	17	0
N-006-B	4.79	45	22.21	6.1	2.9	3.5
Samples Near Heater Surface						
K-054-B	3.18	55.74	12.74	0.1	0.3	0.7
Q-092-B	5.74	32.01	19.86	2.1	16.5	3.2
Anaerobic Incubation: Samples Near Buffer / Granite Interface						
G-008-B	1.25	19.84	19.85	48.5	5.8	0.1
K-006-B	3.18	39.67	20.55	18	10.2	0
N-006-B	4.79	45	22.21	n.m.	n.m.	n.m.
Samples Near Heater Surface						
K-054-B	3.18	55.74	12.74	0	0.5	0.6
Q-092-B	5.74	32.01	19.86	n.m.	n.m.	n.m.

* Measured in 15 mL (1/10 wet clay weight/volume) slurries ^{14}C - amino acid mixture (specific activity 1.92 GBq/mg) added to a concentration of 5 mg C/L
n.m. = Not measured

Table 22 Genera and species identified in metal tube samples at the GRAM Laboratory.

Backfill-Buffer Interface Sample

F -002-IB-F :

- Heterotrophic aerobic bacteria :10 strains = *Pseudomonas stutzeri*
- None specialized heterotrophic anaerobic bacteria : presence of two species of *Clostridium*
- SRB : presence of *Desulfovibrio*
- Sulphur-oxidizing bacteria : presence of *Thiobacillus denitrificans*

Granite-Buffer Interface Sample

L -003-IB-F :

- Heterotrophic aerobic bacteria : 9 strains = *Pseudomonas stutzeri* 1 strain = *Nocardia*
- Sulphur-oxidizing bacteria : presence of *Thiobacillus denitrificans*

Hollow Stem Auger Sample

N -001-AB-F :

- SRB : presence of two species of *Desulfovibrio*

Metal Tube Samples

G -008-B :

- Heterotrophic aerobic bacteria :
  6 strains = *Cellulomonas* (?)
- 4 strains = *Bacillus*
- None specialized heterotrophic anaerobic bacteria : presence of *Clostridium*
- SRB : presence of *Desulfomonas*
- Sulphur-oxidizing bacteria : presence of *Thiobacillus denitrificans*

G -092-B :

- Heterotrophic aerobic bacteria :
  6 strains = *Curtobacterium albidum*
  2 strains = *Pseudomonas stutzeri*
  1 strain = *Bacillus*
  1 strain = *Cellulomonas (?)*
- None specialized heterotrophic anaerobic bacteria : presence of *Clostridium*
- Sulphur-oxidizing bacteria : presence of *Thiobacillus denitrificans*
- Methanogenic bacteria : are present but in numbers too low to make identification possible

H -008-B :

- Heterotrophic aerobic bacteria :
  6 strains = *Pseudomonas stutzeri*
  2 strains = *Curtobacterium albidum*
  1 strain = *Bacillus*
- None specialized heterotrophic anaerobic bacteria : presence of *Clostridium*
- SRB : presence of two species of *Desulfotomaculum* and a few *Desulfovibrio*
- Sulphur-oxidizing bacteria : presence of *Thiobacillus denitrificans*

H -092-B :

- Heterotrophic aerobic bacteria :
  5 strains = *Pseudomonas stutzeri*
  4 strains = *Bacillus*
  1 strain = *Bradyrhyzobium japonicum*
- SRB : presence of *Desulfovibrio*
- Sulphur-oxidizing bacteria : presence of *Thiobacillus denitrificans*

I -008-B :

- Heterotrophic aerobic bacteria :
  6 strains = *Pseudomonas stutzeri*
  2 strains = *Bacillus*
  2 strains = *Cellulomonas* (?)
- Sulphur-oxidizing bacteria : presence of *Thiobacillus denitrificans*

I -092-B :

- Heterotrophic aerobic bacteria :
  6 strains = *Bacillus*
  4 strains = *Cellulomonas* (?)
- Sulphur-oxidizing bacteria : presence of *Thiobacillus denitrificans*

K -006-B :

- Heterotrophic aerobic bacteria :
  7 strains = *Bradyrhyzobium japonicum*
  2 strains = *Cellulomonas* (?)
  1 strain = *Bacillus*
- SRB : presence of *Desulfovibrio*
- Sulphur-oxidizing bacteria : presence of *Thiobacillus denitrificans*

L -006-B

- Heterotrophic aerobic bacteria :
  5 strains = *Bacillus*
  4 strains = *Nocardia*
  1 strain = Actinomycete (probably Nocardioform)
- None specialized heterotrophic anaerobic bacteria : presence of *Clostridium*
- SRB : presence of two species of *Desulfotomaculum*
- Sulphur- oxidizing bacteria : presence of *Thiobacillus denitrificans*

N -006-B :

- Heterotrophic aerobic bacteria :
  5 strains = *Bacillus*
  3 strains = *Actinomycete* (probably Nocardioform)
  2 strains = *Acinetobacter (Iwoffii?)*
  1 strain = *Curtobacterium flaccumfaciens* ?
- None specialized heterotrophic anaerobic bacteria : presence of *Clostridium*
- SRB : presence of *Desulfotomaculum*
- Sulphur-oxidizing bacteria : presence of *Thiobacillus denitrificans*

O -006B :

- Heterotrophic aerobic bacteria:
  9 strains = *Pseudomonas stutzeri*
  1 strain = *Bacillus*
- None specialized heterotrophic anaerobic bacteria : presence of *Clostridium*

- SRB : presence of *Desulfotomaculum*
- Sulphur-oxidizing bacteria : presence of *Thiobacillus denitrificans*

P -008-B :

- Heterotrophic aerobic bacteria :
  9 strains = *Pseudomonas stutzeri*
  1 strain = *Bacillus*
- SRB : presence of *Desulfotomaculum* and two species of *Desulfovibrio*
- Sulphur-oxidizing bacteria : presence of *Thiobacillus denitrificans* and *Thiobacillus thiooxydans*

P -092-B :

- Heterotrophic aerobic bacteria :
  8 strains = *Bacillus*
  2 strains = *Cellulomonas*
- Sulphur-oxidizing bacteria : presence of *Thiobacillus denitrificans*

Q -092-B :

- Heterotrophic aerobic bacteria :
  10 strains = *Bacillus*
- Sulphur-oxidizing bacteria : presence of *Thiobacillus denitrificans*

Table 23 The numbers of indentical clones shared between the metal tube samples H-97-B, M-18-B AND P-16-B.

	H-97-B	M-18-B	P-16-B
H-97-B	23	11	12
M-18-B		22	12
P-16-B			22

Table 24 Summary of PLFA analysis results*.

Sample	**P-001-AB**	**M-001-AB**	**H-001-AB**	**Lab. Blanc**
Sample Weight	**75.37 g**	**75.39 g**	**75.49 g**	**NA**
Water Content	**15.84%**	**11.08%**	**14.64%**	**NC**
Dry Weight	**63.43 g**	**67.04 g**	**64.44 g**	**NC**
Total pmoles of PLFA	**1,861**	**2,211**	**405**	**35**
Data Summary Sheet				
Biomass:				
PLFA/gram of dry soil	29.3	33.0	6.3	
Cells/gram of dry soil	1.73×10^6	1.95×10^6	3.71×10^5	
Nutritional Status:				
cy 17:0/16:1w7c	1.3	0.4	0.9	
cy 19:0/18:1w7c	2.5	0.3	1.1	
16:1w7t/16:1w7c	ND	ND	ND	
18:1w7t/18:1w7c	0.2	NC	NC	
nmoles PHA	ND	0.6	ND	
PHA/PLFA	NC	0.28	NC	
Community Structure:				
TerBr Sats	12.6	7.2	8.9	13.4
Monos	38.7	11.6	30.9	11.5
Polys	9.7	20.9	15.4	11.3
BrMonos	1.3	0.0	0.0	0.0
MidBrSats	9.2	2.2	4.7	0.0
NSats	28.6	58.1	40.1	63.8

* For complete results see Table A-6-1 (Appendix 6)

Table 25 Results from an analysis of variance of effects hypothesized to influence the CFU of the buffer masses sampled.

Source	Degrees of Freedom	Type III Sum of Squares (SS)	Mean Square (MS)	F-Test	F	F.99	Effect is Significant at the 99%Level
T2	3	54.05	18.02	MS[W2(T2)] / MS[T2]	1.57	28.2	No
W2(T2)	5	141.51	28.30	MS[W2(T2)] / MS[Residual]	19.65	3.78	Yes
T3*ox* medium *lab	10	383.77	38.38	MS[T3*ox* medium*lab] / MS[Residual]	26.65	2.32	Yes
Residual	375	539.12	1.44				

The total number of observations was 394, distributed over 4 temperature classes, 4 water content classes, 2 culturing temperature classes, aerobic or anaerobic culturing conditions, 4 different media and 3 laboratories. T2 is the effect from the insitu sample temperature, w2(t2) is the effect from the water content of the sample nested within the in situ sample temperature, t3*ox*medium*lab is the crossed (interacting) effect from culturing temperature, oxygen condition at culturing, the medium used and the laboratory doing the analysis.

Table 26 Results from the variance component analysis of the effects hypothesized to influence the CFU of the buffer masses sampled.

Source	Type III Expected Mean Square Value	Variance Component	Estimate	Part of Total Variance Component Estimate (%)
T2	Var(Residual) + 85.307 Var(T2) + Q(W2(T2))	Var(T2)	0.174	4.9
W2(T2)	Var(Residual) + Q(W2(T2))	Var (W2(T2))	1.160	32.4
T3*ox* medium* lab	Var(Residual)+ 33.744 Var(T3*ox*medium*T)	Var (T3*ox*medium *lab)	1.158	32.4
Residual			1.102	30.8

Numbers of observations and symbols as in Table 25. The effect from each variable on the CFU numbers is reflected by the variance component estimates

Table 27 The mean values of CFU distributed over the four class levels of in situ temperatures used for the statistical analysis N is the number of observations at each level.

Level of T2, C^o	N	10log(CFU gdw-1)	SD
25	109	2.76	1.91
35	98	2.41	1.62
45	71	1.68	1.48
55	116	0.94	1.51

Table 28 The mean values of CFU distributed over the different class levels of water contents nested within the in situ itu temperatures used for the statistical analysis N is the number of observations at each level.

T,C^o	Level of W2, % Water gdw^{-1}	N	$^{10}log(CFU\ gdw^{-1})$	SD
25	16.5	48	2.67	1.68
25	19.5	61	2.83	2.08
35	16.5	23	1.95	1.48
35	19.5	30	2.32	1.47
35	22.5	45	2.71	1.76
45	22.5	71	1.68	1.48
55	13.5	75	0.17	0.62
55	16.5	20	2.02	1.37
55	19.5	21	2.67	1.83

Table 29 The mean values of CFU distributed over the different class levels of laboratory, medium oxygen conditions and the medium used for the statistical analysis N. Is the number of observations at each level.

Level of						
Lab.	**Medium**	**Ox**	**T3, C^o**	**N**	**^{10}log(CFU gdw^{-1})**	**SD**
WL, Canada	Heterotrophic	Aerobic	20	72	3.13	1.57
WL, Canada	Heterotrophic	Aerobic	50	72	2.14	1.73
WL, Canada	Heterotrophic	Anaerobic	20	66	1.12	1.43
WL, Canada	Heterotrophic	Anaerobic	50	43	0.28	0.78
GRAM, France	Heterotrophic	Aerobic	20	32	3.44	2.09
GRAM, France	Heterotrophic	Anaerobic	20	16	3.26	1.49
GRAM, France	Sulphur reducers	Anaerobic	20	16	0.60	0.75
GRAM, France	Sulphur reducers	Anaerobic	20	16	2.68	1.94
U of G, Sweden	Fermenters	Anaerobic	50	20	1.78	1.27
U of G, Sweden	Methanogenes	Anaerobic	50	21	1.12	1.03
U of G, Sweden	Sulphate reducers	Anaerobic	50	20	1.30	0.99

Table 30 Water activity of several materials with some microorganisms growing at that water activity in comparison with NA-BENTONITE, MX-80 with different water contents.

Water Activity	Material	Some Organisms Growing at Stated Water Activity
1.000	Pure Water	*Caulobacter, Spirillum*
0.995	Human Blood	*Streptococcus, Escherichia*
0.990	Ground Water (500 m)	*Bacillus*, SRB, *Pseudomonas*
0.980	Sea Water	
0.960	MX-80, 25% Water	*Pseudomonas*, SRB, *Vibrio*
0.950	Bread	Most gram-positive rods
0.920	MX-80, 20% Water	
0.900	Maple Syrup, Ham	Gram-positive cocci
0.850	Salami	*Saccharomyces rouxii* (yeast)
0.800	Fruit Cake, Jams	*Saccharomyces bailii, Penicillium* (Fungi)
0.780	MX-80, 15% Water (start)	
0.750	Salt Lake, Salt Fish	*Halobacterium, Halococcus*
0.700	Cereals, Dried Fish	Xerophilic fungi

Table 31 The amount of leucine assimilated by bacteria in the BMC buffer mass test and in groundwater and on surfaces exposed to flowing groundwater in Swedish granitic rock at the Stripa research mine (Pedersen and Ekendahl 1992a) and the Äspö Hard Rock Laboratory environments(Pedersen and Ekendahl 1992b).

Environment	10^{14} Mole Leucine Per g dw	10^{14} Mole Leucine Per ml	10^{14} Mole Leucine Per cm^2
BMC	1 - 7	-	-
Stripa	-	1 - 5	160 - 280
Äspö	-	3 - 66	16 - 150

13 FIGURES

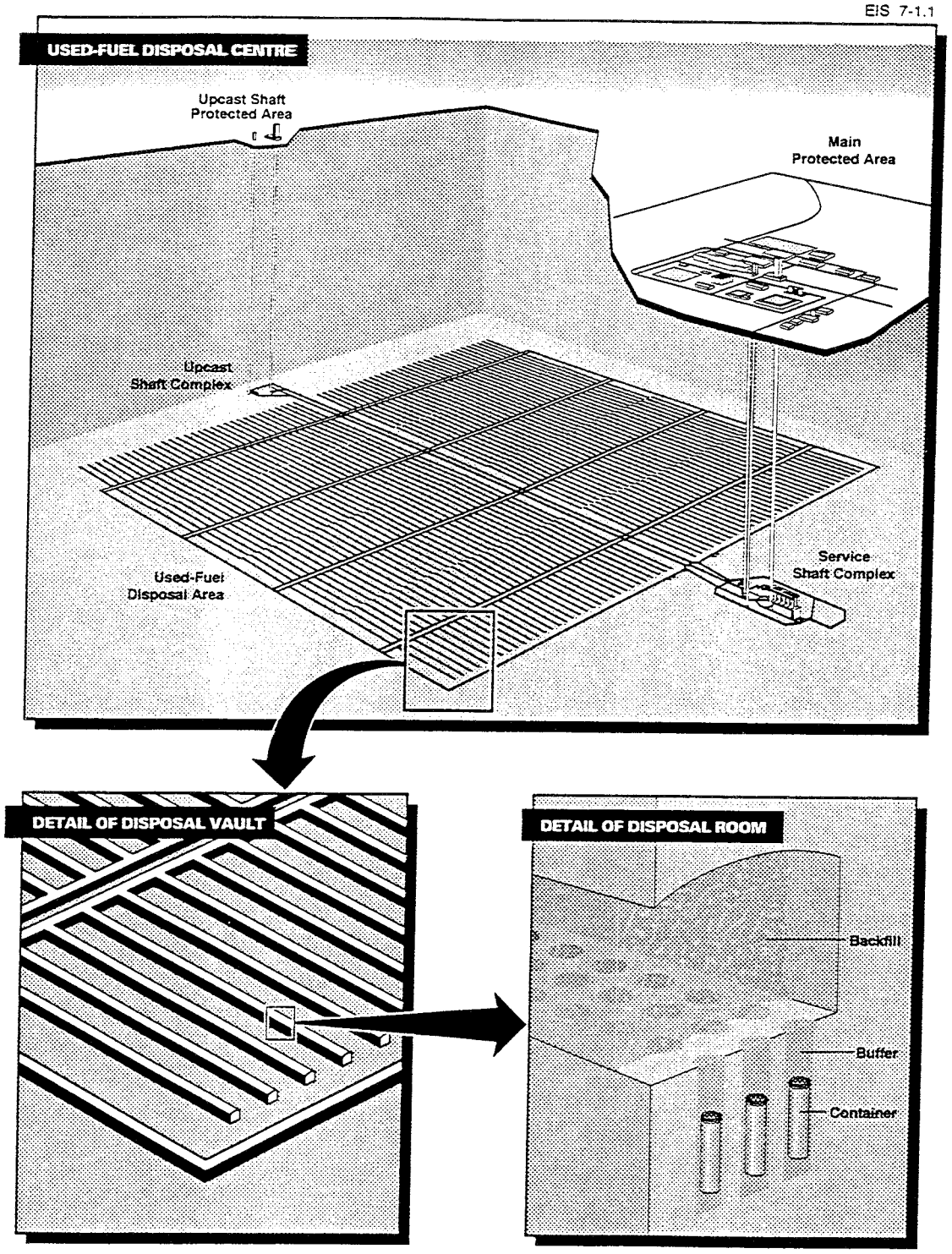


Figure 1 *Schematic representation of a nuclear fuel waste disposal facility with surface and underground structures. Enlarged insert shows a section through an emplacement room with containers emplaced boreholes.*

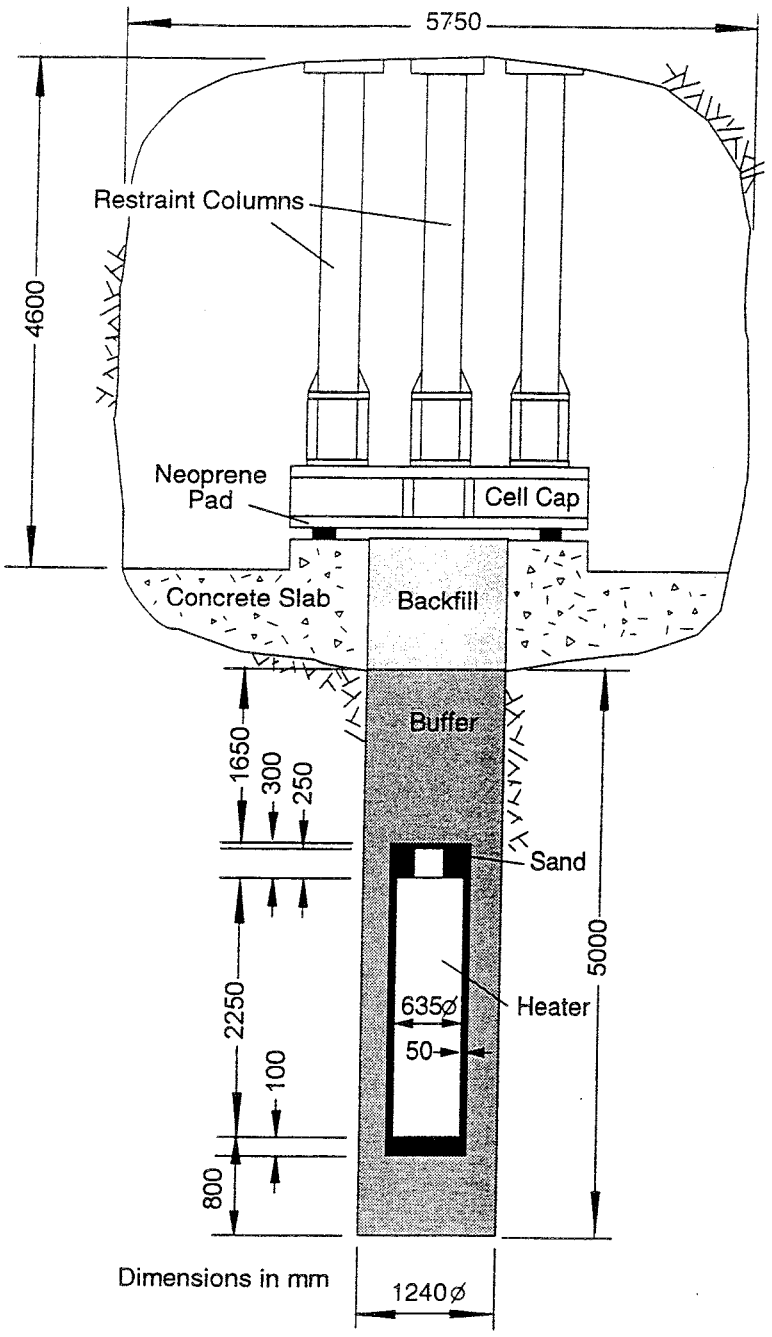


Figure 2 *Schematic representation of the Buffer-Container Experiment (BCE) at the URL.*

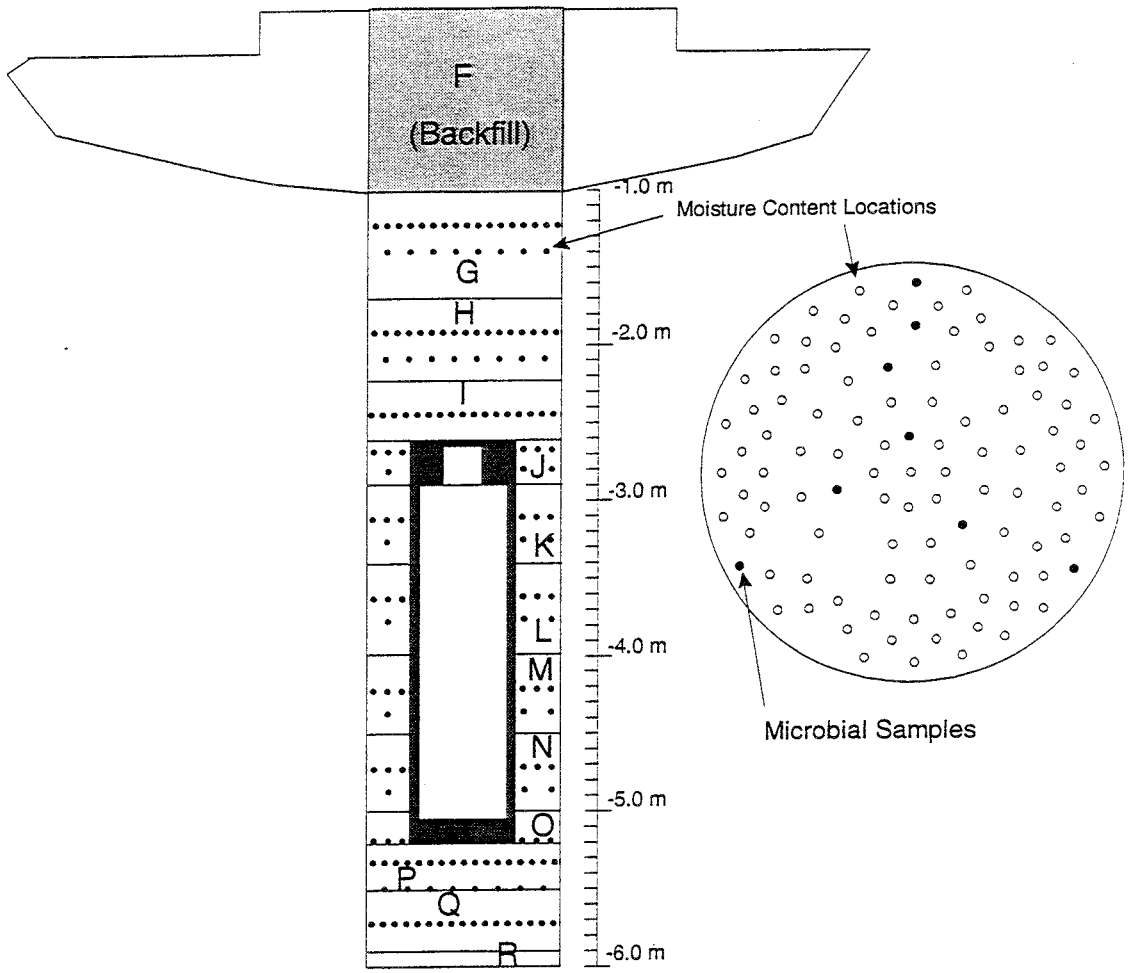


Figure 3 *Schematic cross section of the BCE showing the layers sampled.*

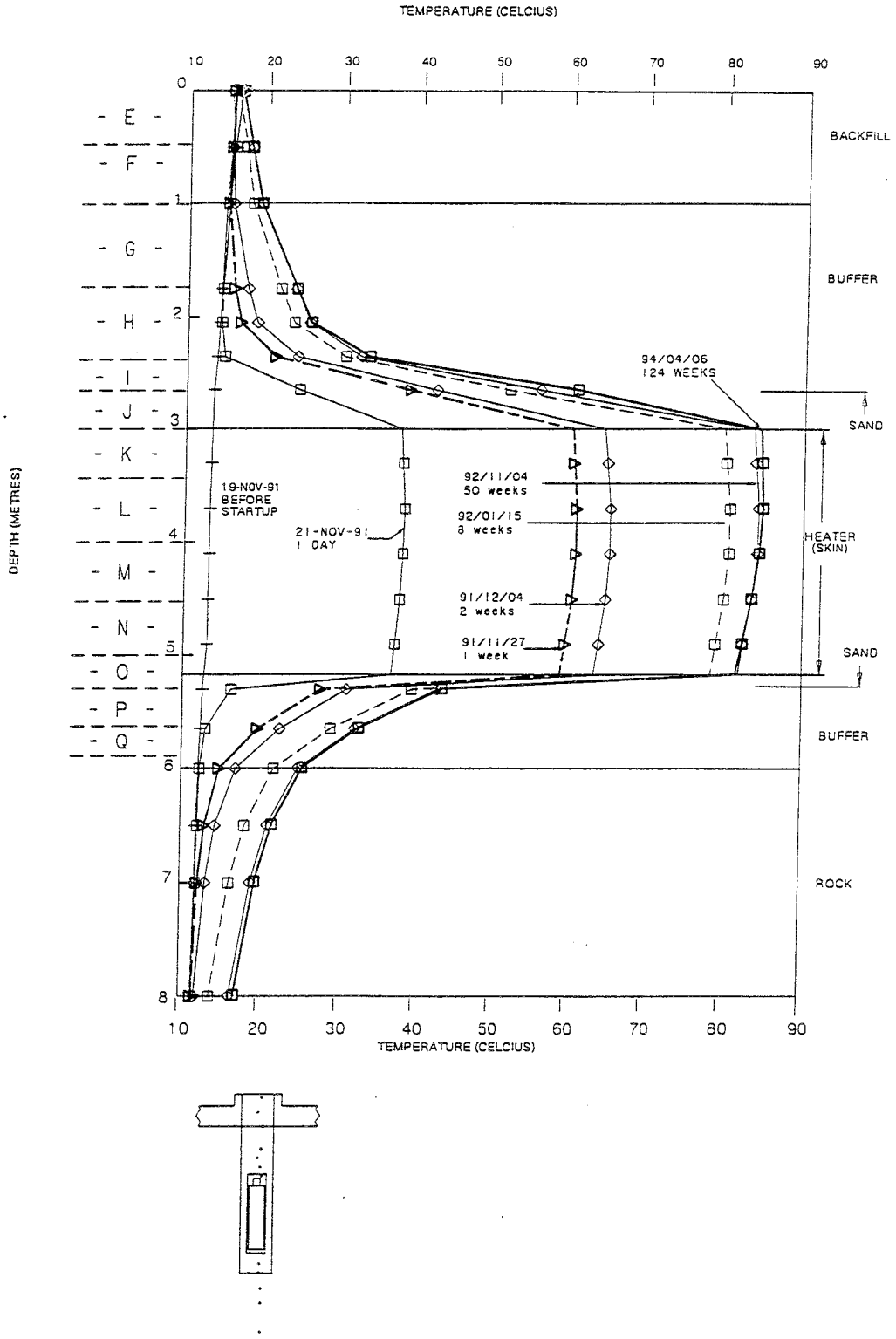


Figure 4 *Vertical temperature profile through the emplacement borehole centre.*

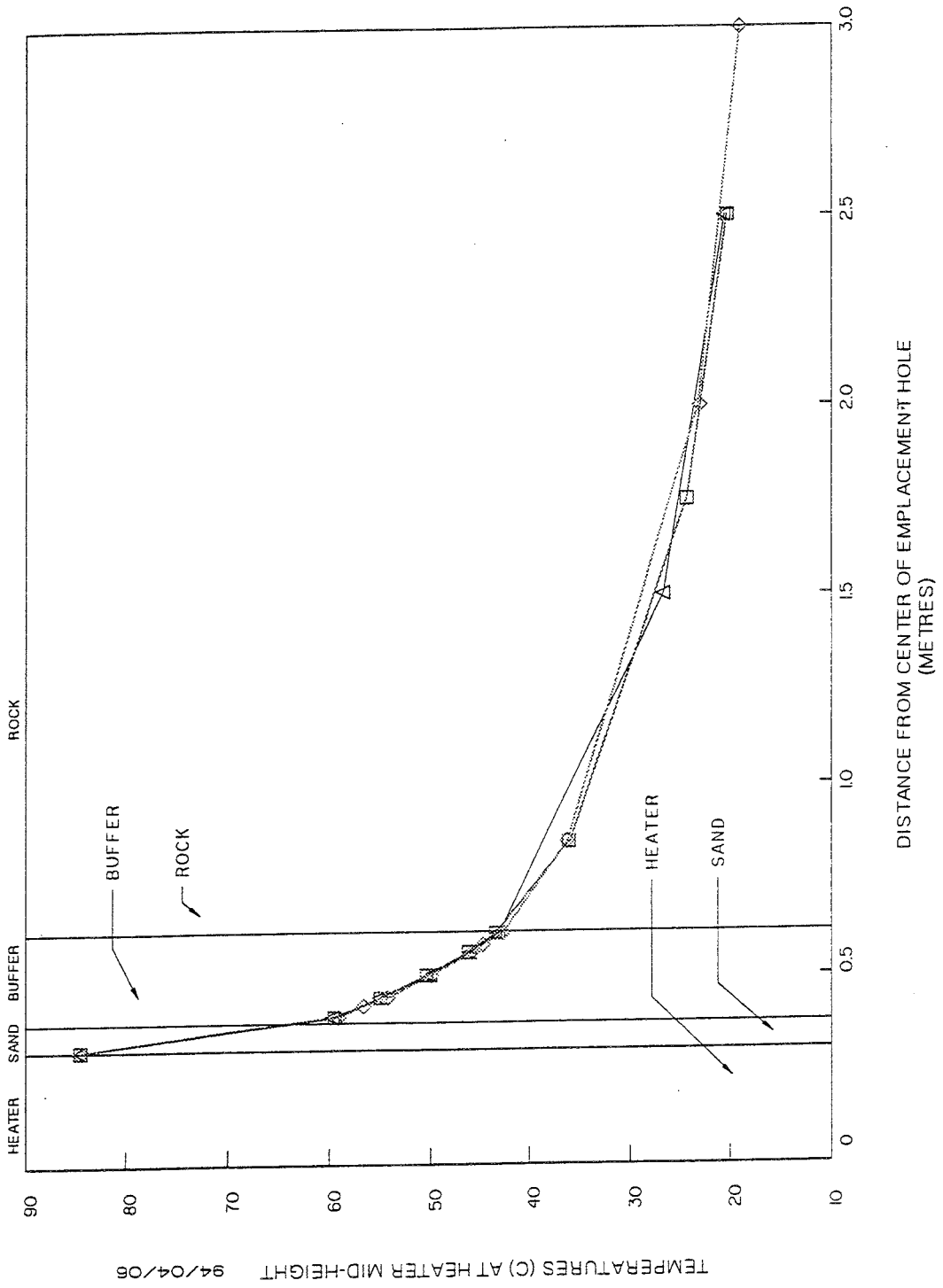


Figure 5 *Temperature t heater mid-height as a function of distance from the emplacement borehole centre.*

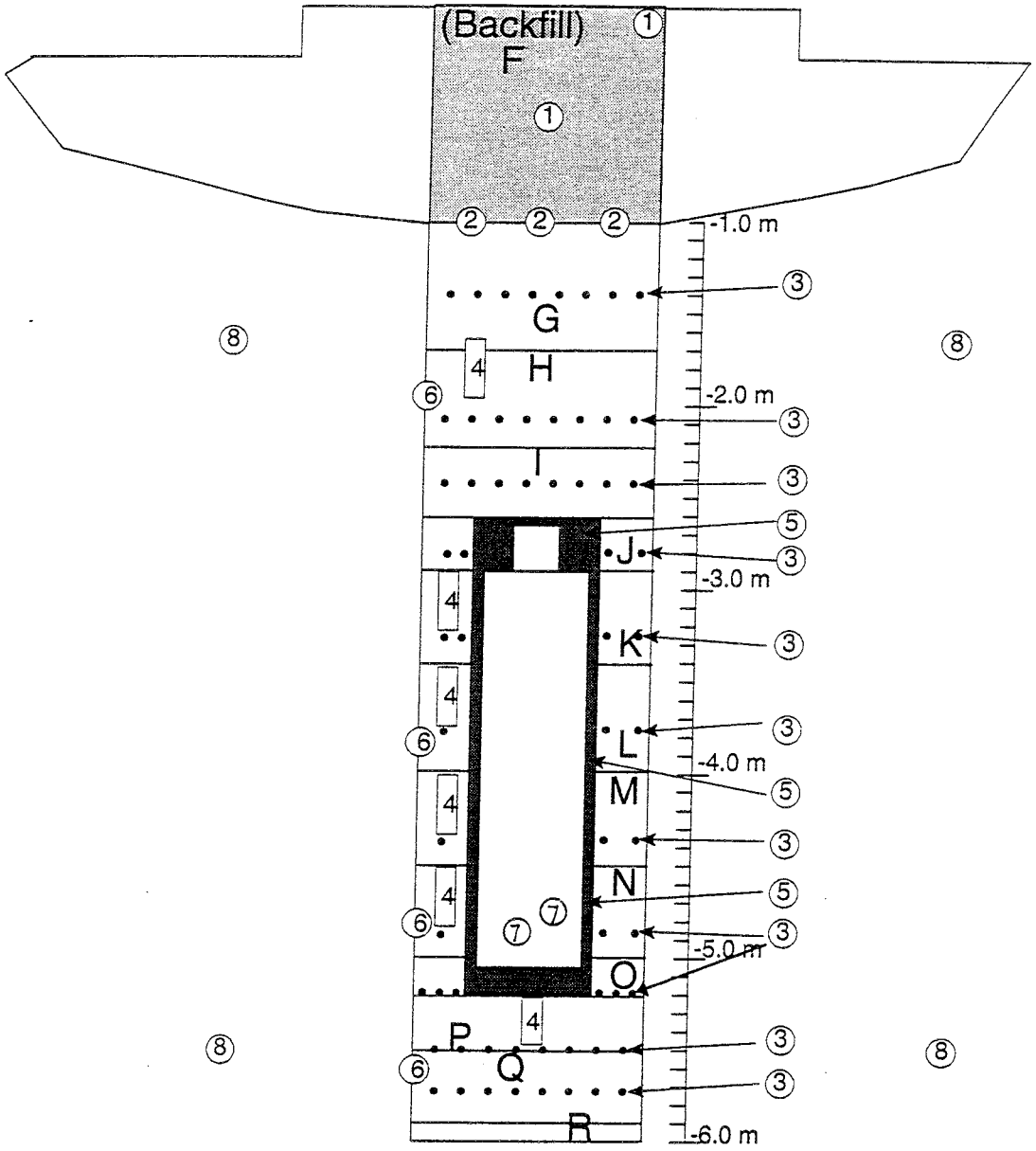


Figure 6 *Schematic cross section of the BCE showing the layers sampled and the locations of the different types of microbiology samples.*

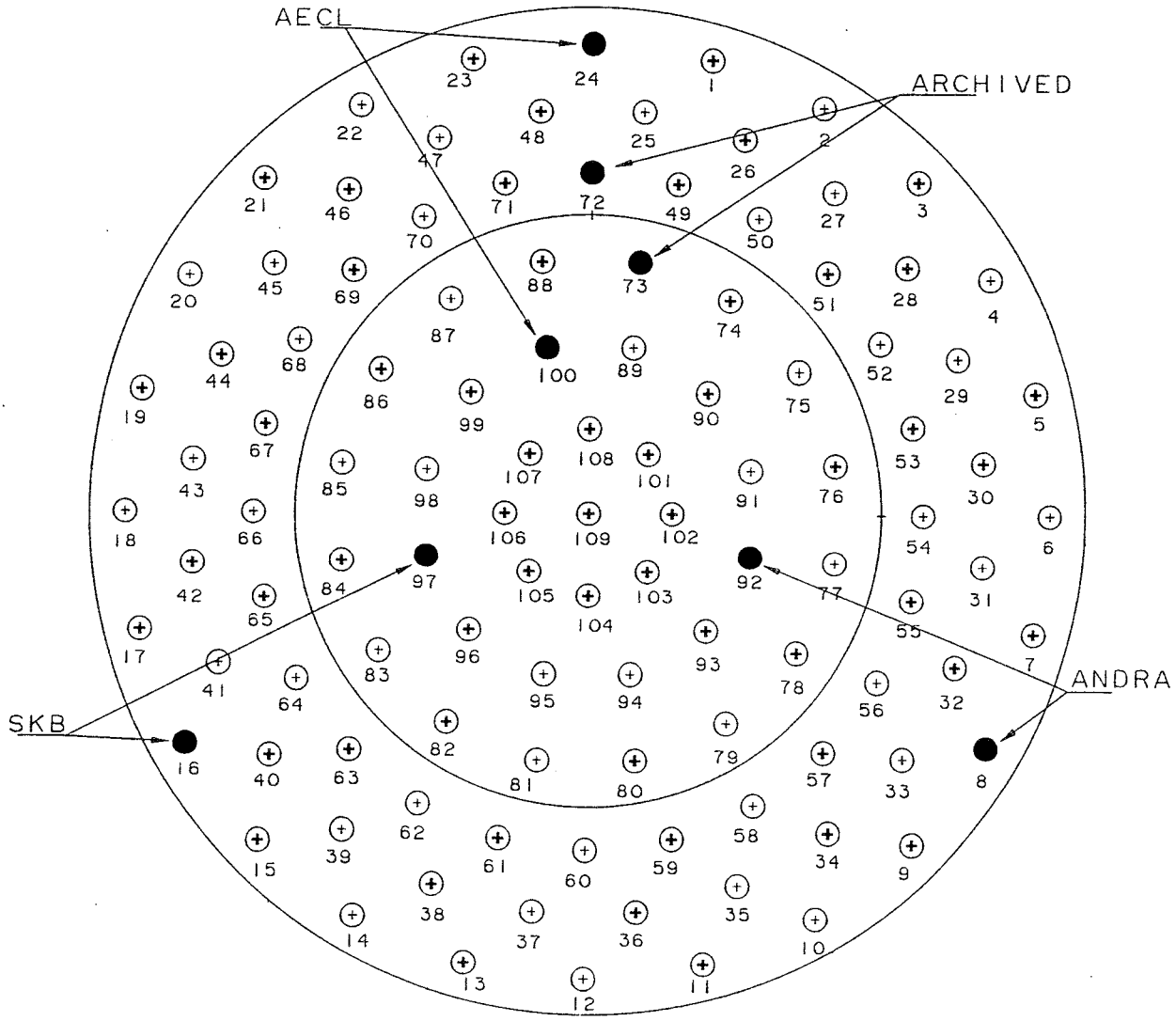


Figure 7 *Location of the metal tube samples in the layers above and below the heater.*

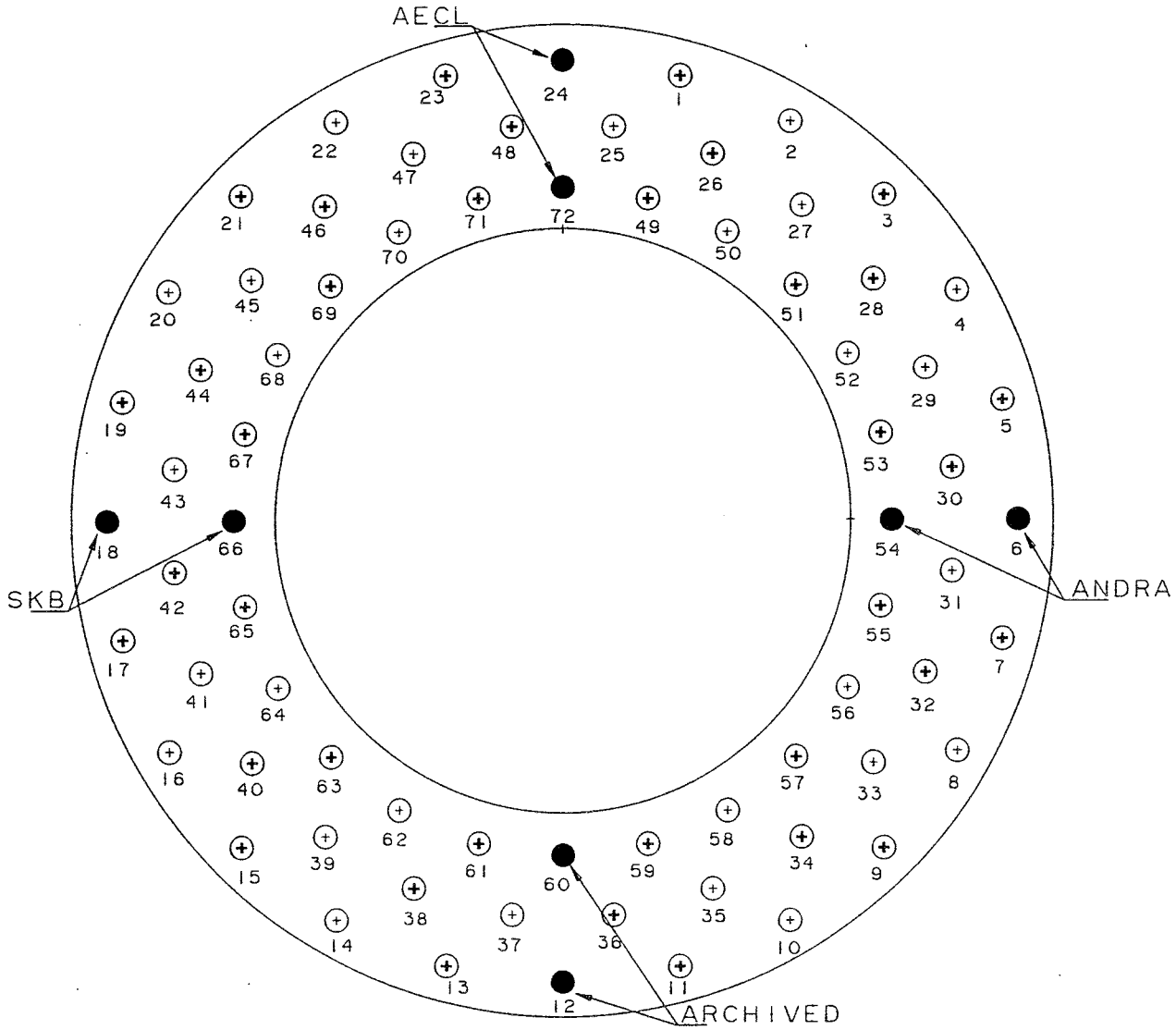


Figure 8 *Location of the metal tube sample in the layers around the heater annulus.*

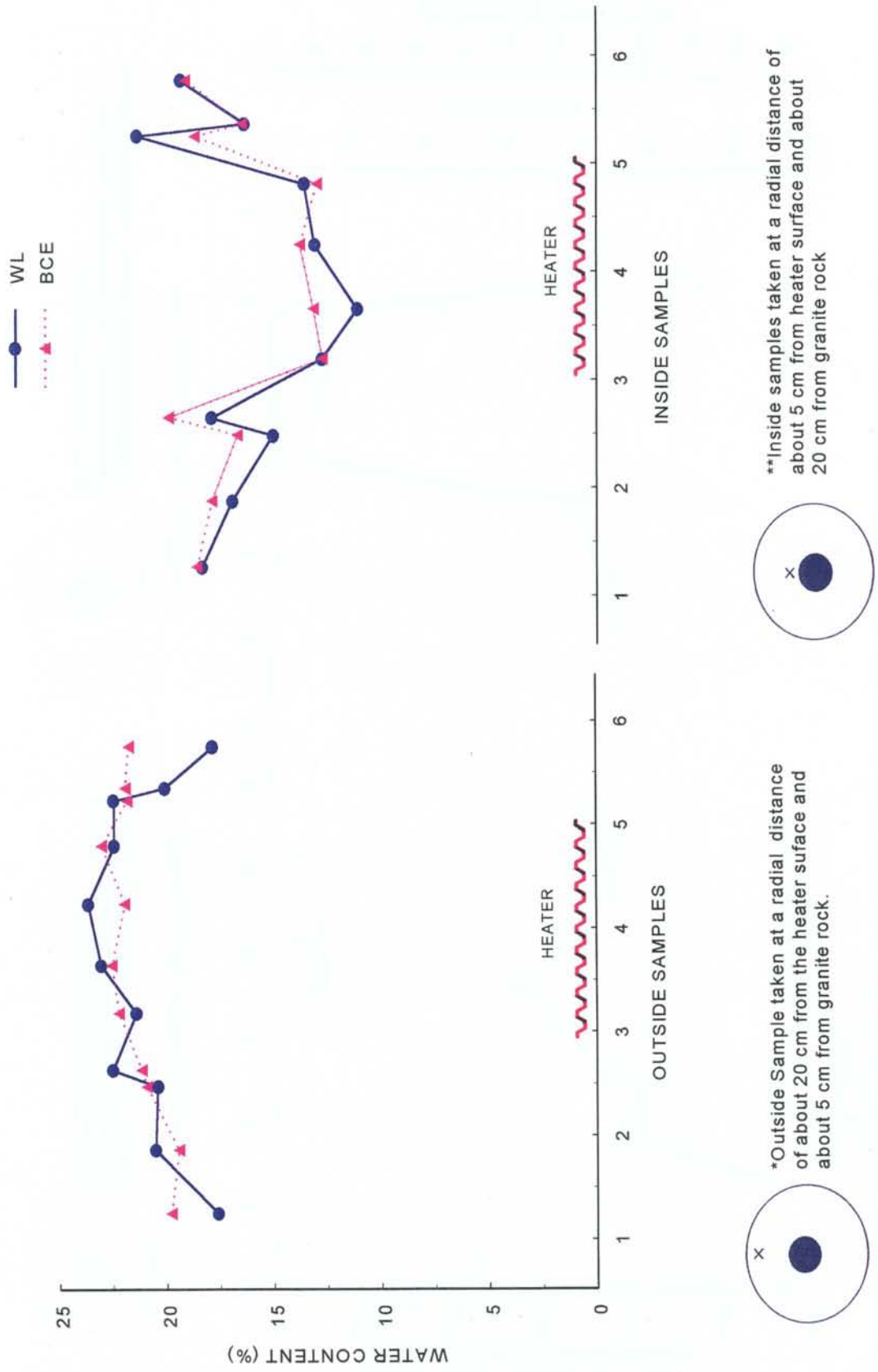


Figure 9 *Comparison of the Moisture Contents Measured in the Moisture Content Samples (BCE) and the Equivalent Microbiology Samples (WL)*

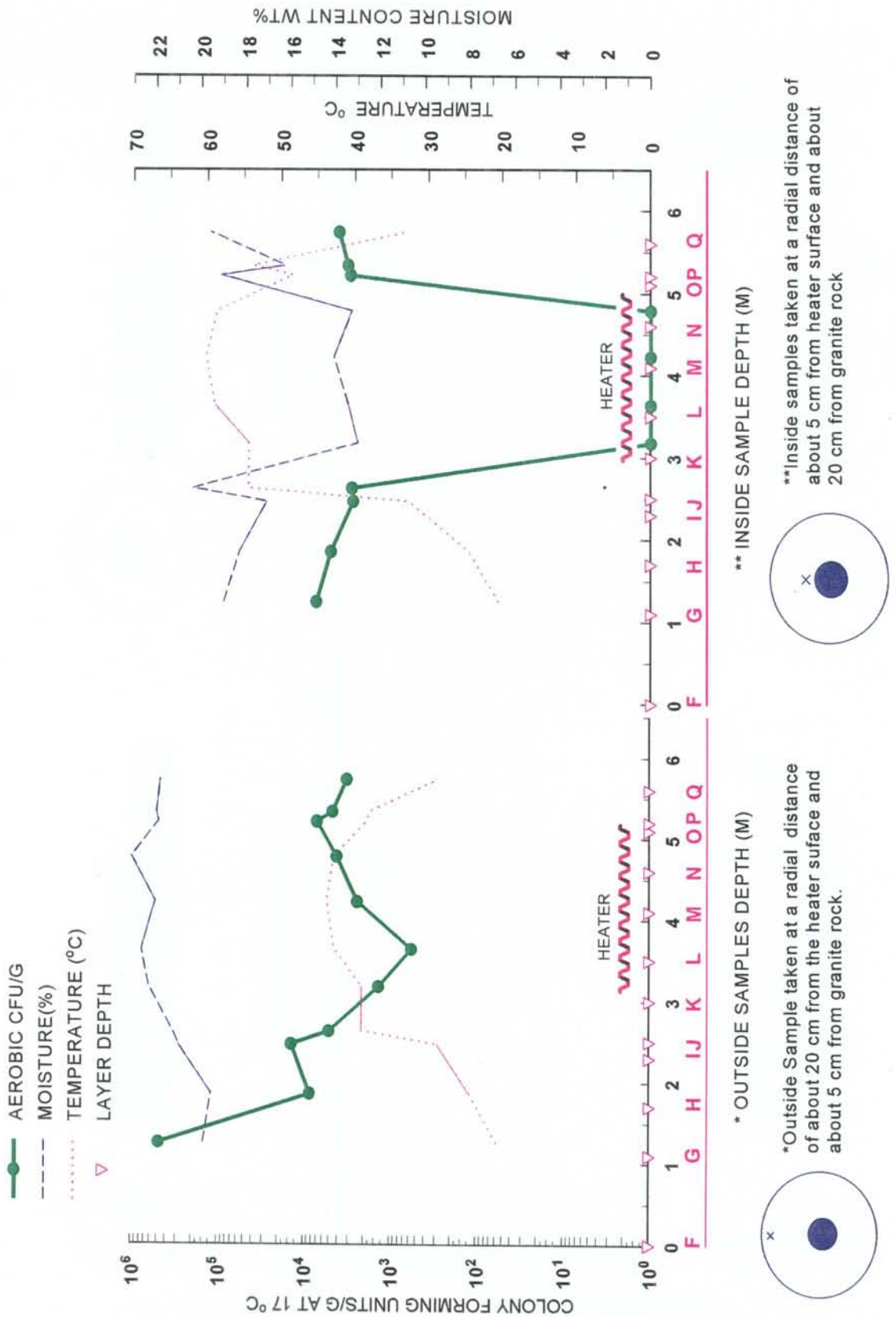


Figure 10 *Aerobic Heterotrophs in Metal Tube Samples Cultured at 17^0 C on R2A Medium at WL*

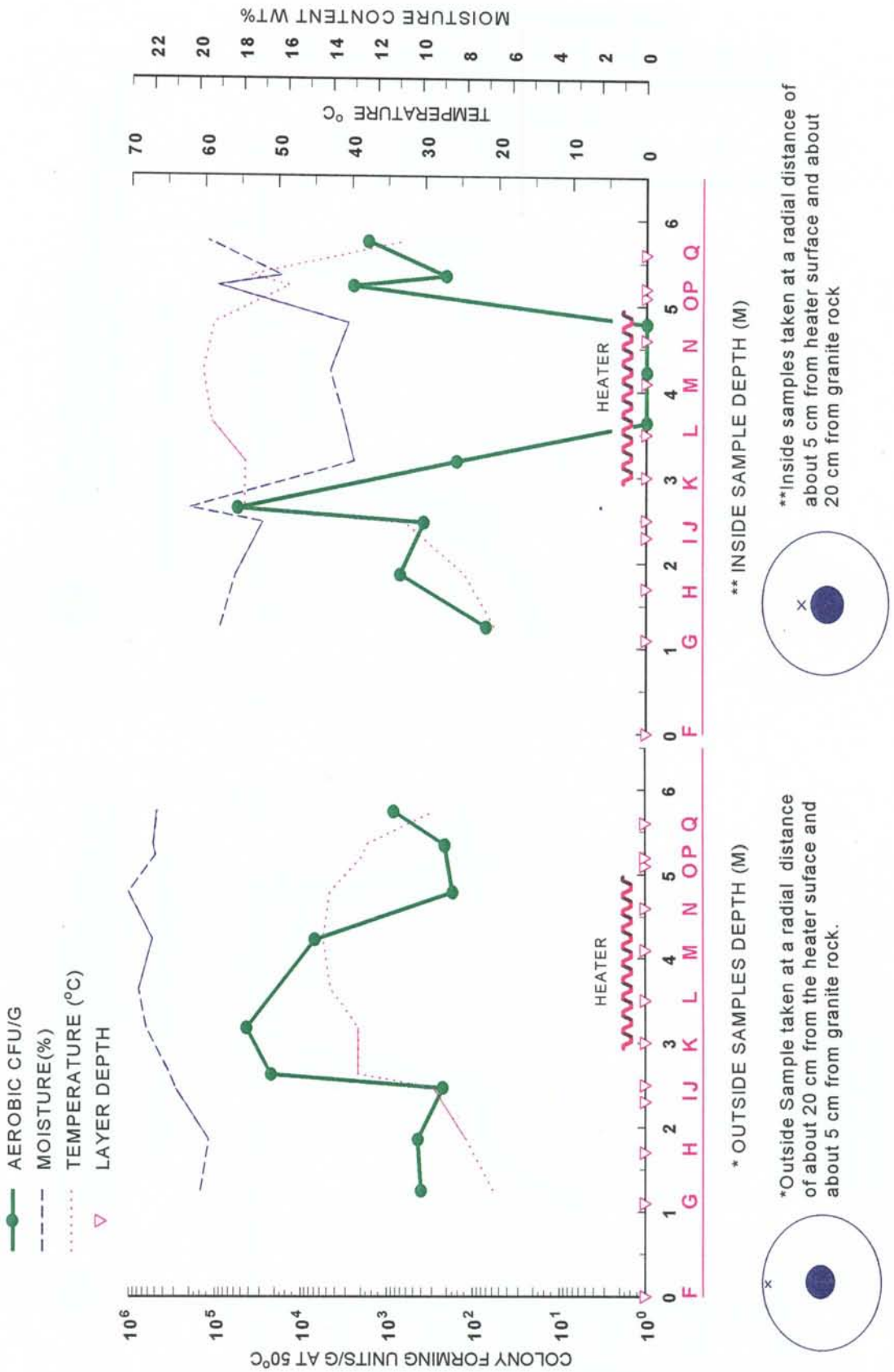


Figure 11 *Aerobic Heterotrophs in Metal Tube Samples Cultured at 50^0 C on R2A Medium at WL.*

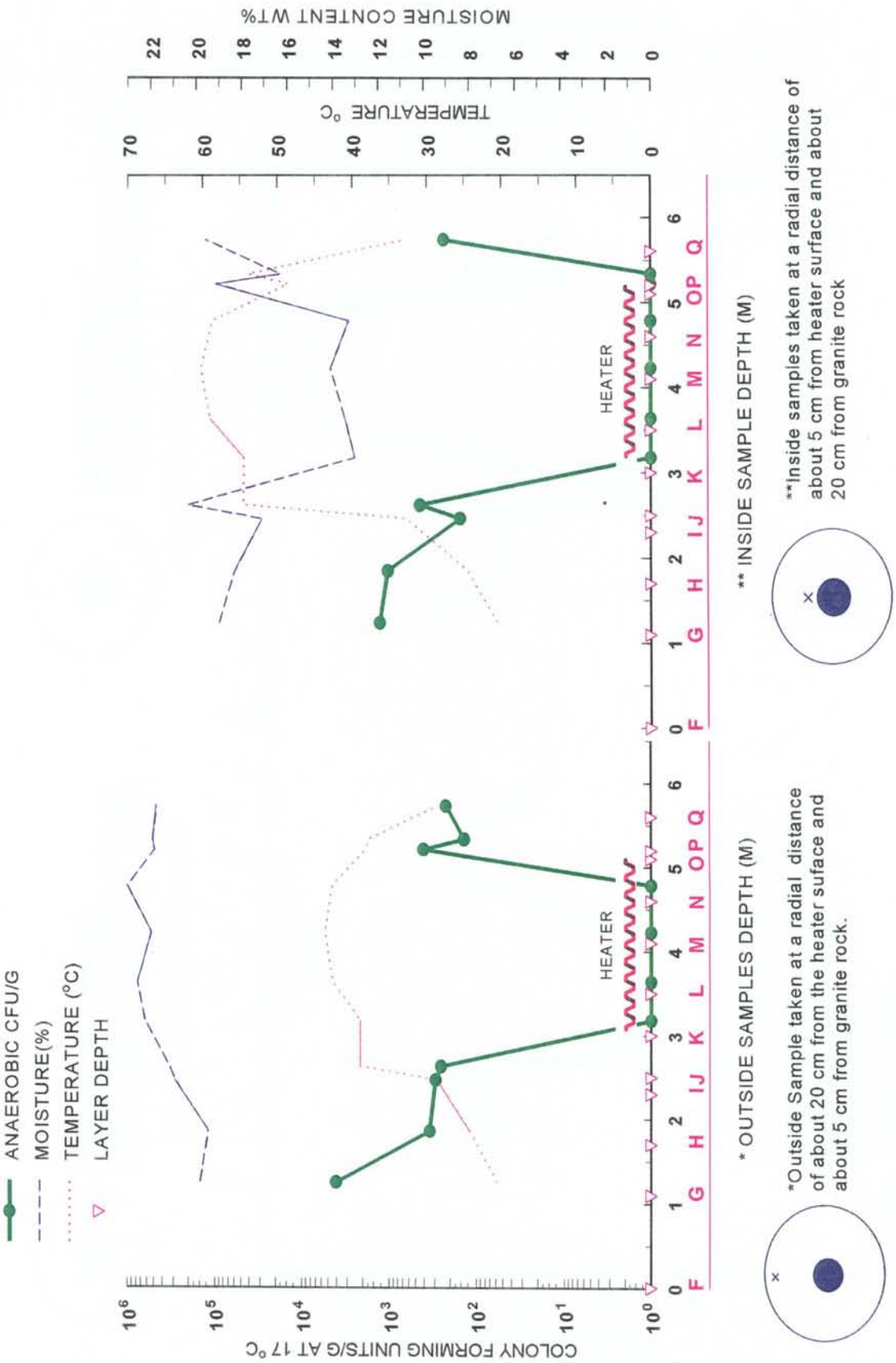


Figure 12 *Anaerobic Heterotrophs in Metal Tube Samples Cultured at 17^{0}C on R2A Medium at WL.*

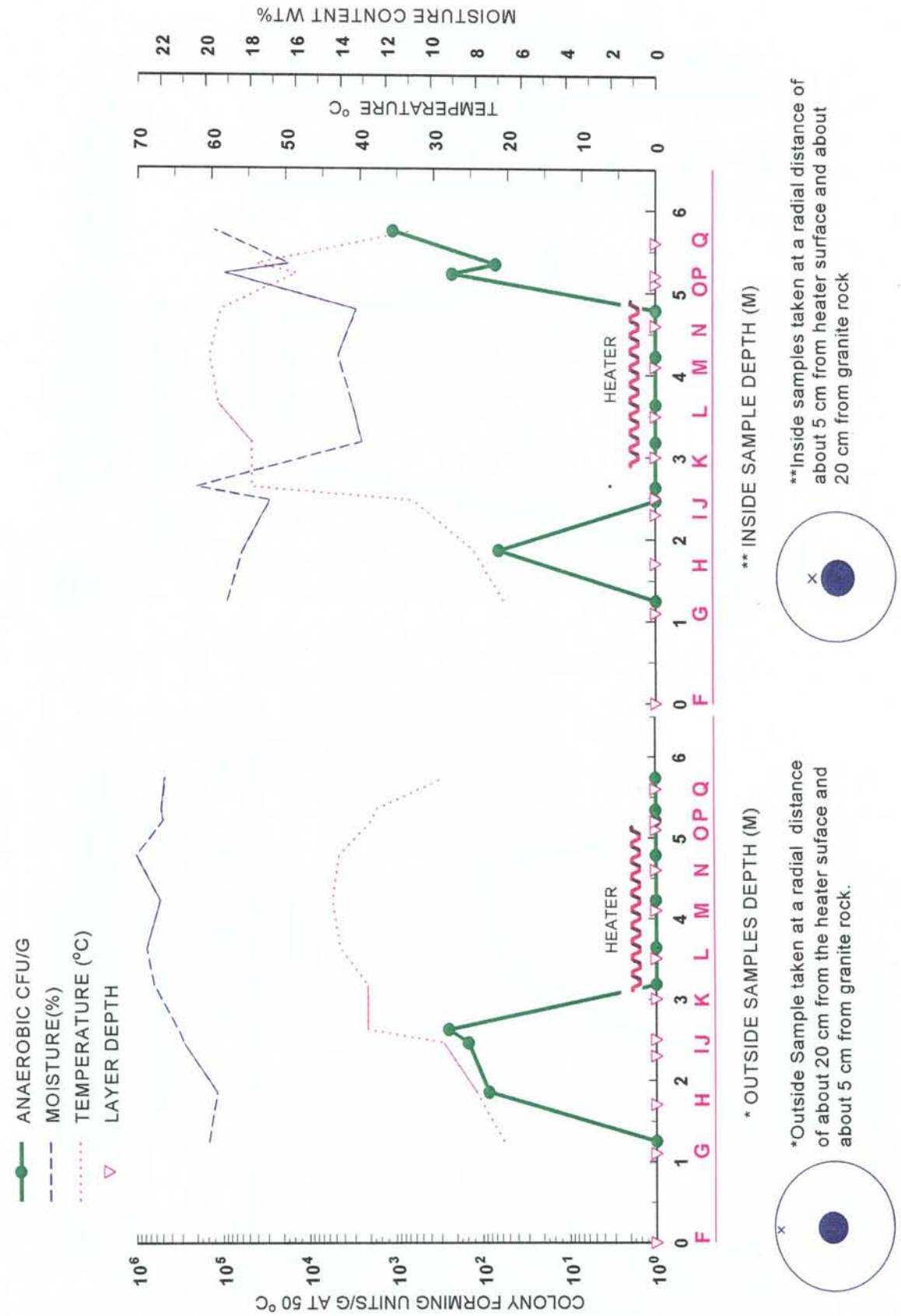


Figure 13 *Anaerobic Heterotrophs in Metal Tube Samples cultured at 50^{0}C on R2A Medium at WL.*

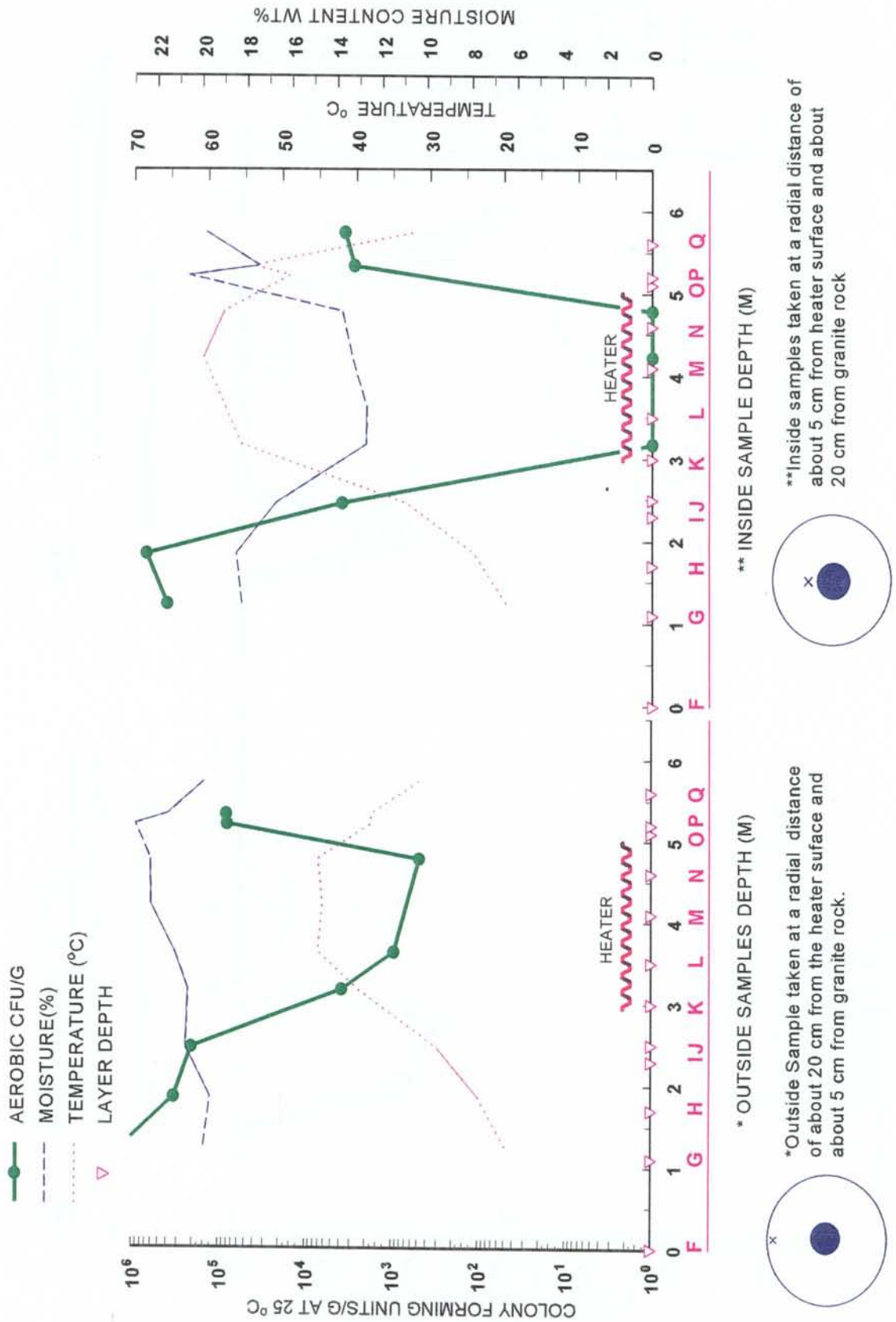


Figure 14 *Aerobic Heterotrophs in Metal Tube Samples Cultured at 25^{0}C on PCA Medium at the GRAM Laboratory.*

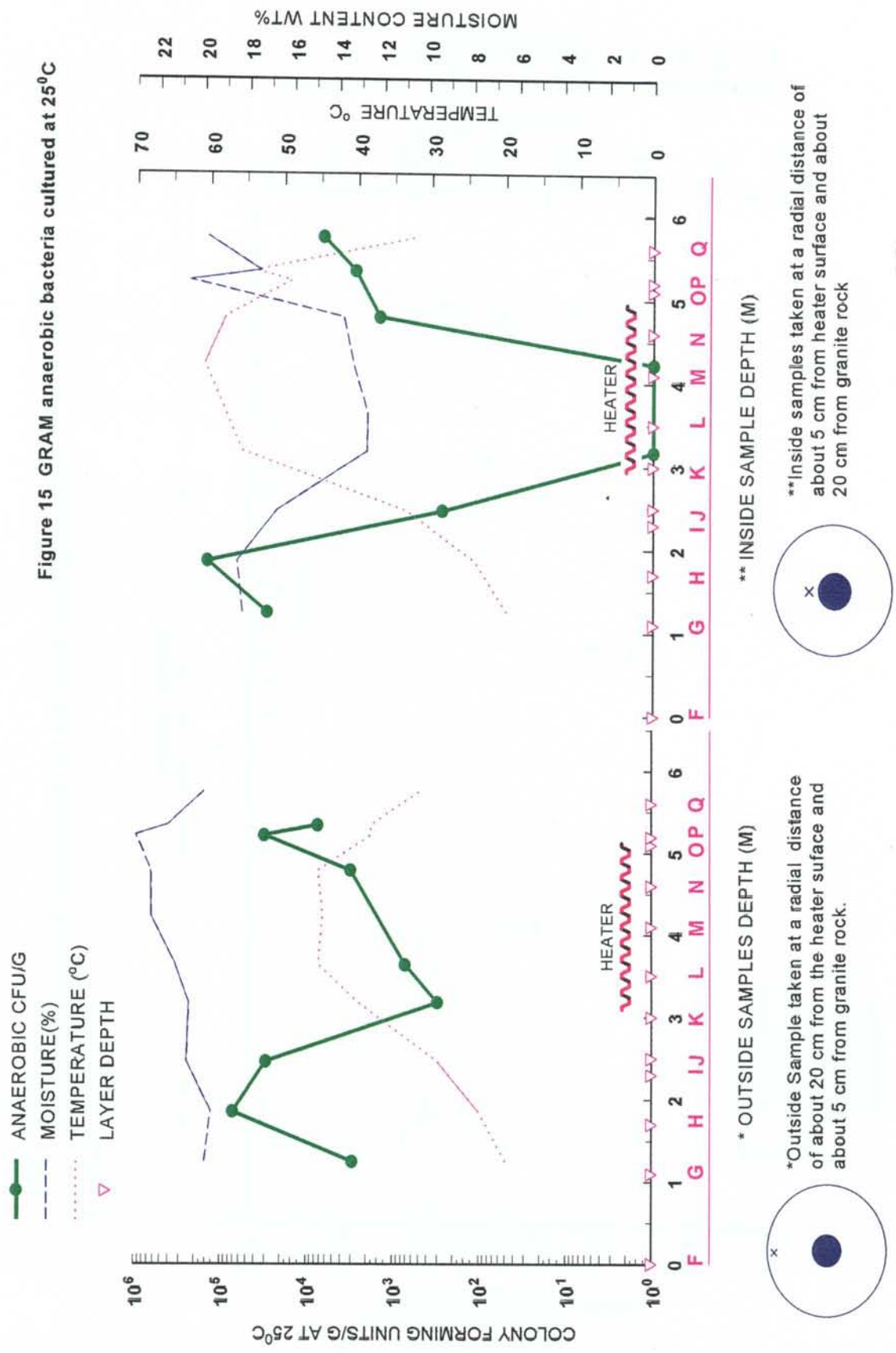


Figure 15 *Anaerobic Heterotrophs in Metal Tube Samples Cultured at 25^0 C on PCA Medium at the GRAM Laboratory.*

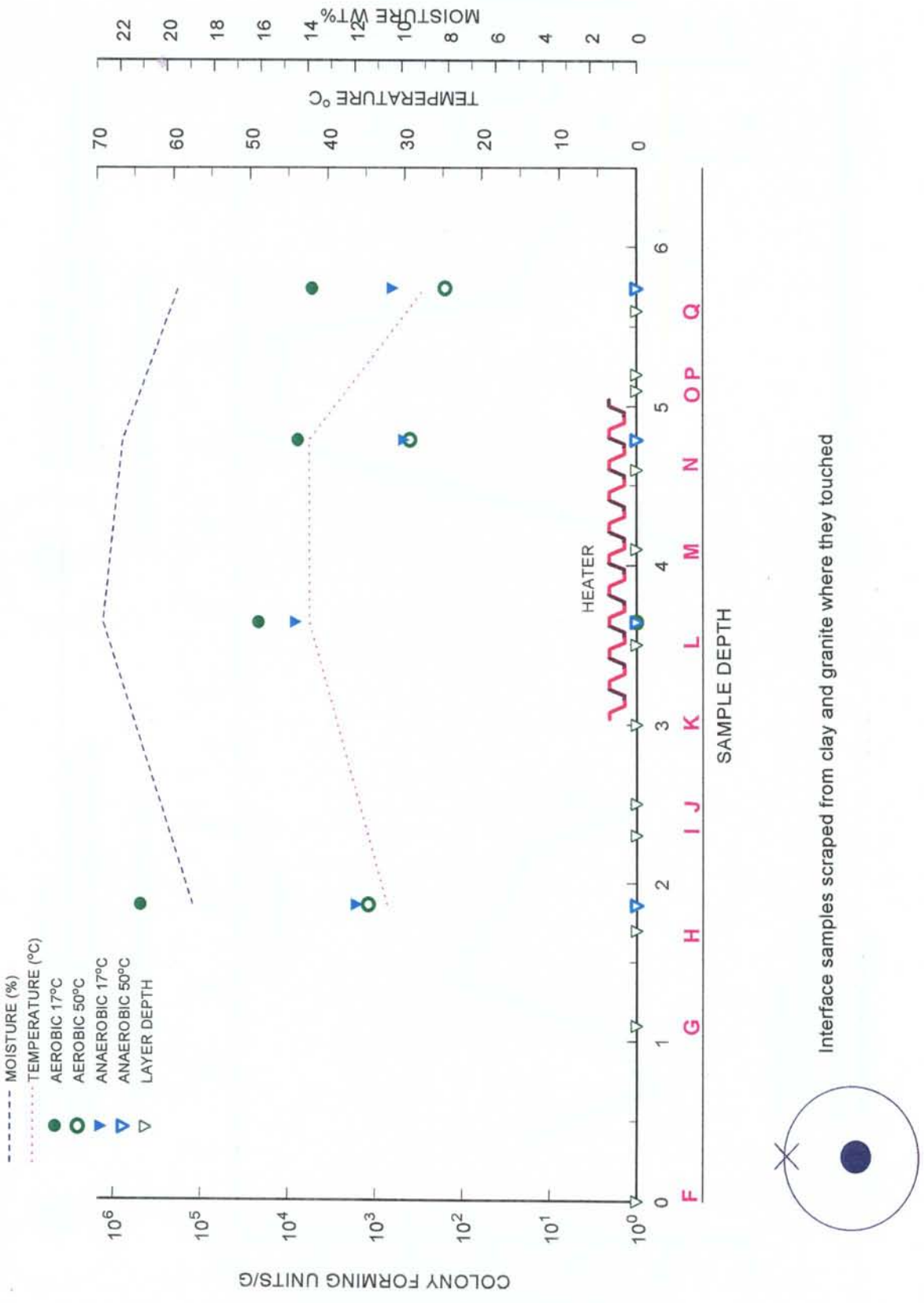


Figure 16 *Heterotrophic Bacteria in the Buffer-Granite Interface Samples analyzed at WL.*

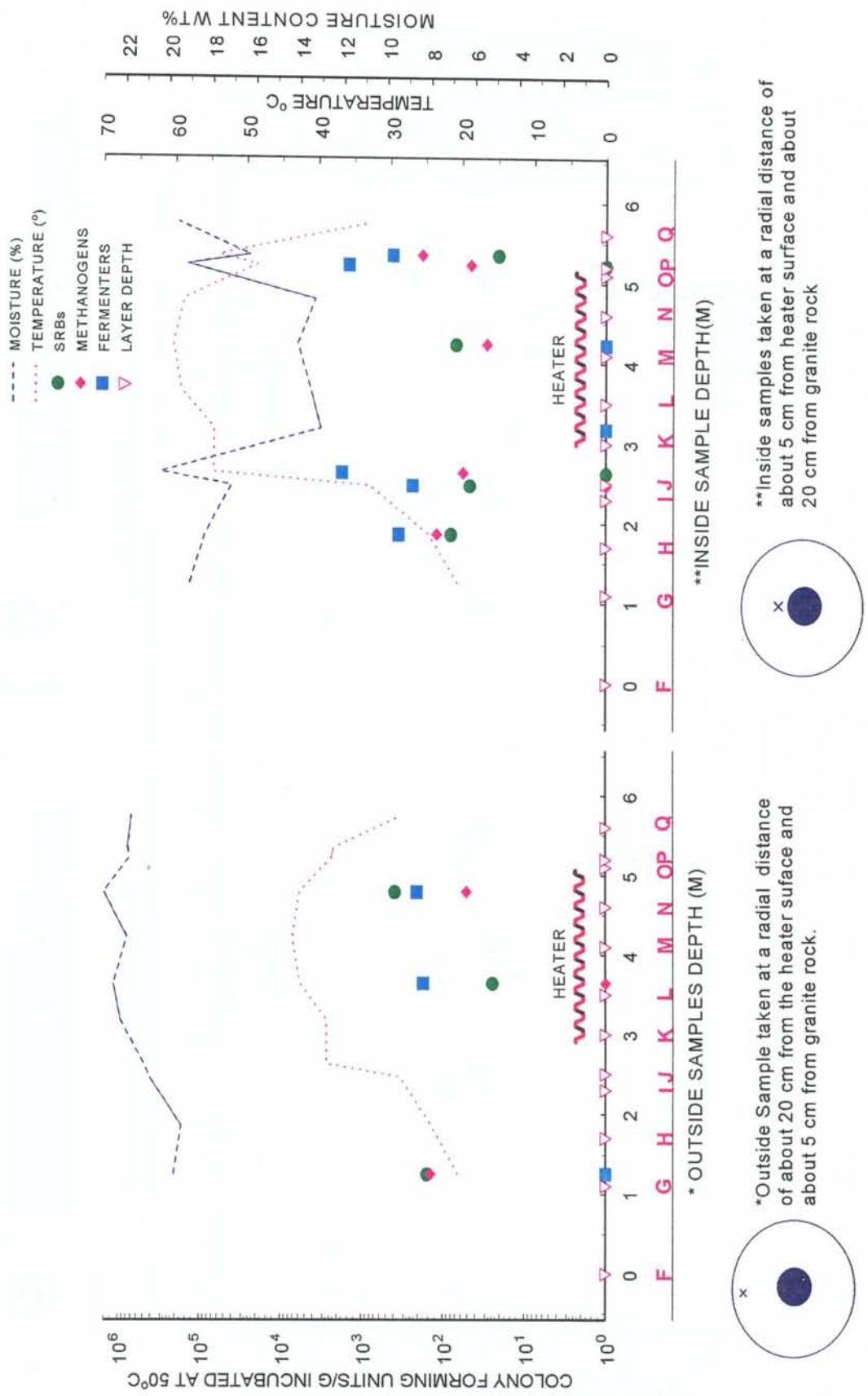


Figure 17 *Sulphate-Reducing Bacteria, Methanogens and Fermenters, in Metal Tube Samples Cultured Anaerobically at 50^{0}C at WL*

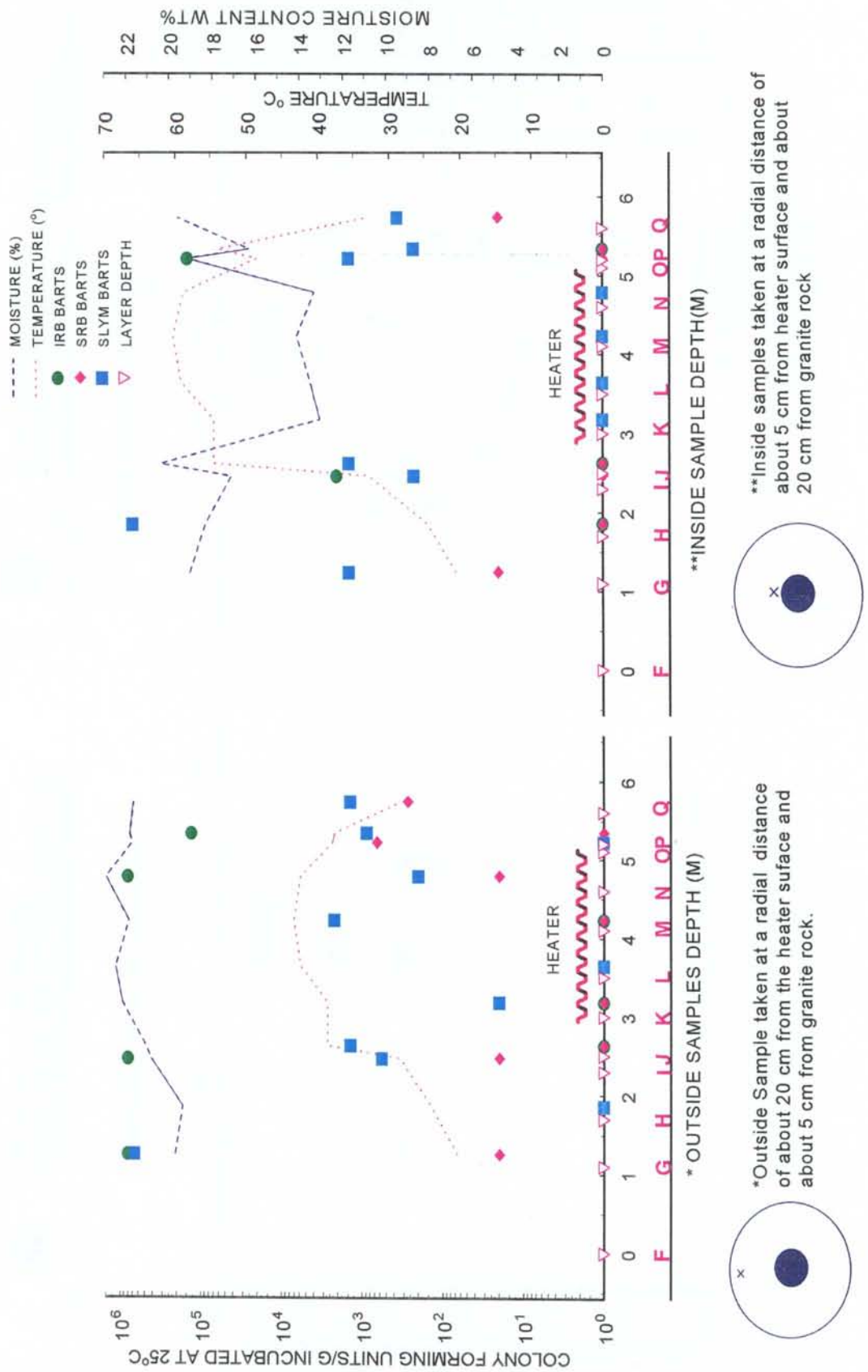


Figure 18 *Sulphate-Reducing (SRB), Iron-Related (IRB) and Slime Forming (SLYM) Bacteria in Metal Tubes Analyzed With BART Tests at 25^{0}C at WL.*

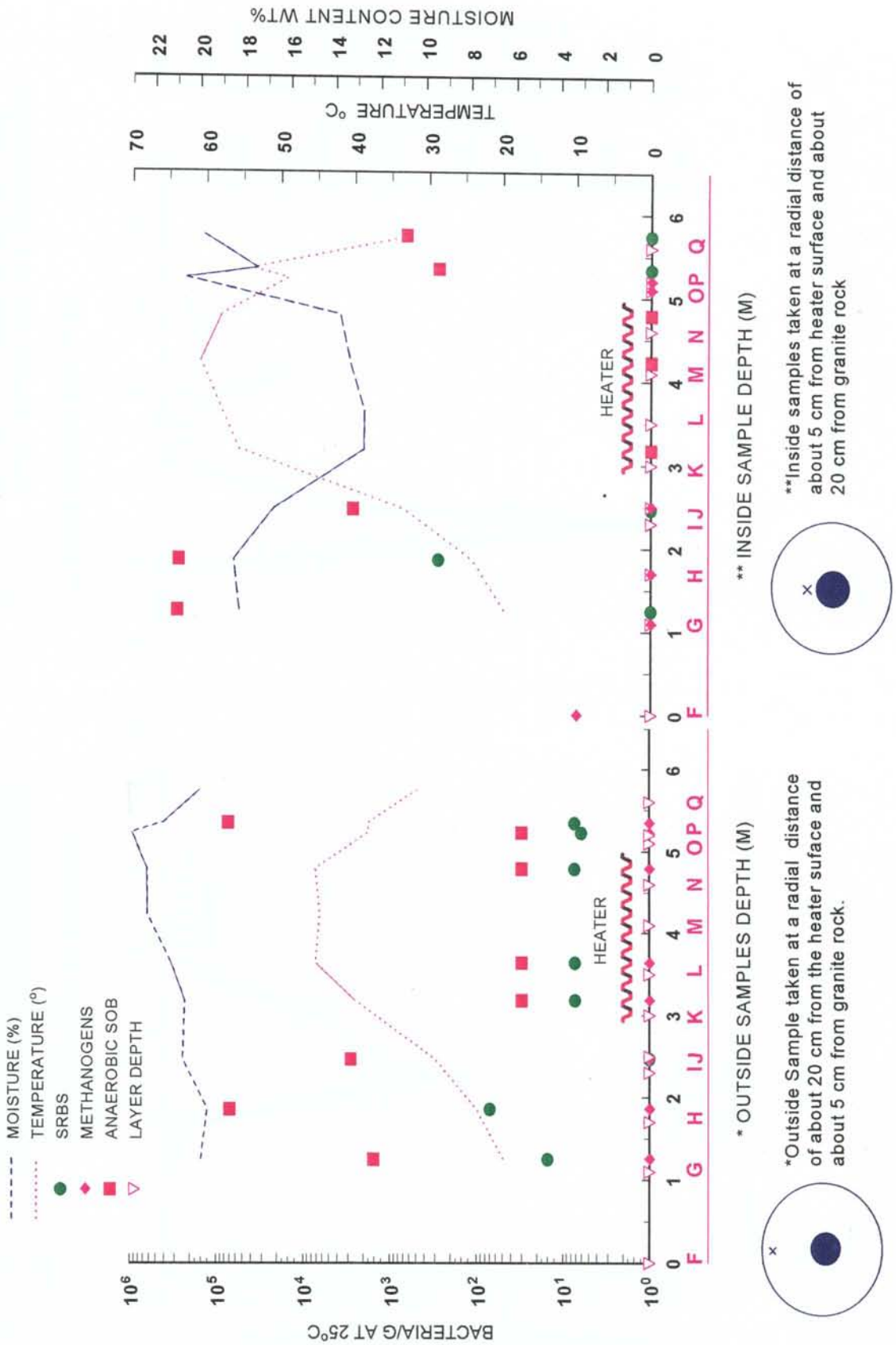


Figure 19 *Sulphate-Reducing Bacteria (SRB), Methanogens and Anaerobic Sulphur Oxidizers in Metal Tube Samples Cultured at 25^{0}C at the GRAM Laboratory.*

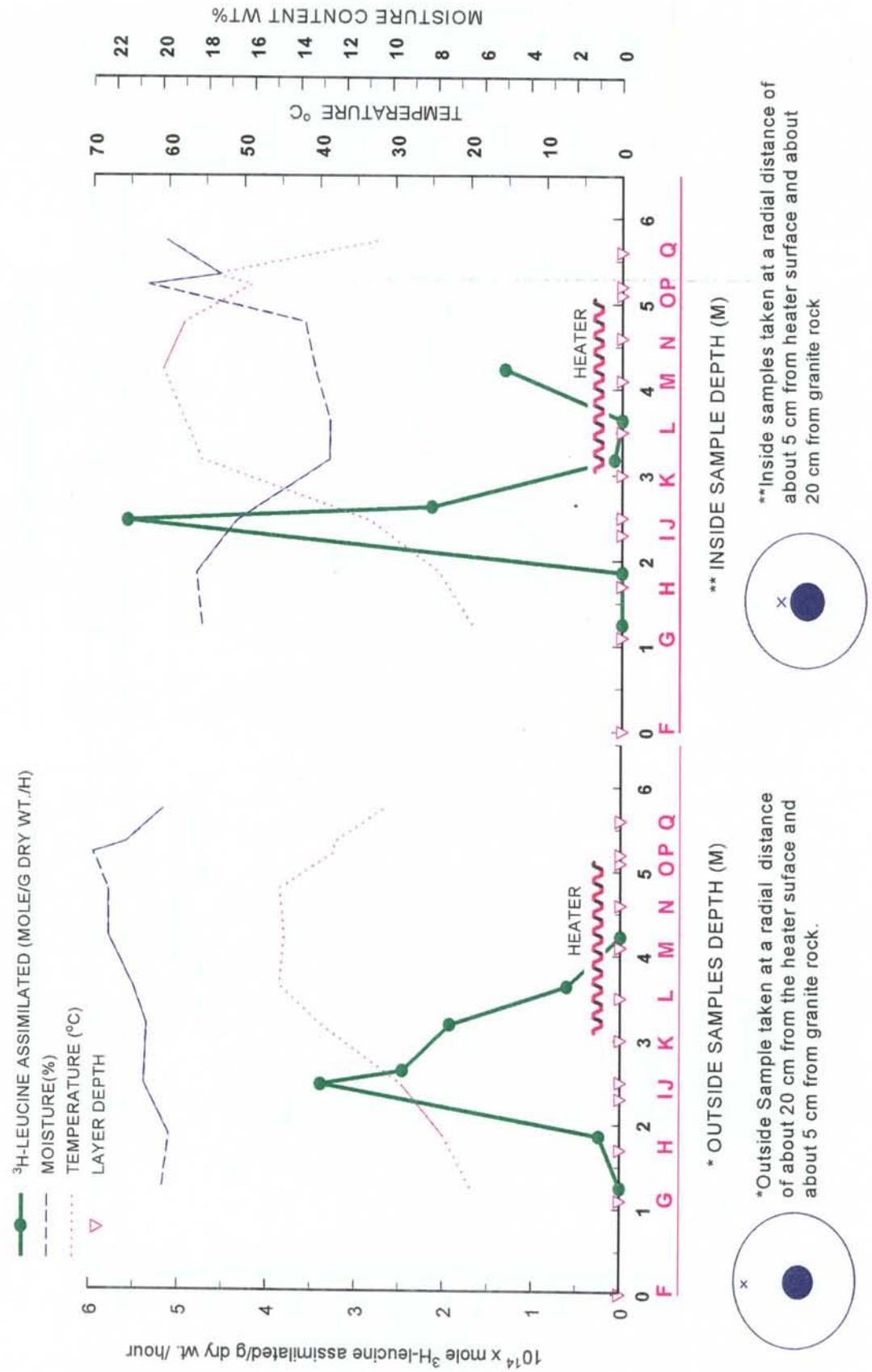


Figure 20 *Microbial Activity in Metal Tube Samples Analyzed at WL, Measured as Anaerobic ^{3}H Leucine Assimilation at 50^{0}C.*

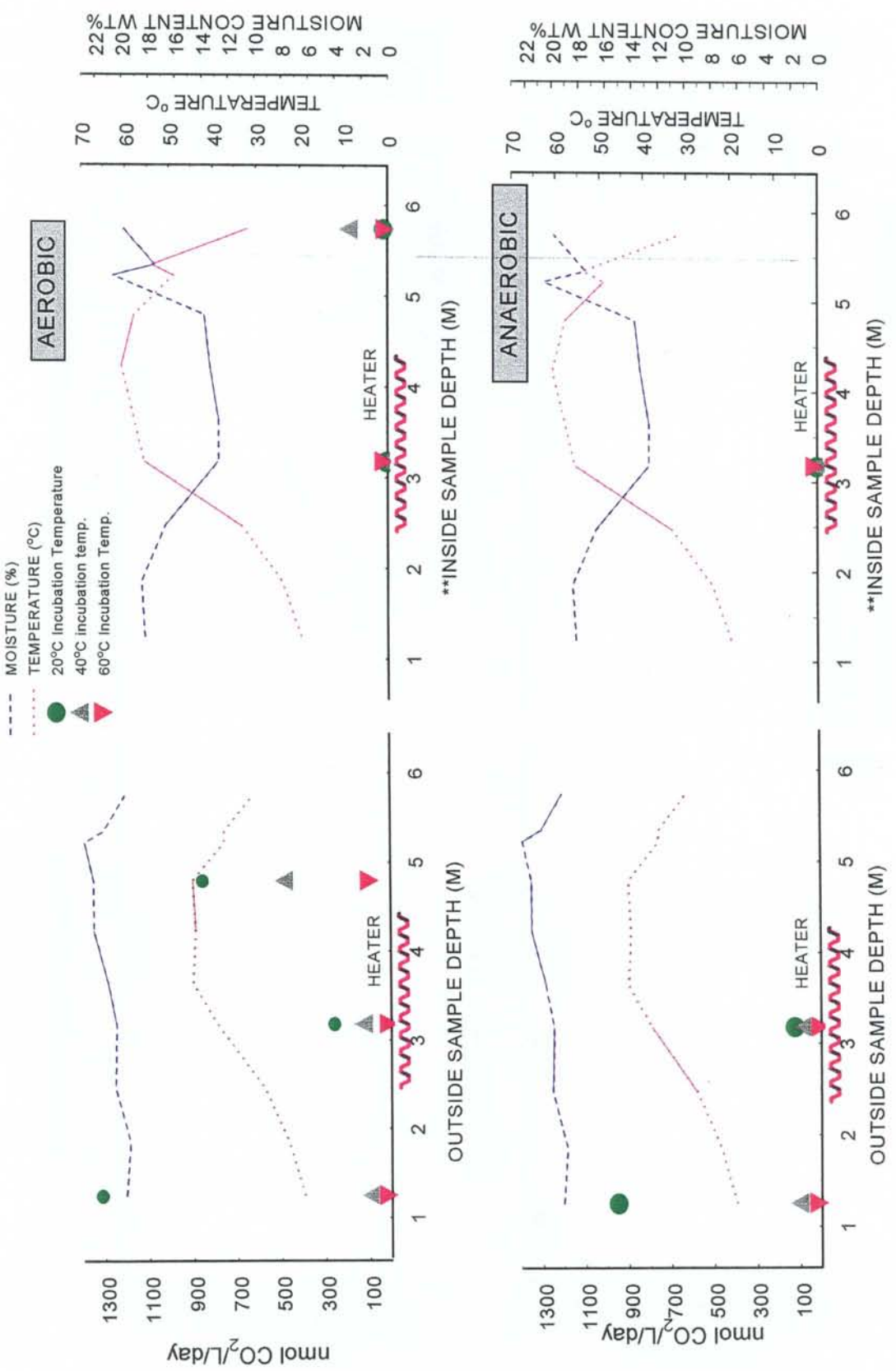


Figure 21 *Microbial Activity in Metal Tube Samples Analyzed at GRAM, Measured as Aerobic and Anaerobic ^{14}C-Labelled Glucose Assimilation at 20, 40 and 60^{0}C.*

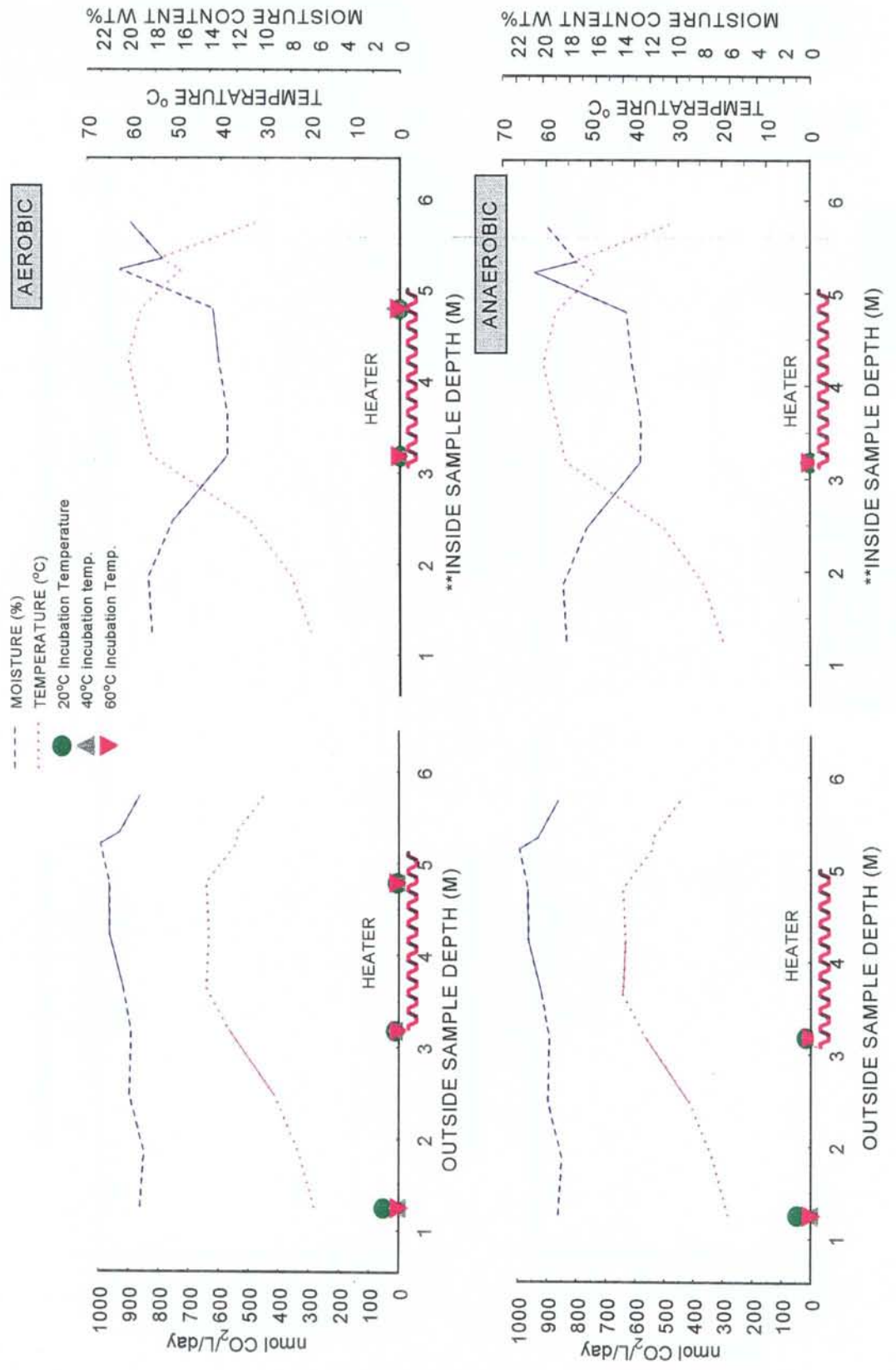


Figure 22 *Microbial Activity in Metal Tube Samples Analyzed at GRAM, Measured as Aerobic and Anaerobic ^{14}C-Labelled Amino Acid Mixture Assimilation at 20, 40 and 60^0C.*

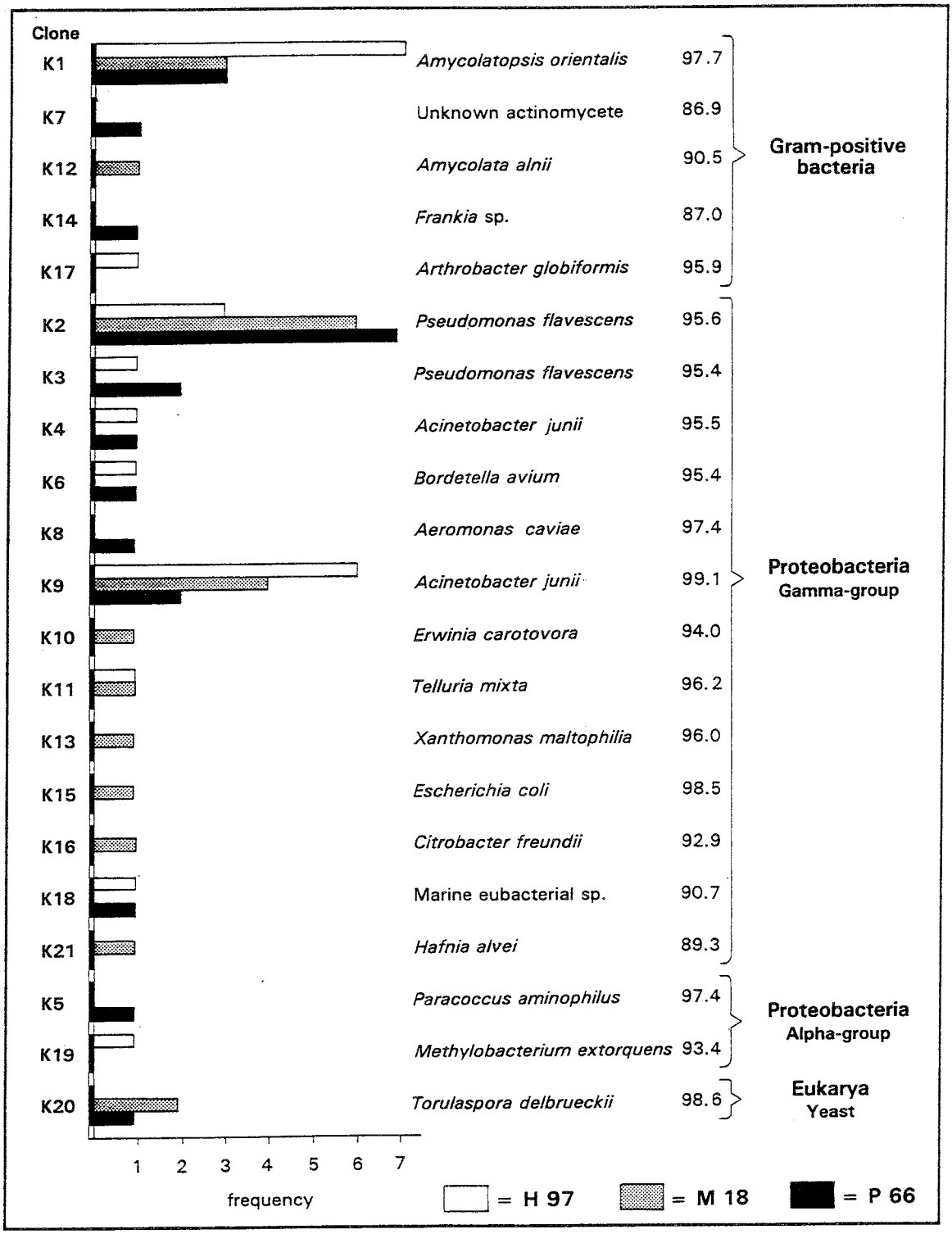


Figure 23 *The distribution of the 21 different 16S rRNA gene clones, K1 - K21, obtained from metal tube samples H-97-B, M-18-B and P-16-B, and the identity values for the closest organism available in the database. The phylogenetic affiliation of the clones is depicted to the right. The total number of clones from each sample were: H- 97-B (23); M-18-B (22) and P-16-B (22).*

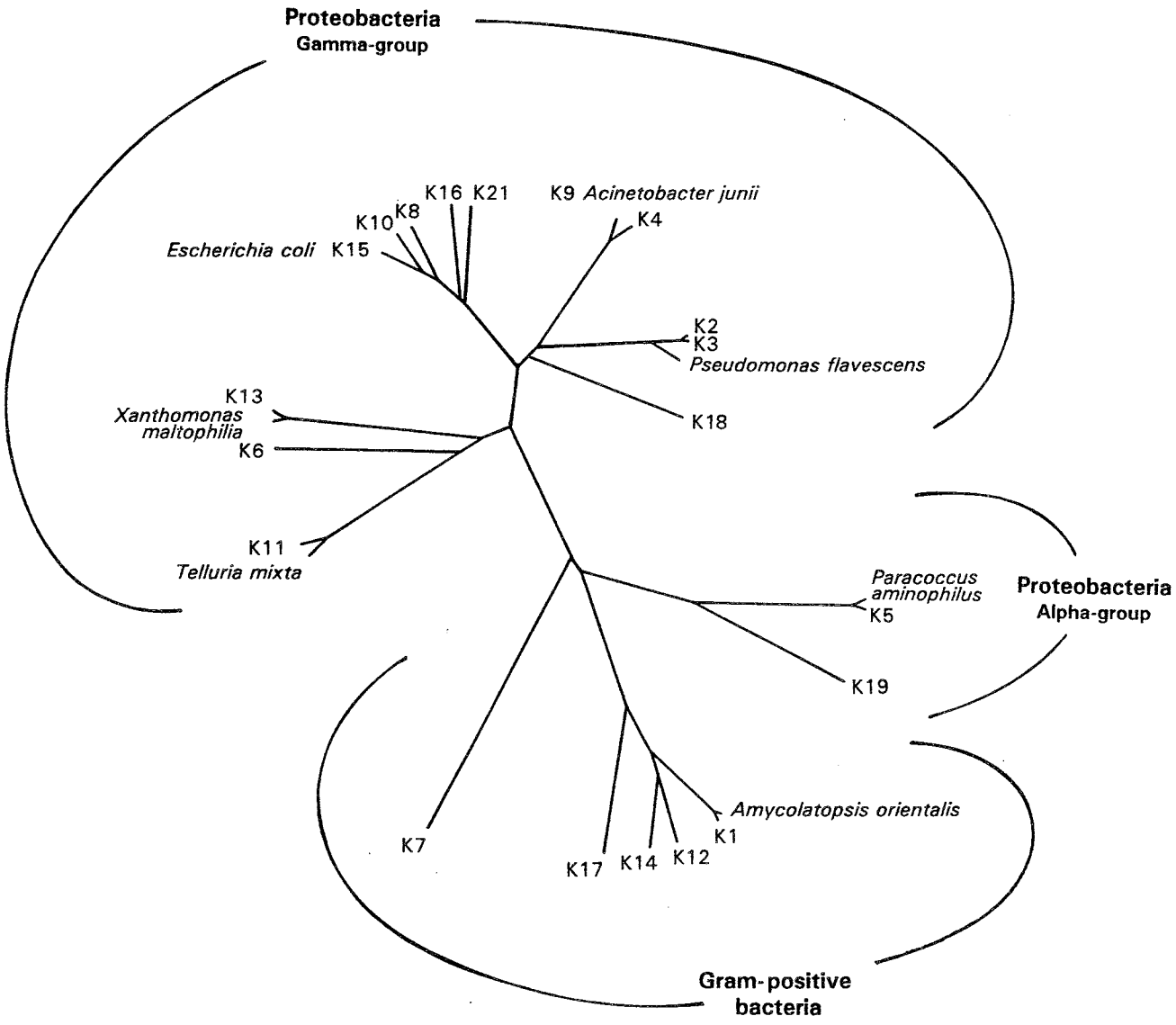


Figure 24 *Evolutionary distance tree based on the 16S rRNA gene sequences of clones from three buffer mass samples, H-97-B, H-18-B and P-16-B. Only sequences for species with an identity >95 % with respective clones are added to the tree.*

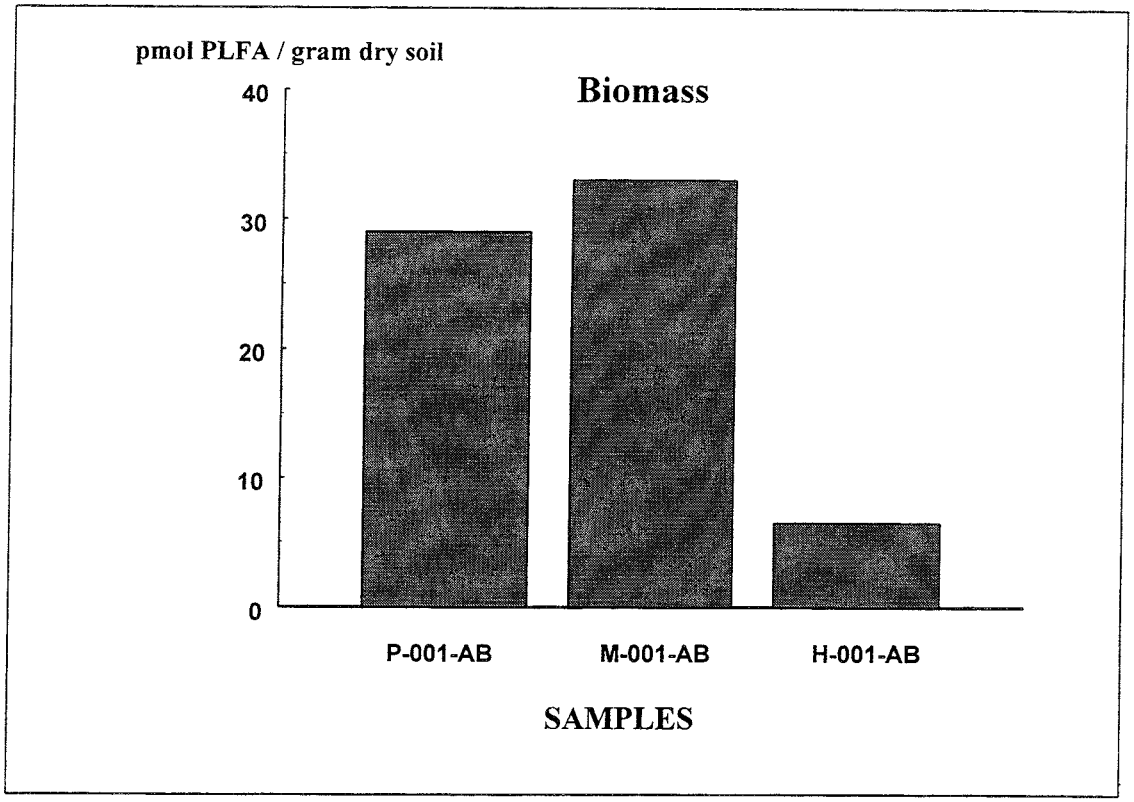


Figure 25 *Biomass in p-mol PLFA/g of Dry Material in Hollow Stem Auger Samples H-001-AB (Temp.~ 60^{0}C) and P-001-AB (Temp. ~ 48 to 50^{0}C)*

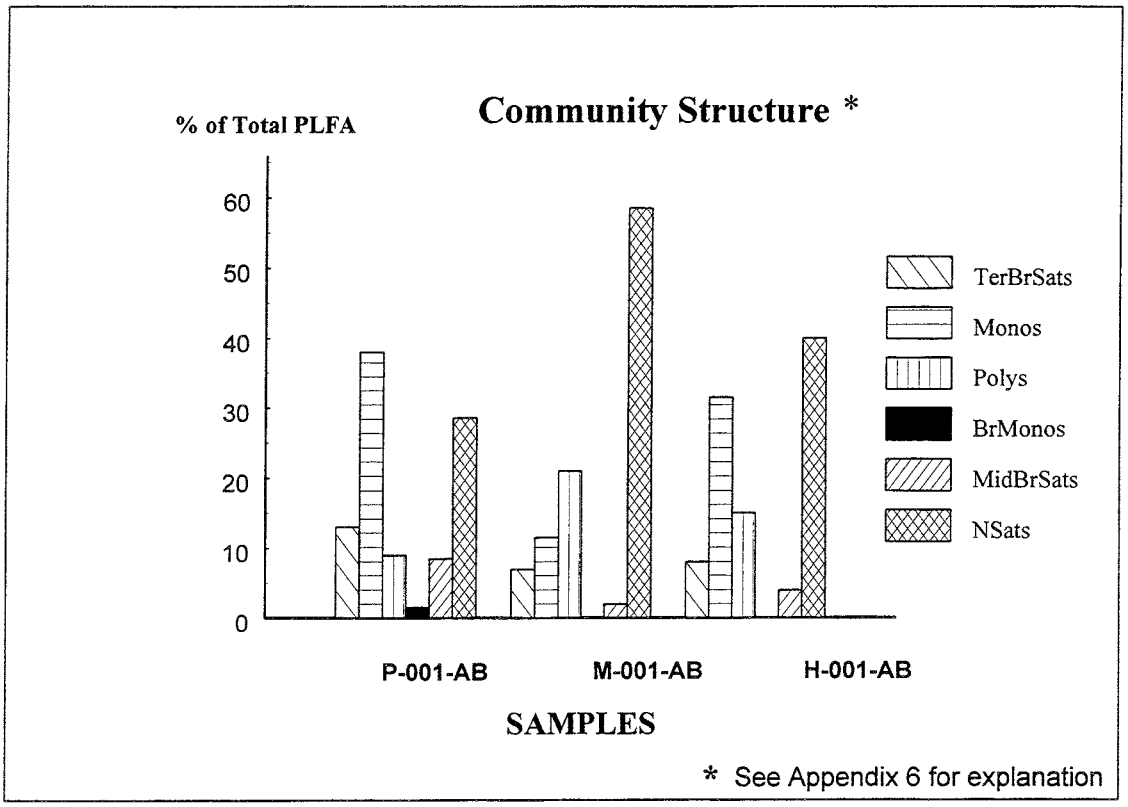
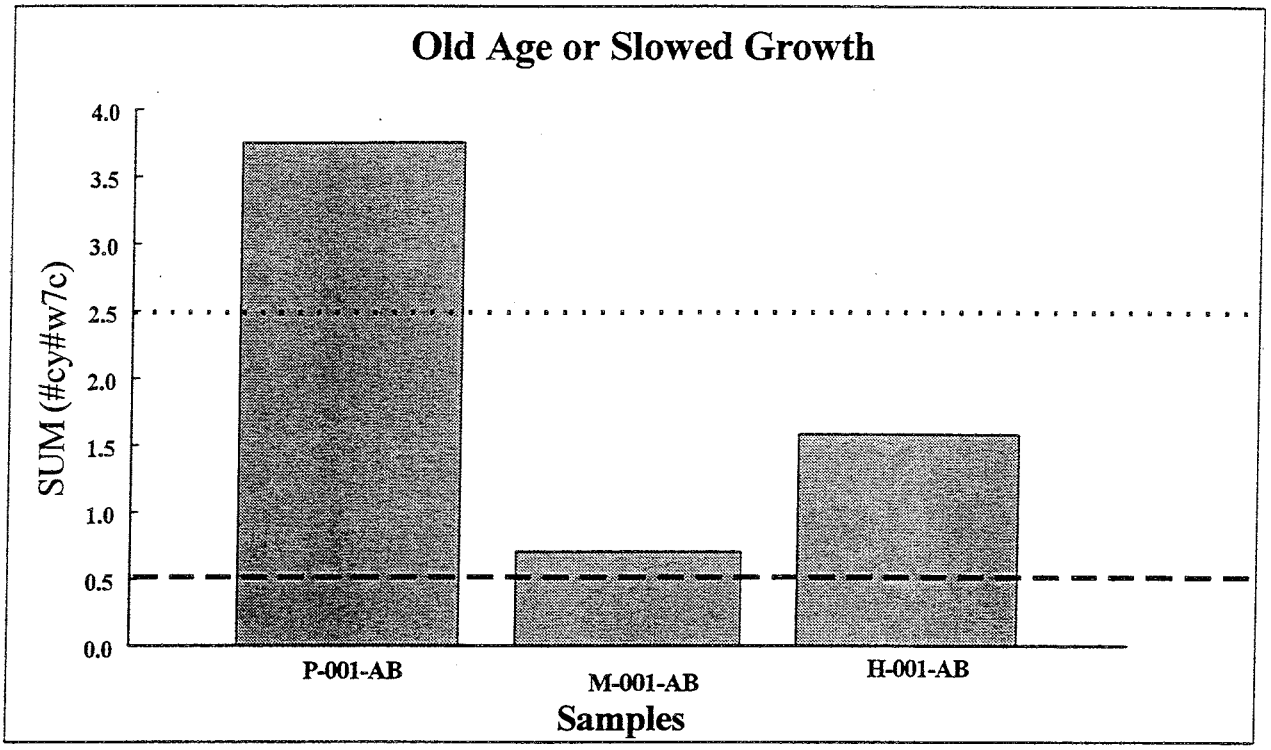


Figure 26 *Community Structure in % of Total PLFA (Appendix 6) in Hollow-Stem Auger Samples H-001-AB, M-001-Ab and P-001-AB.*



P-001-AB T = 48 - 59 °C
M-001-AB T = 60°C
H-001-AB T = 25°C

---------- Ratio below which log phase growth (see Appendix 6)

..... Ratio below which stationary phase (see Appendix 6)

Figure 27 *PLFA Data from Hollow Stem Auger Samples H-001-AB, M-001-AB and P-001-AB indicating that Bacteria found in These Samples are in Stationary Growth Phase or Worse (Appendix B).*

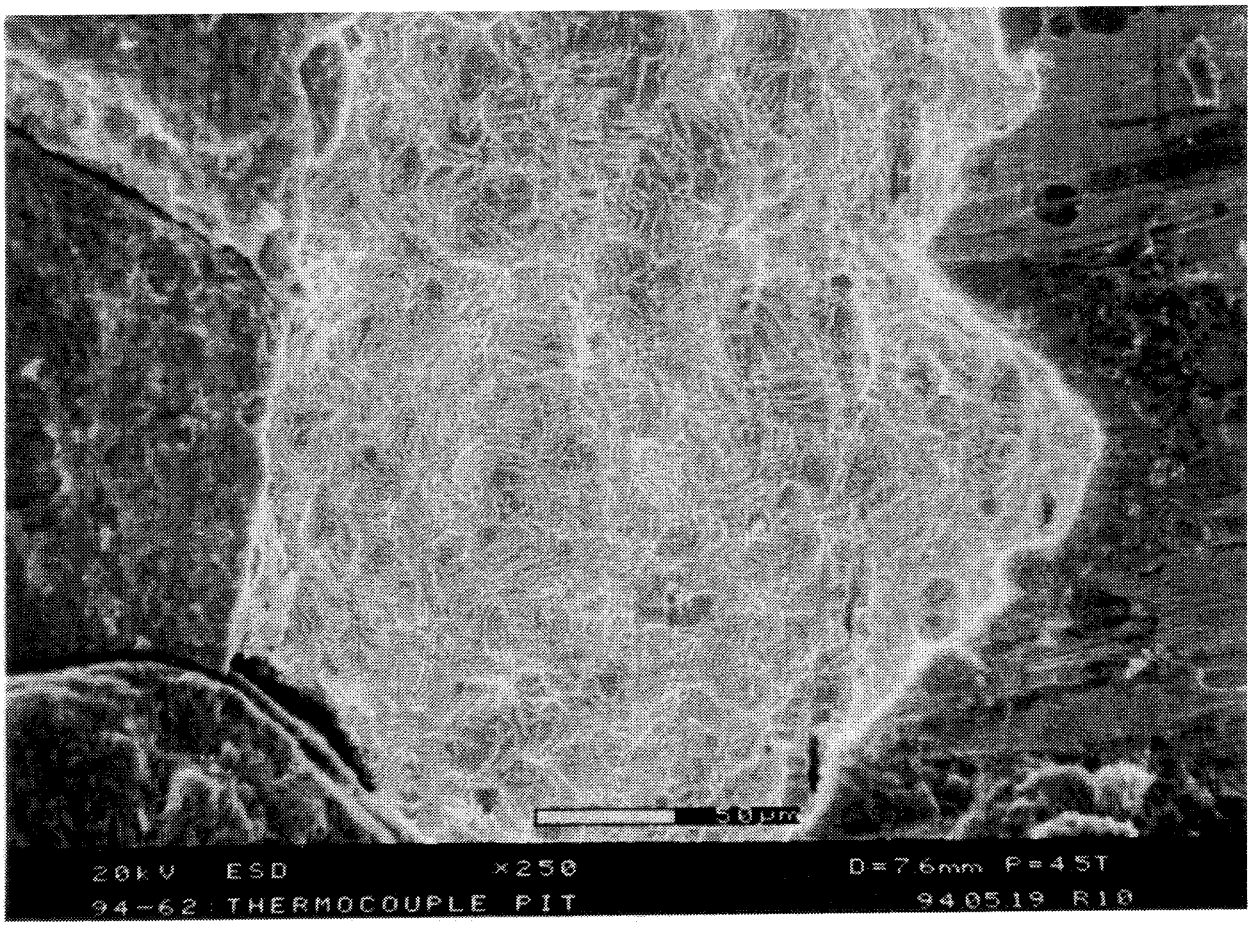


Figure 28 *ESEM Micrograph of a pit in the segment of corroded thermocouple from Layer N. No evidence of bacteria present.*

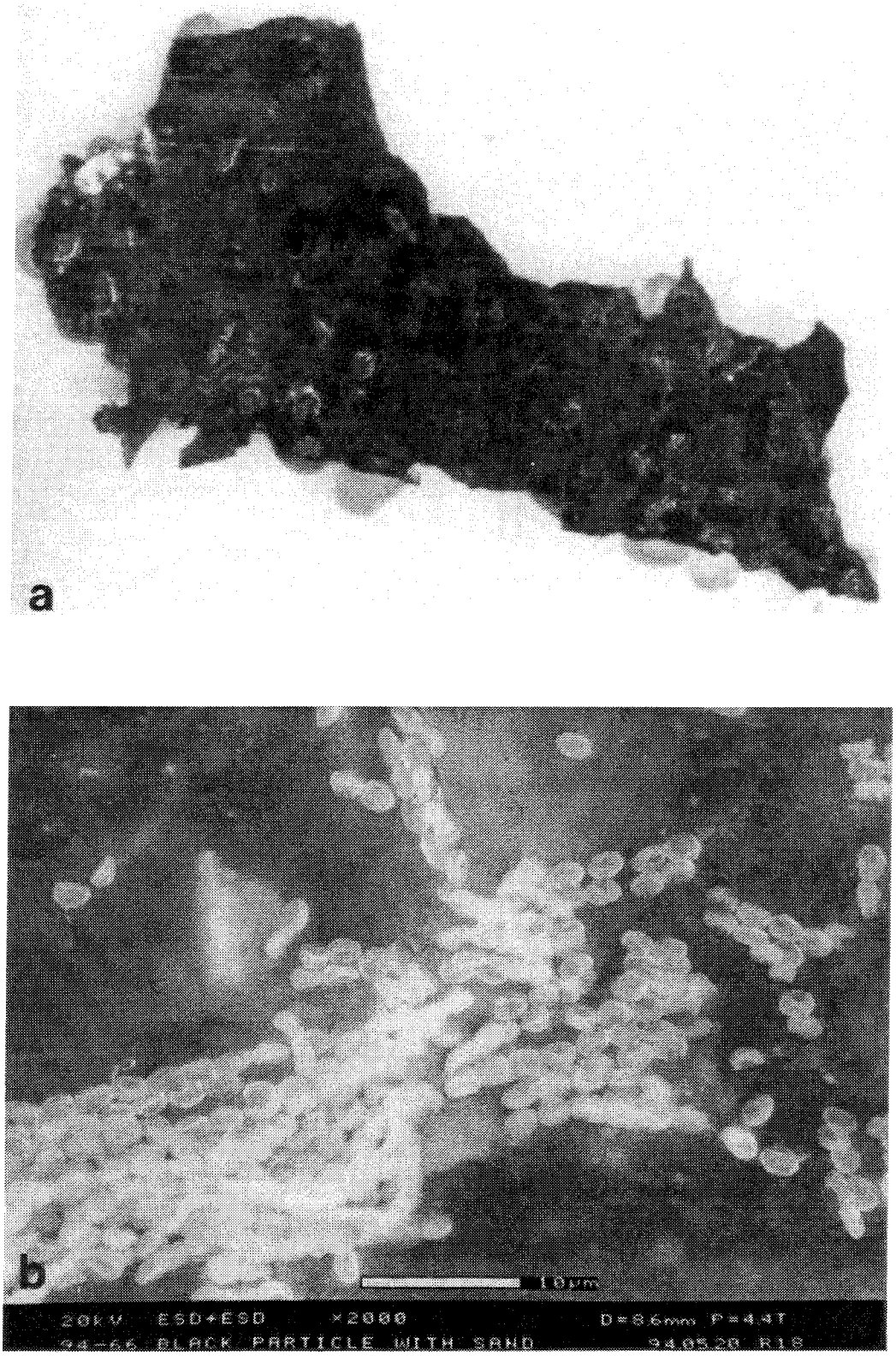


Figure 29 *a) Piece of black tape taken from the Teflon cloth around the heater (mag. 17x), b) ESEM Micrograph of string of bacteria (cocci) clearly visible.*

Figure 30 *TEM Micrograph of metal tube sample N-024-B washed 4x with 0.05 M HEPES Buffer. Few bacteria and difficult to find.*

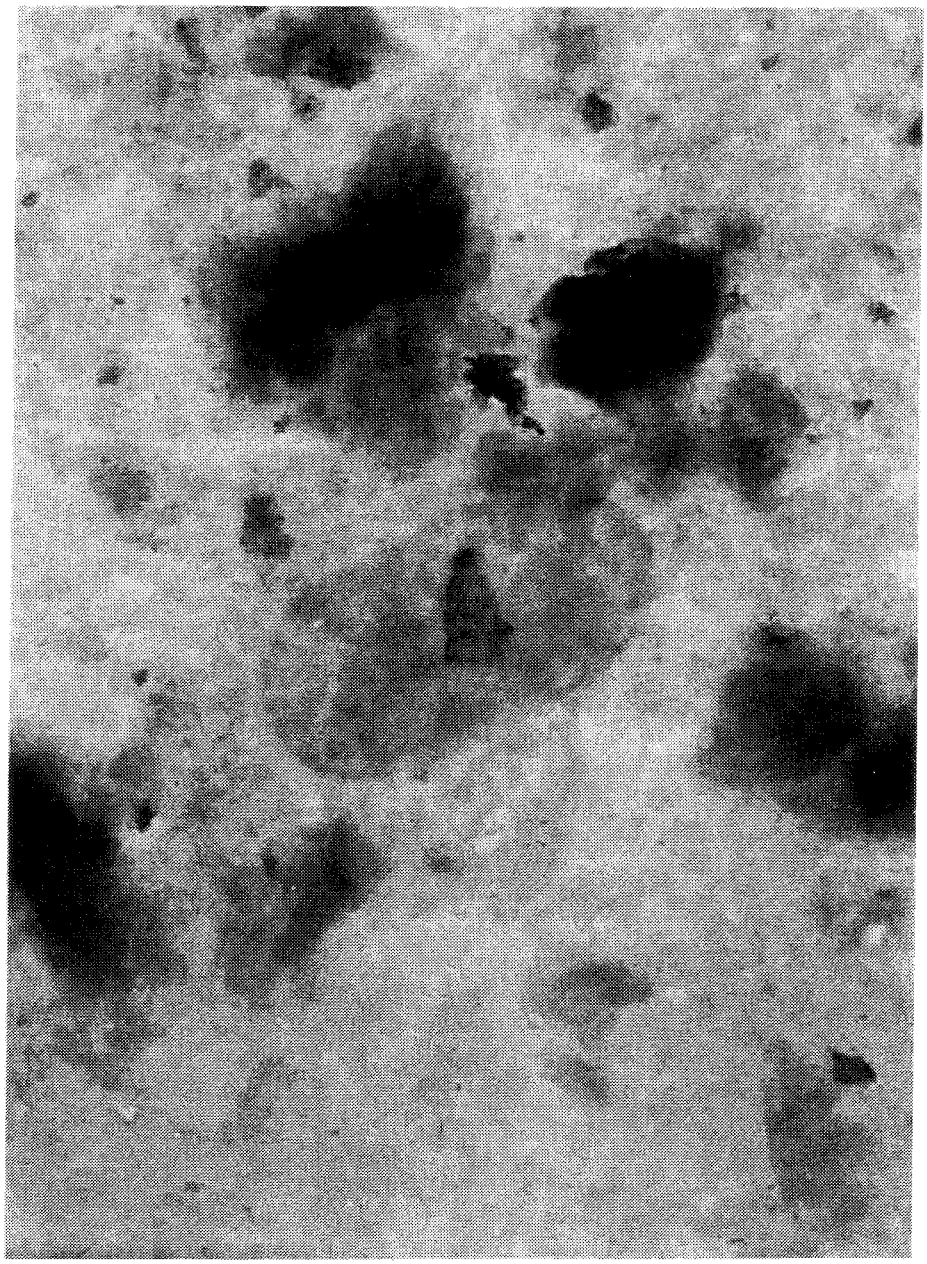


Figure 31 *TEM Micrograph of metal tube sample K-072-B washed 8x with 0.15 M HEPES Buffer. Bacteria still difficult to find.*

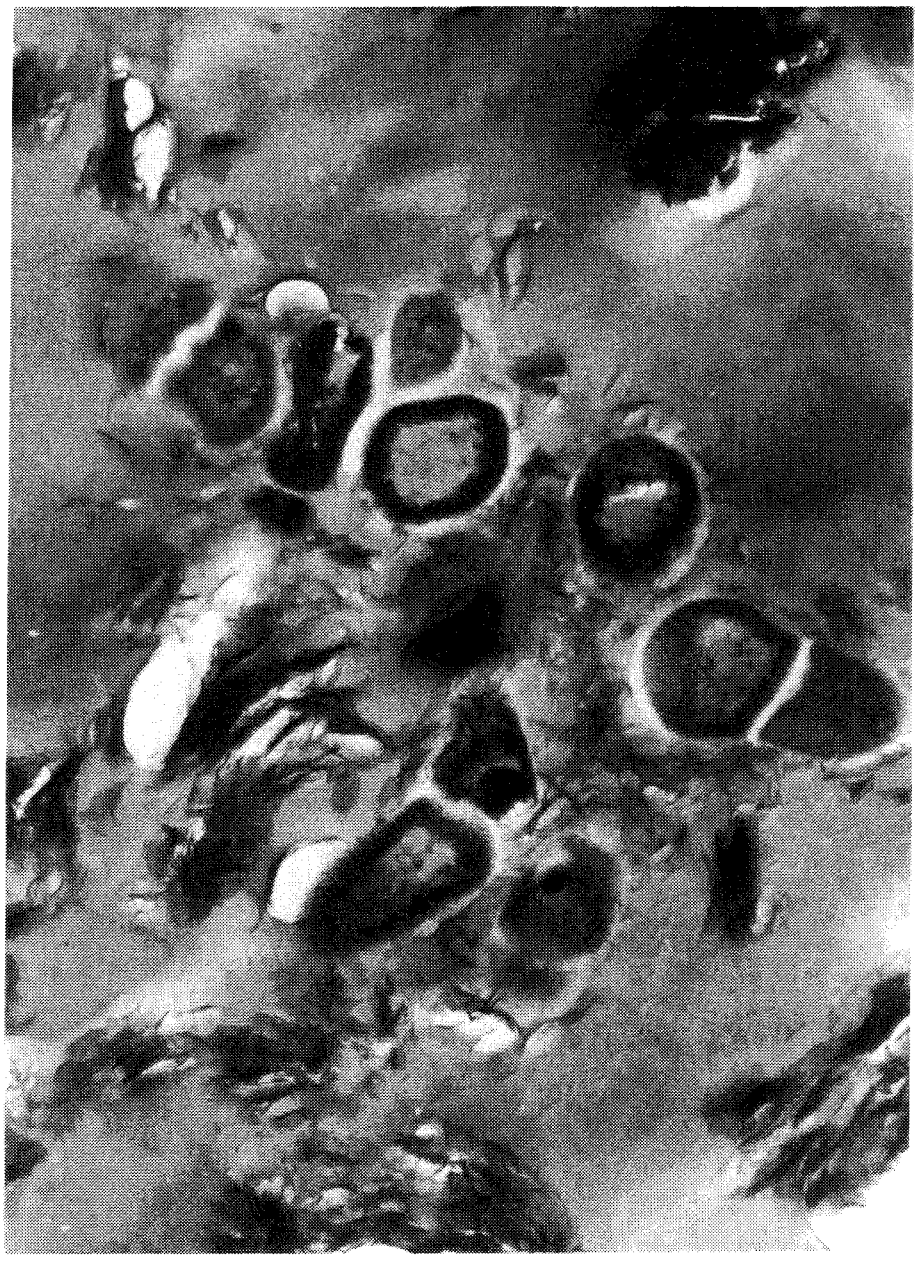


Figure 32 *TEM Micrograph of metal tube sample K-072-B, washed 8x with 0.15 M HEPES Buffer embedded in Nanoplast and stained with Uranyl (17k by 2.8).*

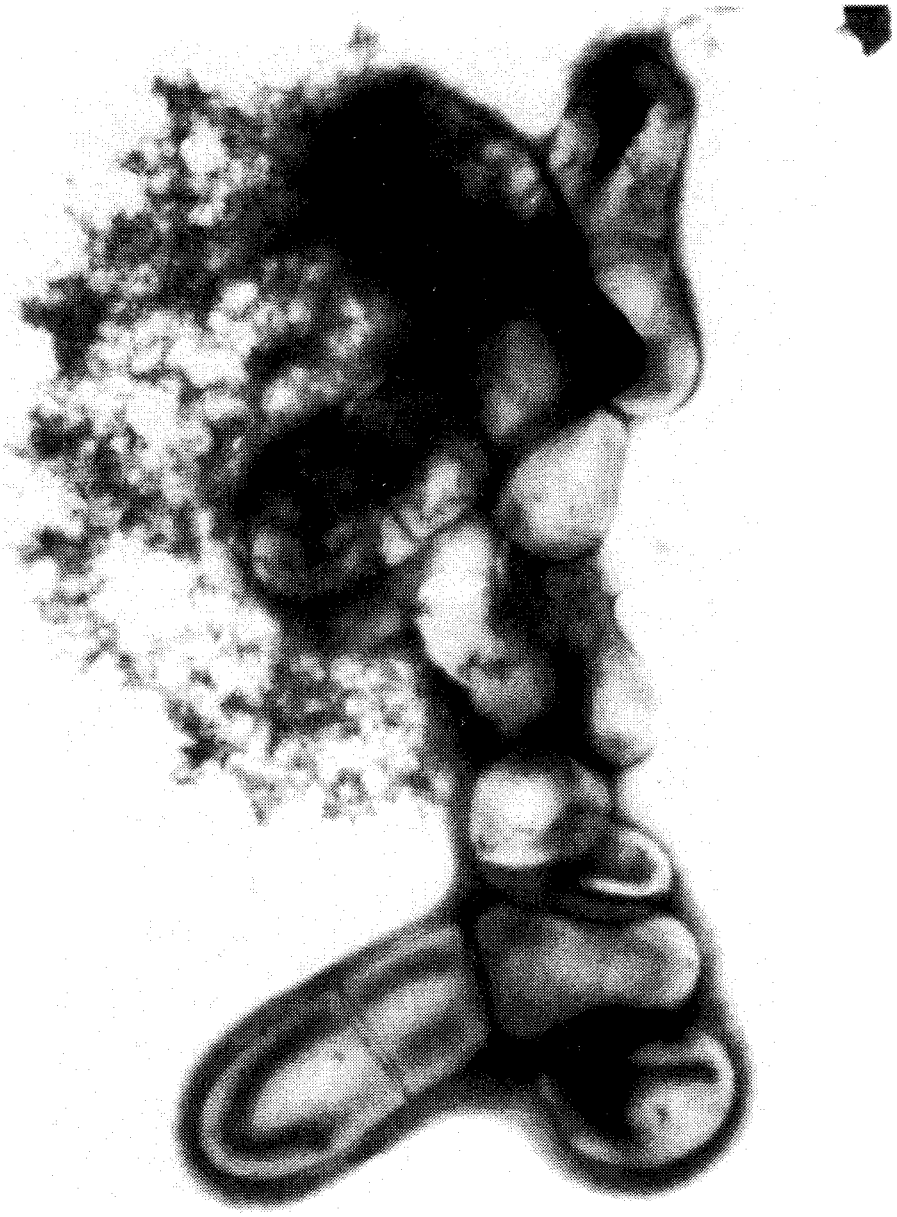


Figure 33 *TEM Micrograph of metal tube sample K-072-B, grown in M-9 and NB media (Appendix 6)(10k by 2.8).*

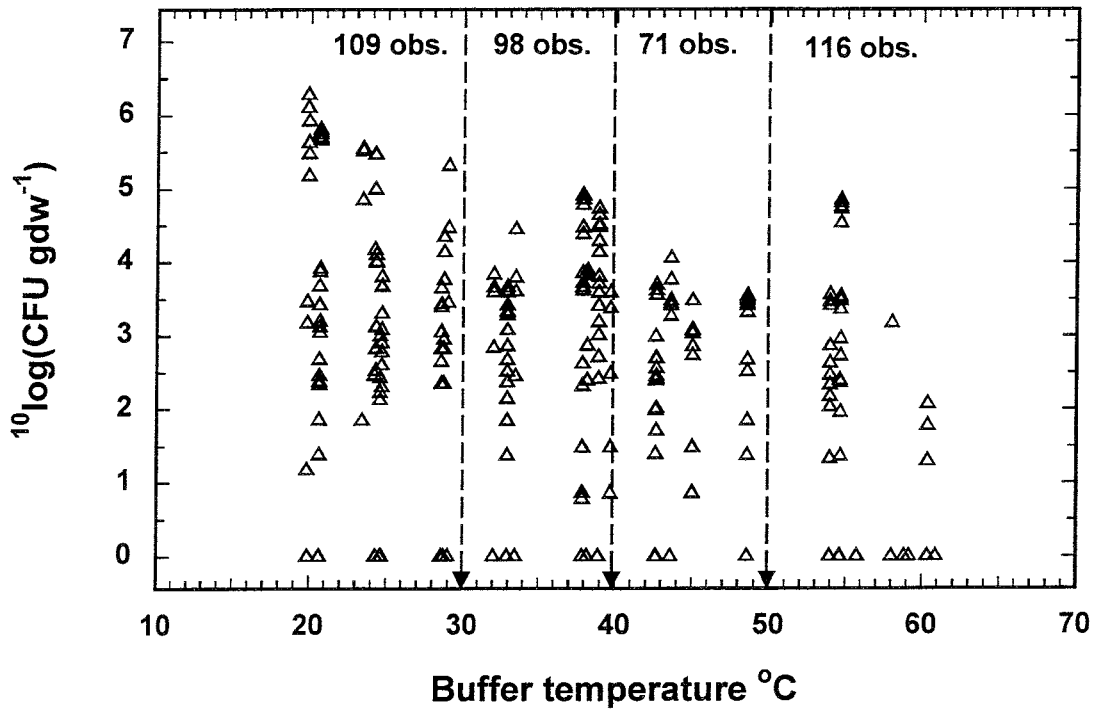


Figure 34 *The distribution of all colony forming unit (CFU) data obtained (394 observations) over the in situ temperatures for the buffer samples investigated. The four temperature classes that were used for the analysis of variance are indicated and the number of observations within each class level is presented.*

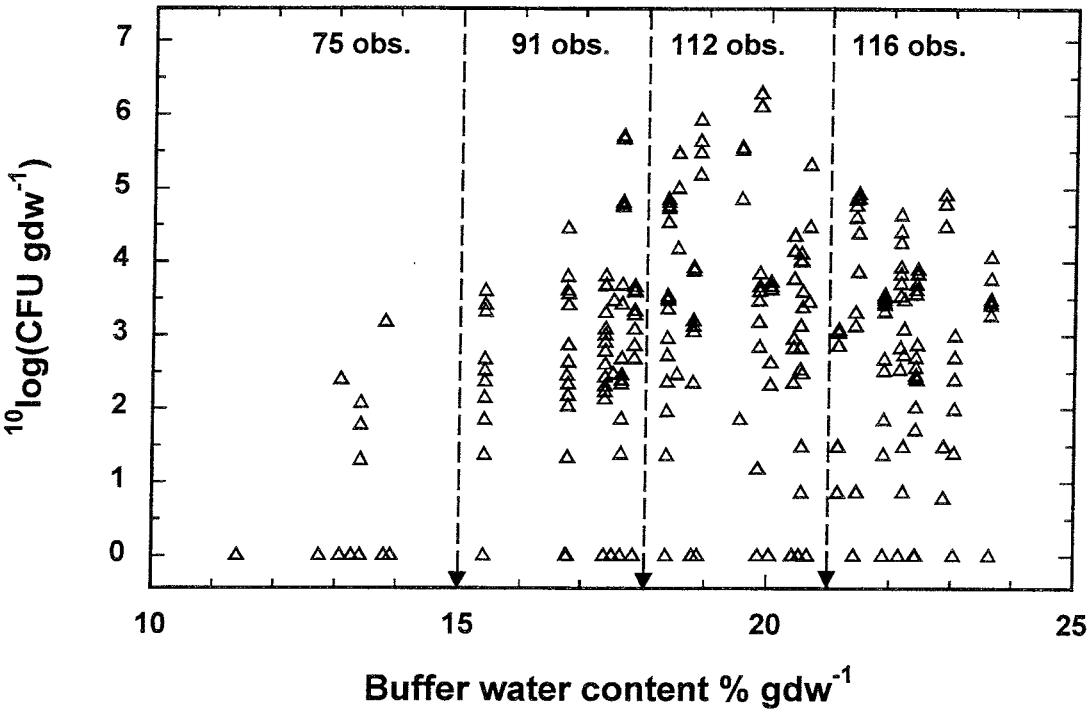


Figure 35 *The distribution of all colony forming unit (CFU) data obtained (394 observations) over the in situ water contents for the buffer samples investigated. The four water content classes that were used for the analysis of variance are indicated and the number of observations within each class level is presented.*

1 APPENDIX 1 - Buffer / container experiment decommissioning sampling procudures for Microbiology sample

1.1 INTRODUCTION

There were 8 types of microbial samples taken during the decommissioning of the Buffer-Container Experiment (BCE). Figure 6 (main text) shows the location of these samples. The step-by-step procedure that was followed for taking each of these samples, the location of the sample and the sample numbering are described in this Appendix. Sample analyses protocols are outlined in separate Appendices (Appendix 2 for protocols at WL (within 24 h of sample retrieval), Appendix 3 for protocol at UoG (16S rRNA analysis) and Appendix 4 for protocols at GRAM).

1.2 SAMPLING PROCEDURE 1

1.2.1 SAMPLE TYPE 1: BACKFILL SAMPLE FROM SECTION F

After the restraining columns are removed at the start of the decommissioning, the backfill at the top part of the experiment will be excavated. One sample of backfill is required from the middle area of the backfill for microbial analysis. This sample cannot be taken with a metal tube (diameter 1") because the backfill contains 3/4" large granite chunks. Instead, the sample (a small block of 10 by 10 by 10 cm approximately) needs to be excavated with sterile chisels (either sterilize by autoclaving prior to use or sterilize on site with alcohol). The sample number is F-001-B.

1. Have sterile knife, chisels, tongs and beaker (diameter at least 12 cm) ready.
2. Cut out ~10 by ~10 by ~10 cm block using sterile chisels.
3. Lift sample using sterile tongs and place in sterile beaker.
4. Cover beaker immediately with sterile lid.
5. Mark beaker with sample number F-001-B.
6. Transport sample immediately to the anaerobic glovebox in the Temporary Microbiology Laboratory (TML) (Room 212 on the 240 Level).
7. Record time between sample F-001-B excavation and arrival in anaerobic glove box.
8. Analyze sample according to Procedure 1 (Appendix 2).

1.3 SAMPLING PROCEDURE 2

1.3.1 SAMPLE TYPE 2: BACKFILL-BUFFER INTERFACE

As the backfill is being excavated and the buffer is almost reached, two samples need to be taken from the backfill-buffer interface to look for biofilms. The sample numbers are: F-002-B and F-002-B-F.

Have ready:

* Sterile chisels and scalpels
* 100 mL sterile PBS each in two sterile bottles containing sterile stirrer bars
* Number bottles F-002-IB and F-002-IB-F
* Preweigh the bottles
* Small sample vial with tight cap for dry weight

1. Chisel a block of backfill adjacent to the buffer out with sterile tools.
2. Scrape the backfill-buffer and buffer-backfill interfaces with a sterile scalpel and put scraped-off material in 100 mL PBS, aim for ~5 g material in bottle. Repeat for second bottle. Weigh bottles to determine added sample weight.
3. Also: Scrape some extra material off both interfaces, place in tightly sealed vial and determine dry weight as soon as possible, by weighing sample and drying to constant weight at 105°C. (F-002-IB-DW)
4. Degas bottles immediately with N2 in gassing station in TML. Cap tightly. Put sample F-002-IB-F in fridge for shipment to France.

   Note: Put green marks on samples to be shipped to France, blue marks on samples to be shipped to Sweden.
5. Place sample F-002-IB in anaerobic glovebox. Record time elapsed between sampling and sample arrival in anaerobic glovebox.
6. Analyze F-002-IB according to Procedure 2 (Appendix 2).

1.4 SAMPLING PROCEDURE 3

1.4.1 SAMPLE TYPE 3: METAL TUBE SAMPLES

These samples will be taken in conjunction with the moisture content metal tube samples in every lift. The microbial samples will be taken with 45-cm-long metal tubes in certain locations indicated in Figure 7 (lifts G,H,I,P,Q) and Figure 8 (lifts K,L,M,N,O), directly after 24-cm-tube samples are taken for moisture content (with sterilized 24-cm tubes) in these locations.

* The metal tube samples will be taken through a specially fabricated 109 hole template.

* The metal tubes will be have a serial number on the middle of the tube according to the following guidelines:

* Lift letter-sample number-sample type e.g., G-024-B (lift G, location 24, for microbiology) G-072-B (lift G, location 72, for microbiology)

* The microbiology tubes will be sterilized (24 h at 550°C)and will be distinguishable by the red sealing cap at the top end of the tube.

* As soon as the 24-cm-tube sample for moisture content is taken in any of the microbiology locations, a microbiology sample is taken immediately afterwards, to reduce any air diffusing into the sample.

* For example, as soon as sample G-024-T has been taken, sample G-024-B will be taken.

* Microbiology samples in lifts G,H,I,P and Q are required from locations: 24, 72, 73, 100 (AECL, WL), 16, 97 (SKB, UoG), 8, 92 (ANDRA, GRAM)

* For lifts K,L,M,N,O microbiology samples are required from locations: 24, 72, 60, 12 (AECL), 18, 66 (SKB) 6, 54 (F).

Procedure for each layer:

1. Have 8 sterilized and numbered 24-cm tubes ready for moisture content.

   Note: Sterilization can be done prior to sampling in autoclave or in situ using alcohol.

2. Have 8 sterilized and numbered 45-cm tubes ready (red caps) for microbiology.

3. Have sterilized spatulas, hot paraffin, alcohol and cloths ready.

4. Attach 24-cm tube to Hilty gun as described in "Procedure for taking metal tube samples" (Roach et al. 1995; Chandler et al. 1995).

5. Wipe outside of tube with alcohol on cloth prior to taking sample; take sample for moisture content.

6. Attach 45-cm tube to Hilty gun as described, wipe with alcohol.

7. Take sample for microbiology.

8. As soon as sample has been taken, cap with sterilized caps.

9. At empty end of tube, pour hot paraffin in to seal, swirl around, cap and let set.

10. Once paraffin has set, turn tube over, with sterile spatula scrape out 1 cm material, seal with hot wax, let set, cap.

11. Seal caps with gray tape, wipe tube with alcohol, put in plastic bag, add small bag with iron filings, squeeze out air and seal with bag sealer, double bag.

12. Put samples in fridge immediately.

13. Store all samples in fridge and prepare shipments to Sweden and France.

14. Store AECL samples (72 and 73 for lifts G,H,I,P,Q; 60 and 12 for lifts K,L,M,N,O) in fridge.

15. Put other AECL samples (24 and 100 for lifts G,H,I,P,Q; 24 and 72 for lifts K,L,M,N,O) in anaerobic glove box and start analytical Procedure 3 (Appendix 2).

16. Record approximate time between sampling time and start of analytical Procedure 3 (Appendix 2).

1.5 SAMPLING PROCEDURE 4

1.5.1 SAMPLE TYPE 4: HOLLOW STEM Auger SAMPLES

A total of 6 hollow stem auger samples are required, one in lift H, near the centre above the heater, one each in layers K, L, M and N around the heater and one in layer P near the centre below the heater (Figure 6, main text).

1. Have sterile hollow tubes (with caps) ready, numbered as follows

   H-001-AB(PLFA).
   K-001-AB
   L-001-AB
   M-001-AB(PLFA)
   N-001-AB-F
   P-001-AB(PLFA)

   Note: Have sterile scalpels ready

2. Attach hollow tube to auger, taking care not to touch the inside of the tube.

3. Wipe off tube with alcohol.

4. Take sample as per URL procedure (Roach et al. 1995; Chandler et al. 1995).

5. Detach tube from auger, cap.

6. Seal each end with hot paraffin (scrape some material out with sterile scalpel if necessary to make room for paraffin) and cap while paraffin is setting.

7. Wipe tube with alcohol.

8. Put in plastic bag to which several small bags of iron filings have been added, squeeze out air and seal. Double bag.

9. Freeze samples H-001-AB(PLFA), M-001-AB(PLFA) and P-001-AB(PLFA) as quickly as possible to -20°C and arrange to ship these to Microbial Insights, Knoxville, Tennessee in the USA for PLFA analysis.

10. Put the other auger tubes in the fridge (K-001-AB, L-001-AB and N-001-AB-F).

11. Ship sample N-001-AB-F to France as soon as possible.

1.6 SAMPLING PROCEDURE 5

1.6.1 SAMPLE TYPE 5: SAND SAMPLES

Four sand samples are required, one from layer J above the heater, two from layer L and one from layer N around the heater. The approximate locations of these samples is shown in Figure 6 (main text). The sand samples will be taken into sterilized glass vials, attached to a long stake, during the removal of the sand from around the heater with a large vacuum cleaner (Roach et al. 1995; Chandler et al. 1995).

1. Have sterile sand sampling vials ready. Number tubes as follows:

   J-001-SB
   L-001-SB
   L-004-SB-F
   N-001-SB

2. Attach vials to stake with tape

3. Take sand samples, measure approximate depth

4. Seal vials immediately upon retrieval

5. Seal in plastic bag

6. Double bag and put L-001-SB in fridge for shipment to France

7. Put other sand samples in anaerobic glove box and process same day using Procedure 4 (Appendix 2)

1.7 SAMPLING PROCEDURE 6

1.7.1 SAMPLE TYPE 6: BUFFER-GRANITE INTERFACE

The buffer-granite interface needs to be sampled in layers H, L, N and Q (Figure 6, main text). Two samples are required from layer L, one to be shipped to France. Sample numbering is as follows H-001-IB, L-001-IB, L-003-IB-F, N-001-IB, Q-001-IB.

Sample procedure:

1. Have sterile chisels and scalpels ready.

   Have 100 mL PBS ready in sterile bottles containing sterile stirrer bars.
   Preweigh the bottles.
   Sample vial with screwcap.

2. Chisel a block of buffer adjacent to the granite out.

3. Scrape the granite-buffer and buffer-granite interfaces with a sterile scalpel and put scraped-off material in 100 mL PBS, aim for ~5 g material in bottle. Weigh bottles to determine weight of sample.

4. Also: Scrape some extra material off both interfaces, place tightly sealed vial and determine dry weight as soon as possible, by weighing sample and drying to constant weight at 105°C. (Number this sample H-001-IB-DW, etc.).

5. Degas bottles immediately with N_2 in gassing station in TML. Cap tightly. Put sample L-003-IB-F in fridge for shipment to France.

6. Place samples H-001-IB, L-001-IB, N-001-IB and Q-001-IB in anaerobic glovebox, stir for 30 minutes.

7. Analyse samples according to Procedure 2 (Appendix 2).

1.8 SAMPLING PROCEDURE 7

1.8.1 SAMPLE TYPE 7: Teflon CLOTH SAMPLES FROM AROUND THE HEATER

Two samples will be cut using a sterile utility knife and/or sterile scissors and a pair of sterile tweezers. The samples should be taken before the crane has fully lifted the heater out of the borehole, to minimize contamination from handling. Sample numbering is as follows: HE-001-B, HE-003-B(SEM).

Have ready:

* Sterile utility knife (extra sterile blades).

* Sterile scissors.

* Sterile tweezers.

* Small sterile bottle containing 4% glutaraldehyde in cacodylate buffer *

* (HE-003-B(SEM).

* 1 small sterile bottle containing 50 mL PBS.

1. Watch carefully when heater is being attached to crane, to pick uncontaminated area for sample cutting.

2. Using sterile tools, cut out two samples (few cm wide and high).

3. Immediately put samples in sterile bottles and degas with N2 (or put in anaerobic glovebox).

4. Send sample HE-003-B(SEM) to Brenda Little in 4% glutaraldehyde in cacodylate buffer for Environmental SEM analysis.

5. Analyze HE-001-B using Procedure 5 (Appendix 2).

1.9 SAMPLING PROCEDURE 8

1.9.1 SAMPLE TYPE 8: ROCK PORE WATER

This sample type could not be taken.

1.10 REFERENCES

Chandler, N.A., A.W.L. Wan, R.A. Hampshire, D.S. Hnatiw, S.G. Keith, C.L. Kohle, P.J. Roach, L.A. Rolleston, V.P. Steiner and S. Stroes-Gascoyne. 1994. Decommissioning Procedures for the Buffer Container Experiment. Atomic Energy of Canada Limited Technical Record, TR-657*, COG-94-508.

Roach, P.J., R.A. Hampshire, S.G. Keith, C.L. Kohle, L.A. Rolleston and V.P. Steiner. 1995. The design, construction, testing and use of equipment for decommissioning the Buffer/Container Experiment. Atomic Energy of Canada Limited Technical Record, TR-695* (in preparation).

Table A-1-1 Overview of all microbial samples.

Sample Number	Sample Type	Sampling procedure (Appendix 1)	Analysis procedure (Appendix 2)
	Layer F		
F-001-B	backfill	1	1
F-002-IB	b/b interface	2	2
F-002-IB-F	b/b interface	2	ship to France
F-003-B	rust	2	2
F-007-B	b/b from underneath earth pressure cell	2	2

Sample Number	Sample Type	Sampling procedure (Appendix 1)	Analysis procedure (Appendix 2)
	Layer G		
G-024-B	metal tube	3	3
G-072-B	metal tube	3	archive in fridge
G-073-B	metal tube	3	archive in fridge
G-100-B	metal tube	3	3
G-016-B	metal tube	3	ship to Sweden
G-097-B	metal tube	3	ship to Sweden
G-008-B	metal tube	3	ship to France
G-092-B	metal tube	3	ship to France

Sample Number	Sample Type	Sampling procedure (Appendix 1)	Analysis procedure (Appendix 2)
	Layer H		
H-024-B	metal tube	3	3
H-072-B	metal tube	3	archive in fridge
H-073-B	metal tube	3	archive in fridge
H-100-B	metal tube	3	3
H-016-B	metal tube	3	ship to Sweden
H-097-B	metal tube	3	ship to Sweden
H-008-B	metal tube	3	ship to France
H-092-B	metal tube	3	ship to France
H-001-AB(PLFA)	hollow stem auger	4	freeze to -20°C ship to USA (PLFA)
H-001-IB	b/g interface	6	2

Sample Number	Sample Type	Sampling procedure (Appendix 1)	Analysis procedure (Appendix 2)
	Layer I		
I-024-B	metal tube	3	3
I-072-B	metal tube	3	archive in fridge
I-073-B	metal tube	3	archive in fridge
I-100-B	metal tube	3	3
I-016-B	metal tube	3	ship to Sweden
I-097-B	metal tube	3	ship to Sweden
I-008-B	metal tube	3	ship to France
I-092-B	metal tube	3	ship to France

Note: Samples in this layer may be taken with the template slightly rotated, because layer I is too thin for extended samples. For instance, I-024-B would be taken at the location between 1 and 24. Use the long, sterilized tubes, it is not necessary to use short tubes.

Sample Number	Sample Type	Sampling procedure (Appendix 1)	Analysis procedure (Appendix 2)
	Layer J		
J-001-SB	sand	5	4
HE-001-B	heater cloth	7	5
HE-003-B	heater cloth	7	ship to USA for Env. SEM analysis(SEM)
J-024-B	metal tube	3	3
J-072-B	metal tube	3	3
J-018-B	metal tube	3	archive
J-066-B	metal tube	3	archive
J-006-B	metal tube	3	archive
J-054-B	metal tube	3	archive

Note: Layer J tube samples were not planned but were taken and analyzed at WL only)

Sample Number	Sample Type	Sampling procedure (Appendix 1)	Analysis procedure (Appendix 2)
	Layer K		
K-024-B	metal tube	3	3
K-072-B	metal tube	3	3
K-060-B	metal tube	3	archive in fridge
K-012-B	metal tube	3	archive in fridge
K-018-B	metal tube	3	ship to Sweden

Sample Number	Sample Type	Sampling procedure (Appendix 1)	Analysis procedure (Appendix 2)
	Layer K		
K-066-B	metal tube	3	ship to Sweden
K-006-B	metal tube	3	ship to France
K-054-B	metal tube	3	ship to France
K-001-AB	hollow stem auger	4	archive in fridge

Sample Number	Sample Type	Sampling procedure (Appendix 1)	Analysis procedure (Appendix 2)
	Layer L		
L-024-B	metal tube	3	3
L-072-B	metal tube	3	3
L-060-B	metal tube	3	archive in fridge
L-012-B	metal tube	3	archive in fridge
L-018-B	metal tube	3	ship to Sweden
L-066-B	metal tube	3	ship to Sweden
L-006-B	metal tube	3	ship to France
L-054-B	metal tube	3	ship to France
L-001-AB	hollow stem auger	4	archive in fridge
L-001-SB	sand	5	4
L-004-SB-F	sand	5	ship to France
L-001-IB	b/g interface	6	2
L-003-IB	b/g interface	6	ship to France

Sample Number	Sample Type	Sampling procedure (Appendix 1)	Analysis procedure (Appendix 2)
	Layer M		
M-024-B	metal tube	3	3
M-072-B	metal tube	3	3
M-060-B	metal tube	3	archive in fridge
M-012-B	metal tube	3	archive in fridge
M-018-B	metal tube	3	ship to Sweden
M-066-B	metal tube	3	ship to Sweden
M-006-B	metal tube	3	ship to France
M-054-B	metal tube	3	ship to France
M-001-AB(PLFA)	hollow stem auger	4	freeze to -20°C ship to USA (PLFA)

Sample Number	Sample Type	Sampling procedure (Appendix 1)	Analysis procedure (Appendix 2)
	Layer N		
N-024-B	metal tube	3	3
N-072-B	metal tube	3	3
N-060-B	metal tube	3	archive in fridge
N-012-B	metal tube	3	archive in fridge
N-018-B	metal tube	3	ship to Sweden
N-066-B	metal tube	3	ship to Sweden
N-006-B	metal tube	3	ship to France
N-054-B	metal tube	3	ship to France
N-001-AB-F	hollow stem auger	4	ship to France
N-001-SB	sand sample	5	4
N-001-IB	b/g interface	6	2

Sample Number	Sample Type	Sampling procedure (Appendix 1)	Analysis procedure (Appendix 2)
	Layer 0		
O-024-B	metal tube	3	3
O-072-B	metal tube	3	3
O-060-B	metal tube	3	archive in fridge
O-012-B	metal tube	3	archive in fridge
O-018-B	metal tube	3	ship to Sweden
O-066-B	metal tube	3	ship to Sweden
O-006-B	metal tube	3	ship to France
O-054-B	metal tube	3	ship to France

Note: This layer is thin, and samples may be taken with template slightly rotated as in layer I.

Sample Number	Sample Type	Sampling procedure (Appendix 1)	Analysis procedure (Appendix 2)
	Layer P		
P-024-B	metal tube	3	3
P-072-B	metal tube	3	archive in fridge
P-073-B	metal tube	3	archive in fridge
P-100-B	metal tube	3	3
P-016-B	metal tube	3	ship to Sweden
P-097-B	metal tube	3	ship to Sweden
P-008-B	metal tube	3	ship to France

Sample Number	Sample Type	Sampling procedure (Appendix 1)	Analysis procedure (Appendix 2)
	Layer P		
P-092-B	metal tube	3	ship to France
P-001-AB	hollow stem auger	4	freeze to -20°C ship to USA (PLFA)

Sample Number	Sample Type	Sampling procedure (Appendix 1)	Analysis procedure (Appendix 2)
	Layer Q		
Q-024-B	metal tube	3	3
Q-072-B	metal tube	3	archive in fridge
Q-073-B	metal tube	3	archive in fridge
Q-100-B	metal tube	3	3
Q-016-B	metal tube	3	ship to Sweden
Q-097-B	metal tube	3	ship to Sweden
Q-008-B	metal tube	3	ship to France
Q-092-B	metal tube	3	ship to France
Q-001-IB	b/g interface	6	2

Note: metal tube samples to be pushed in as far as the rock. These samples may therefore have less material in, because layer is thin. Alternatively, sampling may be done through a slightly rotated template, as for layer I.

2 APPENDIX 2 - Buffer container experiment decommissioning Microbial analyses procedures and protocols for analysis performed at Whiteshell Laboratories within 24 h after sample retrieval

2.1 ANALYSIS PROCEDURE 1

2.1.1 MICROBIAL ANALYSIS OF BACKFILL SAMPLE:

1. Record time between sample F-001-B excavation and arrival in anaerobic glovebox in TML.

2. Have ready (in glovebox):

   Sterile scalpel and hammer.
   Sterile mortar (preweighed) and pestle.
   mL sterile PBS with sterile stirrer bar (mark bottle F-001-B-1).
   Sterile spatulas.
   mL sterile sample bottles (with 0.2 mL 50% glutaraldehyde) for:
   AO counting (F-001-B-2).
   DAPI counting (F-001-B-3).
   mL sample bottle for archiving (F-001-B-4).
   mL sample bottle for BARTS (F-001-B-5).
   Sterile plates.
   R2A medium.
   Sterile dilution tubes with 18 mL PBS.
   Sterile pipettes for dilutions.

3. Place sample block on sterile surface; break sample open, using sterile tools.

4. Take sample from innermost part of backfill block using sterile spatula and place in mortar. Weigh mortar to determine weight of sample (should be around 5 g). Store rest of sample in beaker until it is known that the analysis has been successful.

5. Crush sample with sterile pestle and transfer sample to bottle with 100 mL sterile PBS. The sample number is F-001-B-1.

6. Stir sample for 30 minutes.

7. Take 5 mL samples F-001-B-2 (AO counting) and F-001-B-3 (DAPI counting) Count AO and DAPI sample when convenient.

8. Fill the 10 mL sample bottle to overflowing and seal. This sample can then be archived in the fridge for future reference if needed. This sample should be marked F-001-B-4.

9. Take 50 mL sample for BART's analysis in 50 mL bottle (F-001-B-5). Do BART's tubes as soon as possible.

10. With remaining 30 mL sample in F-001-B-1 bottle, make 10-1, 10-2, 10-3 dilutions in dilution tubes (2 mL sample in 18 mL PBS). Number these dilutions F-001-B-1(10-1), F-001-B-1(10-2) and F-001-B-1(10-3), mix well.

11. Using pour plate technique, plate 1 ml of each dilution out on R2A medium. Make 12 plates per dilution. Number plates as the dilutions. Incubate 3 plates anaerobically at room temperature (in glovebox), 3 plates anaerobically at 50°C in anaerobic jar or bag, 3 plates aerobically at room temperature (cover with foil) and 3 plates aerobically at 50°C. Enumerate plates after 5 days.

    If 5 days incubation is not enough, count after 10 days, to be decided as the need arises.

12. Take a sample from backfill block to determine dry weight, by weighing sample accurately and drying to constant weight in 105°C oven, as soon as possible after backfill sample F-001-B-1 has been taken (F-001-B-DW).

2.2 ANALYSIS PROCEDURE 2

2.2.1 MICROBIAL ANALYSIS OF INTERFACE SCRAPING SAMPLES:

1. Record time between sample taking and bottle with sample arriving in anaerobic glovebox.
2. Have ready in glovebox:

   mL sterile sample bottles (with 0.2 mL 50% glutaraldehyde) for:
   AO counting (X-00y-IB-2).
   DAPI counting (X-00y-IB-3)).
   mL sample bottle for archiving (X-00y-IB-4).
   mL sample bottle for BARTS (X-00y-IB-5).
   Sterile plates.
   R2A medium.
   Sterile dilution tubes with 18 mL PBS.
   Sterile pipettes for dilutions.
3. Stir bottle (X-00y-IB) for 30 minutes (X = layer letter; y is sample number; I = interface sample; B = biology sample).(For sample numbers see Appendix 1).
4. Take 5 mL samples X-00y-IB-2 (AO counting) and X-00y-IB-3 (DAPI counting). Count AO and DAPI sample when convenient.
5. Fill the 10 mL sample bottle to overflowing and seal. This sample can then be archived in the fridge for future reference if needed. This sample should be marked X-00y-IB-4.
6. Take 50 mL sample for BART's analysis in 50 mL bottle (X-00y-IB-5). Do BART's tubes as soon as possible.
7. With remaining sample in X-00y-IB bottle, make 10-1, 10-2, 10-3, dilutions in dilution tubes (2 mL sample in 18 mL PBS). Number these dilutions X-00y-IB(10-1), X-00y-IB(10-2), X-00y-IB(10-3), mix well.
8. Using pour plate technique, plate 1 ml of each dilution out on R2A medium. Make 12 plates per dilution. Number plates as the dilutions.

   Incubate 3 plates anaerobically at room temperature (in glovebox),
   3 plates anaerobically at 55°C in anaerobic jar or bag,
   3 plates aerobically at room temperature (cover with foil) and
   3 plates aerobically at 55°C. Enumerate plates after 5 days.
   If 5 days incubation is not enough, count after 10 days, to be decided as the need arises.
9. Determine dry weight on sample X-00y-IB-DW, taken during procedure 2 or 6, Appendix 1.

2.3 ANALYSIS PROCEDURE 3

2.3.1 MICROBIAL ANALYSIS OF METAL TUBE SAMPLES:

Have ready in anaerobic glove box:

* Sterile PBS, 200 mL per bottle, containing sterile stirrer bar.
* Magnetic stirrer.
* Sample extruder.
* Sterile scalpels, knives, hammer.
* Scale.
* Sterile mortar and pestle.
* Alcohol + kim wipes.
* 10 mL sterile bottles with 0.2 mL 50% glutaraldehyde (2x).
* 10 mL sterile bottle for archive sample.
* 50 mL sterile bottles for subsamples (BART's, Leucine, Hungate) (3x).
* Plates.
* Pipettes.
* Dilution tubes with PBS (18 mL).

1. Transfer tube sample to anaerobic glovebox port immediately after sample has been taken and sealed.
2. Transfer sample to glove box, record time of arrival.
3. Clamp sample in extruder and extrude sample.
4. Discard first ~ 3 cm, use for dry weight determination (X-0yy-B-DW).
5. Cut off ~2 cm material with scalpel or hammer, drop in mortar, seal tube with paraffin and archive in fridge sealed in plastic.
6. Grind sample.
7. Transfer ~ 10 g of sample to (preweighed) 200 mL PBS, stir for 30 minutes on magnetic stirrer. Weigh to determine sample weight.Sample number is X-0yy-B-1. (X = layer letter, yy = shelby tube location number).

8. Take 5 mL for AO counting in 0.2 mL of a 50% Glutaraldehyde solution (Sample number is X-0yy-B-2). Count sample when convenient.

9. Take 5 mL for DAPI counting in 0.2 mL glutaraldehyde (50%) (Sample number is X-0yy-B-3). Count sample when convenient.

10. Fill 10 mL sterile bottle to overflowing and cap for archived sample (Sample number is X-0yy-B-4). Archive in fridge.

11. Fill 50 mL sterile bottle for BART tests. Sample bottle number is X-0yy-B-5. Do BARTS as soon as possible.

12. Fill 50 mL sterile bottle for 3H Leucine test. Sample bottle number is X-0yy-B-6. Start test as soon as possible.

13. Fill 50 mL sterile bottle for SRB, Methanogens and Fermenters. Sample bottle number is X-0yy-B-7. Start cultures as soon as possible.

14. With remaining 30 mL of sample X-0yy-B-1 make dilutions. Add 2 mL to 18 mL PBS, then 2 mL of that to the next 18 mL PBS, etc. Make 3 dilutions: 10-1, 10-2 and 10-3. Number dilutions. X-0yy-B-1(10-1), X-0yy-B-1(10-2) and X-0yy-B-1(10-3). Using pour plate technique, plate 1 mL of each dilution on R2A medium. Make 12 plates of each dilution. Number plates as the dilutions.

    Incubate 3 plates anaerobically at room temperature (in glovebox),

    3 plates anaerobically at 50°C in anaerobic jar or bag,
    3 plates aerobically at room temperature (cover with foil) and
    3 plates aerobically at 50°C. Enumerate plates after 5 days.

    If 5 days incubation is not enough, count after 10 days, to be decided as the need arises.

15. Determine dry weight on sample X-0yy-B-DW.

2.4 ANALYSIS PROCEDURE 4

2.4.1 MICROBIAL ANALYSIS OF SAND SAMPLES:

Have ready in glove box:

* Sterile PBS, 100 mL per bottle, containing sterile stirrer bar.
* Magnetic stirrer.
* Sterile spoon and spatula.
* Scale.
* Alcohol + kim wipes.
* 10 mL sterile bottles with 0.2 mL 50% glutaraldehyde (2x).
* 10 mL sterile bottle for archive sample.
* 50 mL sterile bottles for BART's.
* Plates.
* R2A medium.
* Pipettes.
* Dilution tubes with PBS (18 mL).

1. Record time between sand sample taken and arrival in glovebox. Open sample vial.

2. Spoon out ~ 5 g sample, put in preweighed 100 mL PBS bottle and weigh.

   Mark sample bottle X-00y-SB-1. (X = layer letter, y = sample number, S = sand, B = biology).

3. Stir for 30 minutes on magnetic stirrer.

4. Take 5 mL for AO counting in 0.2 mL of a 50% glutaraldehyde solution. Mark bottle X-00y-SB-2. Count when convenient.

5. Make 5 mL for DAPI counting in 0.2 mL glutaraldehyde (50%) (Sample number is X-00y-SB-3). Count sample when convenient

6. Fill 10 mL sterile bottle to overflowing and cap for archived sample (Sample number is X-00y-SB-4). Archive in fridge.

7. Fill 50 mL sterile bottle for BART tests. Sample bottle number is X-00y-SB-5. Do BARTS as soon as possible.

8. With remaining 30 mL of sample X-00y-SB-1 make dilutions :

   Add 2 mL to 18 mL PBS, then 2 mL of that to the next 18 mL PBS, etc.

   Make 3 dilutions: 10^{0}, 10^{-1} and 10^{-2}. Number dilutions.

   X-00y-SB-1(10^{0}), X-00y-SB-B-1(10^{-1}) and X-00y-SB-1(10^{-2}). Using pour plate technique, plate 1 mL of each dilution on R2A medium. Make 12 plates of each dilution. Number plates as the dilutions.

   Incubate 3 plates anaerobically at room temperature (in glovebox),

   3 plates anaerobically at 50°C in anaerobic jar or bag,

   3 plates aerobically at room temperature (cover with foil) and

   3 plates aerobically at 50°C. Enumerate plates after 5 days.

   If 5 days incubation is not enough, count after 10 days, to be decided as the need arises.

9. Take another sample from the sand sample tube, number it X-00y-SB-DW and determine the dry weight of this sample.

2.5 ANALYSIS PROCEDURE 5

2.5.1 MICROBIAL ANALYSIS OF HEATER TEFLON CLOTH SAMPLE:

Have ready in anaerobic glovebox in TML:

* 100 mL sterile PBS with sterile stirrer bar (mark bottle HE-001-B-1).
* Sterile tweezers.
* 10 mL sterile sample bottles (with 0.2 mL 50% glutaraldehyde) for:
* AO counting (HE-001-B-2).
* DAPI counting (HE-001-B-3).
* 10 mL sample bottle for archiving (HE-001-B-4).
* 50 mL sample bottle for BARTS (HE-001-B-5).
* Sterile plates.
* R2A medium.
* Sterile dilution tubes with 18 mL PBS.
* Sterile pipettes for dilutions.

1. With sterile tweezers, transfer sample of cloth (HE-001-B) to 100 mL PBS (HE-001-B-1).
2. Stir sample for 30 minutes.
3. Take samples HE-001-B-2 (AO counting) and HE-001-B-3 (DAPI counting) Count AO and DAPI sample when convenient.
4. Fill the 10 mL sample bottle to overflowing and seal. This sample can then be archived in the fridge for future reference if needed. This sample should be marked HE-001-B-4.
5. Take 50 mL sample for BART's analysis in 50 mL bottle (HE-001-B-5). Do BART's tubes as soon as possible.
6. With remaining sample in HE-001-B-1 bottle, make 10^0, 10^{-1}, 10^{-2}, dilutions in dilution tubes (2 mL sample in 18 mL PBS). Number these dilutions HE-001-B-1(10^0), HE-001-B-1(10^{-1}) and HE-001-B-1(10^{-2}), mix well.
7. Using pour plate technique, plate 1 ml of each dilution out on R2A medium. Make 12 plates per dilution. Number plates as the dilutions. Incubate 3 plates anaerobically at room temperature (in glovebox), 3 plates anaerobically at 50°C in anaerobic jar or bag, 3 plates aerobically at room temperature (cover with foil) and 3 plates aerobically at 50°C. Enumerate plates after 5 days.

   If 5 days incubation is not enough, count after 10 days, to be decided as the need arises.

2.6 TOTAL VIABLE HETEROTROPHS

Total viable heterotrophs were determined on R2A medium, aerobically and anaerobically at ambient laboratory temperature (17°C) and 50°C, by pour-plate method. R2A (Reasoner and Geldreich 1985) was originally formulated for the enumeration and isolation of bacteria from wastewater, but has given excellent results for groundwater samples as well. R2A gave the best results for enumerating archived buffer materials (Table 1, main text) (Haveman et al. 1995).

Composition of R2A medium:

Ingredient	**Concentration (g/L Distilled Deionized Water)**
Difco Bacto Peptone	0.50
Casamino Acids	0.50
Yeast Extract	0.50
Glucose	0.50
Soluble Starch	0.50
K_2HPO_4	0.30
$MgSO_4.7H_2O$	0.05
Sodium Pyruvate	0.30
Agar	15.0

2.7 BIOLOGICAL ACTIVITY AND REACTION TEST(BART) FOR IRON-RELATED BACTERIA (IRB), SULPHATE-REDUCING BACTERIA (SRB) AND SLIMEFORMING BACTERIA (SLYM)

BART™ (Droycon Bioconcepts Inc., Regina, Saskatchewan) tests are a quick diagnostic test designed to identify groups of microorganisms present in a water sample, as well as their aggressiveness within the sample (Cullimore 1993). Three different types of BART tests were used: Iron-Related Bacteria (IRB), Suphate-Reducing Bacteria (SRB) and Slime-Forming Bacteria (SLYM). Each BART tube contains a small amount of dried medium as well as a sterile plastic ball. When 15 mL of the undiluted water sample is added to each BART tube, the ball floats at the top of the sample and minimizes the diffusion of oxygen into the sample, creating an oxygen gradient throughout the tube. The tubes are incubated in the dark at room temperature for 21 days and monitored daily for changes in appearance (e.g., colour, turbidity, gas formation). Reactions are entered into the BART-SOFT computer program that gives the aggressiveness and possible population of each group of bacteria as well as a list of possible genera of bacteria present in the sample.

2.8 SULPHATE-REDUCING BACTERIA

2.8.1 University of Göteborg method and medium

An anoxic mineral medium was used to enrich sulphate reducing bacteria. It consisted of $CaCl_2$ x $6H_2O$, 0.88 g; KCl, 1.26 g; KH_2PO_4, 0,4 g; $MgCl_2$ x $6H_2O$, 0.82g; NaCl, 17.54 g; NH_4Cl, 0.54 g; deionized water, 1 litre. This solution was autoclaved and cooled under N_2/CO_2 (90%/10%). To the cooled medium was added sterile: 2 ml trace metal solution, 2 ml selenite and tungsten solution, 1 ml mixed vitamin solution, 2 ml vitamin B_{12} solution, 60 ml $NaHCO_3$ (8.4 g/100 ml), 6 ml Na_2S (9g/100 ml) and pH was adjusted to 7.0 to 7.4.

The trace metal solution consisted of $FeCl_2$, 1.5 g; $ZnCl_2$, 0.07 g; $MnCl_2$ x $2H_2O$, 0.08 g; H_3BO_3, 0.006 g; $CoCl_2$ x 6 H_2O, 0.19 g; $CuCl_2$ x $2H_2O$, 0.002 g; $NiCl_2$ x $6H_2O$, 0.024 g; Na_2MoO_4 x $2H_2O$, 0.036 g and double distilled water, 1 litre.

The selenite and tungsten solutions consisted of NaOH, 0.5 g; Na_2SeO_3 x $5H_2O$, 0.003 g; Na_2WO_4 x $2H_2O$, 0.004 g and double distilled water. Both solutions were autoclaved at 121°C for 20 min.

The mixed vitamin solution consisted of p-aminobenzoic acid, 0.008 g; D(+)-biotin, 0.002 g; nicotinic acid, 0.02 g; Ca-D(+)-pantotenic acid, 0.01 g; pyridoxamine dihydrochloride, 0.03 g; thiaminiumdichloride, 0.02 g and double distilled water, 100 ml.

The vitamin B_{12} solution consisted of cyanocobalamin, 0.05 g in 1 litre double distilled water. The vitamin solutions were sterile filtered through 0.2 mm pore sized filters.

The $NaHCO_3$ solution (8.4g/100 mL) was infused with CO_2 for 5 min. and autoclaved in a screw-capped bottle.

The Na_2S solution (9g/100 mL) was autoclaved under nitrogen atmosphere.

The medium was divided into pre-gassed (N_2) serum bottles with 0.5 ml 50% sterile lactate solution and 10 ml 350 mM sterile Na_2SO_4 solution. The bottles were gassed with N_2 during the replenishment and sealed with aluminium crimp-sealed butyl rubber stoppers.

To autoclaved, tempered (60°C) Hungate tubes with 5 ml melted 30% (w/v) agar (Bacto) were added 6 ml of the salt medium described above and the tubes were gassed with N_2/CO_2 (90/10) for several minutes. The tubes were sealed with aluminium crimp-sealed butyl rubber stoppers and the temperature lowered to 40°C. The tubes were inoculated and subsequently cooled while rotating (so called roller-tubes).

2.9 METHANE-PRODUCING BACTERIA

2.9.1 University of Göteborg method and medium

This medium is described by Whitman et al. (1992). Its composition is per litre of medium: Yeast extract, 2.0 g; trypticase peptones, 2.0 g; salt solution A, 10 ml; phosphate solution (200 g/l of K_2HPO_4 x $3H_2O$), 2 ml; resazurin solution(0.5 g l^{-1}), 2 ml; sodium acetate solution (136 g l^{-1} Na-acetate x 3 H_2O) 10 ml; trace element solution, 10 ml; vitamin solution, 10 ml; $NaHCO_3$, 5.0 g; cysteine hydrochloride, 0.5 g; sulphide solution, 20 ml.

Salt solution A is composed of (per litre): NH_4Cl, 100 g; $MgCl_2$ x $6H_2O$, 100 g and $CaCl_2$ x $2H_2O$, 40 g. After dissolving the salts, the pH was adjusted to 4 with HCl. Sterilized at 121°C for 20 min.

The trace element solution is modified from Wolin et al. (1963)and is composed of (per litre): Nitrilotriacetic acid, 1.5 g; $Fe(NH_4)_2(SO_4)_2$ x $6H_2O$, 0.2 g; Na_2SeO_3, 0.2 g; $CoCl_2$ x $6H_2O$, 0.1 g; $MnSO_4$ x $6H_2O$, 0.1 g; Na_2MoO_4 x $2H_2O$, 0.1 g; Na_2WO_4 x $2H_2O$, 0.1 g; $ZnSO_4$ x $7H_2O$, 0.1 g; $AlCl_3$ x $6H_2O$, 0.04 g; $NiCl_2$ x $6H_2O$, 0.025 g; H_3BO_3, 0.01 g; $CuSO_4$ x $5H_2O$, 0.01 g. The solution was prepared by dissolving nitrilotriacetic acid in 800 ml of water and adjusting the pH to 6.5 with KOH, dissolving the minerals in order, adjusting the pH to 7.0 and bringing the volume to 1 litre. The solution was sterilized at 121°C for 20 min.

The vitamin solution was modified from Bryant et al. (1971)and is composed of (per litre): *p*-aminobenzoic acid, 10 mg; nicotinic acid, 10 mg; calcium pantothenate, 10 mg; pyridoxine hydrochloride, 10 mg; riboflavin, 10 mg; thiamine hydrochloride, 10 mg; biotin, 5 mg; folic acid, 5 mg; *a*-lipoic acid, 5 mg; Vitamin B_{12} (stored in dark at 5 °C), 5 mg. The solution was filter sterilized, pore size 0.22 mm.

The sulphide solution was prepared as follows: 250 ml double distilled water was boiled for at least 2 min. The Erlenmeyer flask was infused with N_2 and capped with a rubber stopper and cooled on ice. 50 ml of the boiled water and 4.5 g Na_2S x 9 H_2O were added to a pregassed (N_2) serum bottle and capped with an aluminium crimped sealed butyl rubber stopper and autoclaved at 121°C for 20 min.

All solutions were added sterile to a base medium consisting of 2.0 g yeast extract and 2.0 g trypticase peptones per litre, which was autoclaved and thereafter cooled under N_2/CO_2 atmosphere (80:20). The medium was divided in 18 ml portions to pregassed 100 ml serum bottles and capped with aluminium crimp-sealed butyl rubber stoppers. Roller-tubes, as described above for the SRB, were prepared with the medium for methane producing bacteria.

2.10 THERMOPHILIC ANAEROBIC FERMENTING BACTERIA

2.10.1 University of Göteborg method and medium

The organic medium consisted of (per litre): glucose, 0.1 g; yeast extract, 0.1 g; peptone, 0.05 g, tryptone, 0.05 g,; $MgSO_4$ x $7H_2O$, 0.6 g; $CaCl_2$ H_2O, 0.6 g; agar, 30 g and finally diluted in PBS buffer as described below. The pH was adjusted to 8.5 to 9.0 after sterilization.

The samples were diluted in a 0.01 M, pH 7.6 phosphate buffer before inoculation. The phospate buffer consists of 100 ml of a stock solution and 900 ml double distilled water. The stock solution consists of (per litre): Na_2HPO_4, 12.36 g; NaH_2PO_4 x H_2O, 1.80 g and NaCl, 85.0 g.

The dilution series were prepared as follows: 5 g of the buffer mass from each sampling site were anaerobically diluted in PBS buffer to a 2 x 10^1 dilution (i.e., 5 g in 100 mL PBS or 10 g in 200 mL PBS) and thereafter 2 x 10^2 and 2 x 10^3 dilution's. Duplicate tubes were inoculated with 1 ml of each dilution and incubated at 51°C.

2.11 BACTERIAL ACTIVITY USING ^{3}H LEUCINE ASSIMILATION AT 50°C

2.11.1 University of Göteborg method

This method has earlier been applied on groundwaters and surfaces from the Stripa and Äspö research areas Pedersen and Ekendahl 1992a,b). A total of 5 g buffer material was diluted in 100 ml anaerobic, sterile PBS (Appendix 1) in sterile serum bottles and properly closed with aluminium crimped sealed butyl rubber stoppers. Three samples of 10 ml were taken from each sampling level: one for determination of total number of bacteria and two for leucine uptake. To the leucine uptake bottles were added 1 ml of a 0.033 µM L-(4,5-^{3}H)-leucine, giving a final concentration of 3 nM and an activity of 4.56 x 10^{-7} Ci ml^{-1}. To the control bottles were immediately added 1 ml concentrated formaldehyde to stop all reactions. Both samples and controls were incubated on a shaker for 6 hours at 50°C in the dark. The reactions were stopped with formaldehyde as for the control. From both controls and samples were 4 portions of a predetermined volume filtered onto sterile Nuclepore filters (0.22 µm pore size). The filters used in the microautoradiography were rinsed with 2 x 1 ml 1 % oxalic acid and the ones used in scintillation measurements were rinsed with 2 x 1 ml PBS buffer.

Scintillation Counting:

The filters for scintillation counts were put in 10 ml Insta-Gel Plus (Packard) scintillation cocktail and the radioactivity was measured in a Packard scintillation counter.

Microautoradiographic studies of bacteria active in Leucine uptake:

The MARGE-E technique (Pedersen and Ekendahl 1992a,b; Tabor and Neihof 1982) was used to determine the leucine uptake activity of individual bacteria in each sample of buffer mass. The exposure time was 48 h under vacuum over silica gel at 4°C. The active bacteria were counted in a Leitz Laborlux 12 Pol epifluorescence microscope. Cells with at least 3 silver grains a maximum of 1 µm from the cell were counted as active.

2.12 REFERENCES

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Table A-2-1 Complete bart test results for all samples analyzes at WL (population numbers are in bacteria/g dry material)

Sample Number:	**G-024-B**
IRB	
Possible population	8.0×10^5
Aggressivity	highly aggressive iron bacteria
Composition	dominated by anaerobic activity
Possible genera	methanogenic bacteria, enteric bacteria, vibrioids
SRB	
Possible population	20
Aggressivity	few SRB recovered
Composition	
Possible genera	*Dsulfovibrio, Desulfotomaculum, Desulfuromonas*
SLYM	
Possible population	6.7×10^5
Aggressivity	very aggressive slime formers
Composition	aerobic slime forming bacteria
Possible genera	*Pseudomonas, Micrococcus, Zoogloea, Bacillus*
Sample Number:	**G-100-B**
IRB	
Possible population	4.9×10^6
Aggressivity	highly aggressive iron bacteria
Composition	dominated by anaerobic activity; pseudomonads present
Possible genera	methanogenic bacteria, enteric bacteria, vibrioids
SRB	
Possible population	20
Aggressivity	few SRB recovered
Composition	SRB bacteria in dense slimes
Possible genera	*Desulfovibrio, Desulfotomaculum, Desulfuromonas*
SLYM	
Possible population	1.4×10^3
Aggressivity	slime bacteria present
Composition	dense aerobic slime generators
Possible genera	*Klebsiella, Enterobacter, Acinetobacter, Bacillus, Lactobacillus, Staphylococcus*

Sample Number:	**H-024-B**
IRB	
Possible population	0
Aggressivity	no reactions
Composition	
Possible genera	
SRB	
Possible population	0
Aggressivity	no reactions
Composition	
Possible genera	
SLYM	
Possible population	0
Aggressivity	no reactions
Composition	
Possible genera	

Sample Number:	**H-100-B**
IRB	
Possible population	0
Aggressivity	no reactions
Composition	
Possible genera	
SRB	
Possible population	0
Aggressivity	no reactions
Composition	
Possible genera	
SLYM	
Possible population	6.7×10^5
Aggressivity	very aggressive slime formers
Composition	mixed enteric + Pseudomonad flora
Possible genera	*Pseudomonas, Micrococcus, Janthinobacterium, Chromobacterium Acinetobacter, Vibrio, Aeromonas, Enterobacter, Serratia, Klebsiella, Escherichia, Proteus, Desulfovibrio*

Sample Number:	**I-024-B**
IRB	
Possible population	8.0×10^5
Aggressivity	highly aggressive iron bacteria
Composition	dominated by anaeorbic activity
Possible genera	methanogenic bacteria, enteric bacteria, virbrioids
SRB	
Possible population	20
Aggressivity	few SRB recovered
Composition	SRB bacteria in dense slime
Possible genera	*Desulfovibrio, Desulfotomaculum, Desulfuromonas*
SLYM	
Possible population	5.7×10^2
Aggressivity	slime bacteria present
Composition	dense aerobic slime generators
Possible genera	*Klebsiella, Enterobacter, Acinetobacter, Bacillus, Lactobacillus, Saphylococcus*

Sample Number:	**I-100-B**
IRB	
Possible population	2.0×10^3
Aggressivity	very low iron bacterial population
Composition	aerobic slime formers
Possible genera	*Pseudomonas, Zoogloea, Janthinobacterium, Chromobacterium, Azotobacter, Bacillus*
SRB	
Possible population	0
Aggressivity	no reactions
Composition	
Possible genera	
SLYM	
Possible population	2.2×10^2
Aggressivity	slime bacteria present
Composition	aerobic slime forming bacteria
Possible genera	*Pseudomonas, Micrococcus, Zoogloea, Bacillus*

Sample Number: **J-024-B**

IRB

Possible population	0
Aggressivity	no reactions
Composition	
Possible genera	

SRB

Possible population	0
Aggressivity	no reactions
Composition	
Possible genera	

SLYM

Possible population	1.4×10^3
Aggressivity	slime bacteria present
Composition	many slime formers in suspensions
Possible genera	*Pseudomonas, Escherichia, Micrococcus, Staphylococcus, Proteus Vibrio, Aeromonas, Arthrobacter*

Sample Number: **J-072-B**

IRB

Possible population	0
Aggressivity	no reactions
Composition	
Possible genera	

SRB

Possible population	0
Aggressivity	no reactions
Composition	
Possible genera	

SLYM

Possible population	1.4×10^3
Aggressivity	slime bacteria present
Composition	aerobic slime forming bacteria
Possible genera	*Pseudomonas, Micrococcus, Zoogloea, Bacillus*

Sample Number:	**K-024-B**
IRB	
Possible population	0
Aggressivity	no reactions
Composition	
Possible genera	
SRB	
Possible population	0
Aggressivity	no reactions
Composition	
Possible genera	
SLYM	
Possible population	20
Aggressivity	few slime formers active
Composition	aerobic slime forming bacteria
Possible genera	*Pseudomonas, Micrococcus, Zoogloea, Bacillus*

Sample Number:	**K-072-B**
IRB	
Possible population	0
Aggressivity	no reactions
Composition	
Possible genera	
SRB	
Possible population	0
Aggressivity	no reactions
Composition	
Possible genera	
SLYM	
Possible population	0
Aggressivity	no reactions
Composition	
Possible genera	

Sample Number: **L-024-B**

IRB
Possible population 0
Aggressivity no reactions
Composition
Possible genera

SRB
Possible population 0
Aggressivity no reactions
Composition
Possible genera

SLYM
Possible population 0
Aggressivity no reactions
Composition
Possible genera

Sample Number: **L-072-B**

IRB
Possible population 0
Aggressivity no reactions
Composition
Possible genera

SRB
Possible population 0
Aggressivity no reactions
Composition
Possible genera

SLYM
Possible population 0
Aggressivity no reactions
Composition
Possible genera

Sample Number: **M-024-B**

IRB

Possible population	0
Aggressivity	no reactions
Composition	
Possible genera	

SRB

Possible population	0
Aggressivity	no reactions
Composition	
Possible genera	

SLYM

Possible population	2.2×10^3
Aggressivity	aggressive slime bacteria
Composition	aerobic slime forming bacteria
Possible genera	*Pseudomonas, Micrococcus, Zoogloea, Bacillus*

Sample Number: **M-072-B**

IRB

Possible population	0
Aggressivity	no reactions
Composition	
Possible genera	

SRB

Possible population	0
Aggressivity	no reactions
Composition	
Possible genera	

SLYM

Possible population	0
Aggressivity	no reactions
Composition	
Possible genera	

Sample Number: **N-024-B**

IRB

Possible population	8.0×10^5
Aggressivity	highly aggressive iron bacteria
Composition	dominated by anaerobic activity, pseudomonas present
Possible genera	methanogenic bacteria, enteric bacteria, vibrioids

SRB

Possible population	20
Aggressivity	few SRB recovered
Composition	SRB bacteria in dense slimes
Possible genera	*Desulfovibrio, Desulfotomaculum, Desulfuromonas*

SLYM

Possible population	2.2×10^3
Aggressivity	aggressive slime bacteria
Composition	aerobic slime forming bacteria
Possible genera	*Pseudomonas, Micrococcus, Zoogloea, Bacillus*

Sample Number: **N-072-B**

IRB

Possible population	0
Aggressivity	no reactions
Composition	
Possible genera	

SRB

Possible population	0
Aggressivity	no reactions
Composition	
Possible genera	

SLYM

Possible population	0
Aggressivity	no reactions
Composition	
Possible genera	

Sample Number:	**O-024-B**
IRB	
Possible population	4.9×10^6
Aggressivity	highly aggressive iron bacteria
Composition	dominated by anaerobic activity, pseudomonads present
Possible genera	methanogenic bacteria, enteric bacteria, vibrioids
SRB	
Possible population	6.5×10^2
Aggressivity	SRB present
Composition	SRB in aerobic consortium
Possible genera	*Desulfovibrio, Desulfotomaculum, Desulfuromonas, Pseudomonas,Micrococcus, Bacillus, Serratia, Chromobacterium, Fungi, Yeasts*
SLYM	
Possible population	0
Aggressivity	no reactions
Composition	
Possible genera	

Sample Number:	**O-072-B**
IRB	
Possible population	1.4×10^5
Aggressivity	significant iron bacterial population
Composition	dominated by anaerobic activity
Possible genera	methanogenic bacteria, enteric bacteria, vibrioids
SRB	
Possible population	20
Aggressivity	few SRB recovered
Composition	aggressive anaerobes, no SRB present
Possible genera	*Bacillus, Staphylococcus, Lactobacillus, Klebsiella, Enterobacter,Clostridium*
SLYM	
Possible population	1.4×10^3
Aggressivity	slime bacteria present
Composition	aerobic slime forming bacteria
Possible genera	*Pseudomonas, Micrococcus, Zoogloea, Bacillus*

Sample Number:	**P-024-B**
IRB	
Possible population	1.3×10^5
Aggressivity	significant iron bacterial population present
Composition	dominated by anaerobic activity
Possible genera	methanogenic bacteria, enteric bacteria, vibrioids
SRB	
Possible population	0
Aggressivity	no reactions
Composition	
Possible genera	
SLYM	
Possible population	5.7×10^2
Aggressivity	slime bacteria present
Composition	dense covert slime formers
Possible genera	*Bacillus, Micrococcus, Staphylococcus, Pseudomononas, Lactobacillus, Acinetobacter, Hyphomicrobium*

Sample Number:	**P-100-B**
IRB	
Possible population	0
Aggressivity	no reactions
Composition	
Possible genera	
SRB	
Possible population	0
Aggressivity	no reactions
Composition	
Possible genera	
SLYM	
Possible population	2.2×10^2
Aggressivity	slime bacteria present
Composition	aerobic slime forming bacteria
Possible genera	*Pseudomonas, Micrococcus, Zoogloea, Bacillus*

Sample Number:	**Q-001-B**
IRB	
Possible population	4.9×10^6
Aggressivity	highly aggressive iron bacteria
Composition	dominated by anaerobic activity
Possible genera	methanogenic bacteria, enteric bacteria, vibrioids
SRB	
Possible population	2.7×10^2
Aggressivity	SRB present
Composition	SRB in aerobic consortium
Possible genera	*Desulfovibrio, Desulfotomaculum, Desulfuromonas, Pseudomonas, Micrococcus, Bacillus, Serratia, Chromobacterium, Fungi, Yeasts*
SLYM	
Possible population	1.4×10^3
Aggressivity	slime bacteria present
Composition	dense aerobic slime generators
Possible genera	*Klebsiella, Enterobacter, Acinetobacter, Bacillus, Lactobacillus, Staphylococcus*

Sample Number:	**Q-100-B**
IRB	
Possible population	8.0×10^5
Aggressivity	highly aggressive iron bacteria
Composition	dominated by anaerobic activity
Possible genera	methanogenic bacteria, enteric bacteria, vibrioids
SRB	
Possible population	20
Aggressivity	few SRB recovered
Composition	SRB bacteria in dense slimes
Possible genera	*Desulfovibrio, Desulfotomaculum, Desulfuromonas*
SLYM	
Possible population	3.5×10^2
Aggressivity	slime bacteria present
Composition	aerobic slime forming bacteria
Possible genera	*Pseudomonas, Micrococcus, Zoogloea, Bacillus*

Sample Number:	**H-001-IB**	**buffer/granite interface layer H**
IRB		
Possible population	0	
Aggressivity	no reactions	
Composition		
Possible genera		
SRB		
Possible population	0	
Aggressivity	no reactions	
Composition		
Possible genera		
SLYM		
Possible population	3.5×10^2	
Aggressivity	slime bacteria present	
Composition	aerobic slime forming bacteria	
Possible genera	*Pseudomonas, Micrococcus, Zoogloea, Bacillus*	

Sample Number:	**L-001-IB**	**buffer/granite interface layer L**
IRB		
Possible population	8.0×10^5	
Aggressivity	highly aggressive iron bacteria	
Composition	dominated by anaerobic activity	
Possible genera	methanogenic bacteria, enteric bacteria, vibrioids	
SRB	20	
Possible population	few SRB recovered	
Aggressivity	SRB bacteria in dense slimes	
Composition	*Desulfovibrio, Desulfotomaculum, Desulfuromonas*	
Possible genera		
SLYM		
Possible population	2.2×10^3	
Aggressivity	aggressive slime bacteria	
Composition	aerobic slime forming bacteria	
Possible genera	*Pseudomonas, Micrococcus, Zoogloea, Bacillus*	

Sample Number:	**N-001-IB**	**buffer/granite interface (layer N)**
IRB		
Possible population	4.9×10^6	
Aggressivity	highly aggressive iron bacteria	
Composition	dominated by anaerobic activity, pseudomonads present	
Possible genera	methanogenic bacteria, enteric bacteria, vibrioids	
SRB		
Possible population	20	
Aggressivity	few SRB recovered	
Composition	SRB bacteria in dense slimes	
Possible genera	*Desulfovibrio, Desulfotomaculum, Desulfuromonas*	
SLYM		
Possible population	5.7×10^2	
Aggressivity	slime bacteria present	
Composition	aerobic slime forming bacteria	
Possible genera	*Pseudomonas, Micrococcus, Zoogloea, Bacillus*	

Sample Number:	**Q-001-IB**	**buffer/granite interface (layer Q)**
IRB		
Possible population	4.9×10^6	
Aggressivity	highly aggressive iron bacteria	
Composition	dominated by anaerobic activity, pseudomonads present	
Possible genera	methanogenic bacteria, enteric bacteria, vibrioids	
SRB		
Possible population	20	
Aggressivity	few SRB recovered	
Composition	SRB in aerobic consortium	
Possible genera	*Desulfovibrio, Desulfotomaculum, Desulfuromonas Pseudomonas, Micrococcus, Bacillus, Serratia, Chromobacterium,Fungi, Yeasts*	
SLYM		
Possible population	1.5×10^4	
Aggressivity	very aggressive slime formers	
Composition	aerobic slime forming bacteria	
Possible genera	*Pseudomonas, Micrococcus, Zoogloea, Bacillus*	

Sample Number: **F-001-IB** **backfill**

IRB

Possible population	8.0×10^5
Aggressivity	highly aggressive iron bacteria
Composition	dominated by anaerobic activity
Possible genera	mixed pseudomonads in flora methanogenic bacteria, enteric bacteria, vibrioids

SRB

Possible population	3.7×10^3
Aggressivity	SRB present
Composition	SRB bacteria in dense slime
Possible genera	*Desulfovibrio, Desulfotomaculum, Desulfuromonas*

SLYM

Possible population	1.9×10^6
Aggressivity	very aggressive slime formers
Composition	aerobic slime forming bacteria
Possible genera	*Pseudomonas, Micrococcus, Zoogloea, Bacillus*

Sample Number: **F-003-B** **backfill (rust)**

IRB

Possible population	5.5×10^4
Aggressivity	significant iron bacterial population
Composition	molds present
Possible genera	*Steptomycetes, Aspergillus, Penicillium, Rhizopus,* various other Fungi, Yeasts

SRB

Possible population	3.7×10^3
Aggressivity	SRB present
Composition	aggressive anaerobes, no SRB present
Possible genera	*Bacillus, Staphylococcus, Lactobacillus, Klebsiella,* *Enterobacter, Clostridium*

SLYM

Possible population	1.9×10^6
Aggressivity	very aggessive slime formers
Composition	
Possible genera	no list given in output

Sample Number: **F-007-B** **backfill (underneath cell, visible water drops)**

IRB	
Possible population	3.3×10^5
Aggressivity	significant iron bacterial population
Composition	dominated by anaerobic activity
Possible genera	methanogenic bacteria, enteric bacteria, vibrioids
SRB	
Possible population	6.5×10^2
Aggressivity	SRB present
Composition	SRB in aerobic consortium
Possible genera	*Desulfovibrio, Desulfotomaculum, Desulfuromonas, Pseudomonas, Micrococcus, Bacillus, Chromobacterium, Yeasts, Fungi*
SLYM	
Possible population	1.4×10^3
Aggressivity	slime bacteria present
Composition	many slime formers in suspension
Possible genera	*Pseudomonas, Escherichia, Micrococcus, Staphylococcus, Proteus, Vibrio, Aeromonas, Arthrobacter*

Sample Number: **F-002-IB** **backfill/buffer interface**

IRB	
Possible population	4.9×10^6
Aggressivity	highly aggressive iron bacteria
Composition	dominated by anaerobic activity
Possible genera	methanogenic bacteria, enteric bacteria, vibrioids
SRB	
Possible population	20
Aggressivity	few SRB recovered
Composition	SRB bacteria in dense slimes
Possible genera	*Desulfovibrio, Desulfotomaculum, Desulfuromonas*
SLYM	
Possible population	2.2×10^3
Aggressivity	aggressive slime bacteria
Composition	aerobic slime forming bacteria
Possible genera	*Pseudomonas, Micrococcus, Zoogloea, Bacillus*

Sample Number: **J-001-SB** **sand sample**

IRB
Possible population 0
Aggressivity no reactions
Composition
Possible genera

SRB
Possible population 0
Aggressivity no reactions
Composition
Possible genera

SLYM
Possible population 2.2×10^3
Aggressivity aggressive slime bacteria
Composition aerobic slime forming bacteria
Possible genera *Pseudomonas, Micrococcus, Zoogloea, Bacillus*

Sample Number: **L-001-SB** **sand sample**

IRB
Possible population 0
Aggressivity no reactions
Composition
Possible genera

SRB
Possible population 0
Aggressivity no reactions
Composition
Possible genera

SLYM
Possible population 0
Aggressivity no reactions
Composition
Possible genera

Sample Number:	**N-001-SB**	**sand sample**
IRB		
Possible population	0	
Aggressivity	no reactions	
Composition		
Possible genera		
SRB		
Possible population	0	
Aggressivity	no reactions	
Composition		
Possible genera		
SLYM		
Possible population	0	
Aggressivity	no reactions	
Composition		
Possible genera		

Sample Number:	**HE-001-B**	**heater-cloth sample**
IRB		
Possible population	0	
Aggressivity	no reactions	
Composition		
Possible genera		
SRB		
Possible population	0	
Aggressivity	no reactions	
Composition		
Possible genera		
SLYM		
Possible population	0	
Aggressivity	no reactions	
Composition		
Possible genera		

Sample Number: **HE-004-B** **sand from underneath heater**

IRB
Possible population 0
Aggressivity no reactions
Composition
Possible genera

SRB
Possible population 0
Aggressivity no reactions
Composition
Possible genera

SLYM
Possible population 0
Aggressivity no reactions
Composition
Possible genera

Sample Number: **HE-005-B** **heater cloth - black tape**

IRB
Possible population 0
Aggressivity no reactions
Composition
Possible genera

SRB
Possible population 0
Aggressivity no reactions
Composition
Possible genera

SLYM
Possible population 0
Aggressivity no reactions
Composition
Possible genera

3 APPENDIX 3 - Protocols for metal tube samples analysis by the 16S rRNA gene sequencing method (University of Göteborg method)

3.1 SAMPLING

The sediment cores used for the 16S rRNA analysis were shipped to Sweden under cold conditions and reached the lab within 72 h. The DNA extractions, PCR reactions, cloning and sequencing were done at the Lundberg Laboratory, Göteborg,Sweden. Two buffer mass samples, each with a weight of 0.30 to 0.33 g, from metal-tube samples H-097-B, M-018-B and P-016-B (Figures 7 and 8 main text) were prepared.

3.2 DNA EXTRACTION

Our protocol for DNA extraction was based on the procedure described by Marmur (1961)and Wallace (1987), but slightly modified. The sediment samples were resuspended in 760 µL of 20 mM Tris-HCl, pH 8.0; 20 mM EDTA; 0.35 M sucrose and incubated with 2 mg/mL lysozyme (Sigma) at 37oC for 1 h to destroy cell walls. Thereafter the cells were lysed by adding 40 ml 20% SDS and the proteins were digested with 250 mg/mL proteinase K (Sigma) during an additional incubation at 60oC for 1 h followed by a centrifugation at 8000 g for 5 min to separate the DNA from the sediment. The supernatant was carefully removed to a new tube and the DNA was extracted with an equal volume phenol : chloroform : isoamyalcohol (25:24:1), and thereafter 3 extractions with chloroform:isoamylalcohol (24:1, called chisam) so that no cell debris was visible. The DNA was precipitated with 1/3 volume of 10 M NH_4Ac (final concentration 2.5 M) an 2.5 volumes of 99% ethanol. To ensure complete precipitation, 50 µg tRNA was added as a coprecipitant and the mixture was incubated at -70°C. The precipitate was washed with 100 µM 70 % ethanol (v/v) and dried in vacuum for 30 s, dissolved in TE-buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) over night and stored at -20°C.

3.3 PCR AMPLIFICATION

One µl of the extracted DNA solution was added to a mixture of 10µl of 10xPCR buffer (Stratagen), 0.2 mM of each nucleotide triphosphate, 0.25µM of each primer and double distilled water to a final volume of 100 µl. The samples were treated with 10 mg/ml of RNase A (Sigma) for 15 min at 37°C and incubated at 95°C for 5 min, before addition of 1 µl Pfu DNA polymerase (Stratagene) and coating with 100µl mineral oil (Sigma). A total of 30 cycles were performed at 95°C (30s), 55°C (1 min), 72°C (2 min) followed by a final incubation at 72°C for 10 min.

The 5' and 3' primers used matched the universally conserved positions 519 to 536 and 1392 to 1404, *E.coli* Brosius numbering (Brosius et al. 1978). These were chosen to ensure that both eubacterial, archaebacterial and possible eukaryotic species could be amplified. The amplification products were purified with the QIAEX agarose extraction kit (Qiagen) following the specification of the manufacturer and were finally diluted in 20 µl TEbuffer and stored at -20°C.

3.4 CLONING

The purified samples were cloned with the pCR-Script SK(+) cloning kit (Stratagene) following the specification of the manufacturer. From each DNA extraction, a total of 12 white colonies containing the insert were randomly picked. The colonies were inoculated in 3.5 ml LB (Luria Broth)+Ampicillin overnight at 37°C. From each culture 0.5 ml was suspended in 0.5 ml of concentrated glycerol and stored at -80°C. The recombinant plasmids were extracted from the bacteria with the Magic miniprep kit (Promega).

3.5 DOUBLE-STRANDED SEQUENCING

The sequencing was made using the Autoread Sequencing kit (Pharmacia Biotech) following the manufacturer's instructions. All clones were sequenced using the primer 926 labelled with fluorescin. The gel electrophoresis was performed on an ALF DNA Sequencer (Pharmacia Biotech).

3.6 SEQUENCE ANALYSIS

The 16S rRNA gene clones were compared to the sequences available in the EMBL database using the FastA procedure in the GCG program package (Genetic Computer Group, Wisconsin, USA). This procedure shows identities between the unknown sequence (clone) and known and sequenced bacteria in the database. The phylogenetic analysis was performed by using the programs contained in the PHYLIP version 3.5c package (Felsenstein 1989) compiled for PC. All nucleotide positions that could be unambiguously aligned for all clones with a species identity larger than 95%, were included in the analysis. The final data set comprised 360 nucleotide positions, position no. 542 to 880 (*E.coli* Brosius numbering), (Brosius et al 1978). The distances were calculated using the DNADIST program and the tree was built running the KITCH program with contemporary tips. The KITCH program was run with a randomised input order of data with 10 jumbles and during the execution 36 634 trees were examined (Felsenstein 1989). The tree was drawn by using the drawing program DRAWTREE, also available in the PHYLIP package.

3.7 REFERENCES

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4 APPENDIX 4 - Analysis of BCE samples at Guigues recherche appliquée en microbiologie (GRAM)

4.1 PREPARING THE "MOTHER SUSPENSION"

To access the samples within the metal tubes, a layer of wax at one end must first be removed with a sterile spatula, which is then resterilized to scrape out the clay to a depth of about 5 cm. Ten grams are then sterily weighed and added to 90 ml of sterile anaerobic physiologic serum (9 g/l NaCl in distilled water). The same dilution method is used for the hollow stem auger and the sand samples. These dilutions by a factor of ten (10^{-1}) are referred to as the "mother suspension". The suspensions are then left in an ultra-sound bath, for 10 minutes, to dislodge the bacteria from the surfaces of the clay particles and to make their distribution throughout the suspension homogeneous. The suspensions are then used to inoculate the various different culture media.

From these mother suspensions, the other dilutions will be made in cascade by factors of ten.

The samples from the backfill-buffer interface and the granite-buffer interface have already been put into suspension before their arrival and thus can be used directly for inoculation, and diluted in cascade by factors of ten for further inoculations.

4.2 DESCRIPTION OF ANALYSES

For each sample, both the non-specialized (total population) and some specialized microorganisms have been investigated. The composition and preparation of the different culture media, mode of inoculation, duration of incubation, observation of results, enumeration of microorganisms and the detection limits are all described below:

The investigated non-specialized groups are:

* Total aerobic bacteria
* Total anaerobic bacteria
* Total microscopic fungi

The specialized groups that can be responsible for the corrosion of metals and the degradation of concrete which are also investigated.

* Sulphate reducing bacteria
* Methanogenic bacteria
* Sulphur-oxidizing bacteria

  - strictly aerobic sulphur-oxidizing bacteria:

* *Thiobacillus thiooxidans*
* *Thiobacillus ferrooxidans*

  - facultatively anaerobic sulphur-oxidizing bacteria:

  Thiobacillus denitrificans

All the microbes tested for are incubated at room temperature (25°C).

In addition, the media inoculated with the ten samples located at the same level as the heater are also incubated at 50° to 55°C to determine the presence of thermophilic microflora.

These ten samples are the:

- 7 metal-tube samples from layers K, L, M, N, and O
- hollow stem auger sample N 001-AB
- sand sample L 004-SB
- granite-buffer interface sample L 003-IB

Solids, like the metal tube samples and the hollow stem auger sample, are more or less dry, depending on their distance from the heater. Their dry weight is determined by drying to a constant weight in a 55°C incubator and then weighing accurately.

4.3 CALCULATION OF ENUMERATION RESULTS

Solid Media:

The number of total aerobic bacteria and fungi is determined by the Colony Forming Unit (C.F.U.) method. It is accepted that one cell gives birth to one, and only one, colony, so every colony is counted on the surface of dishes having between 30 and 300 colonies. This number is multiplied by the dilution factor and then converted into the number of microorganisms/ml or g of the sample.

Liquid Media:

The number of total anaerobic, sulphate reducing, sulphur oxidizing, and methanogenic bacteria is determined by the Most Probable Number (MPN) technique. It is a statistical method based on the dilution of the sample. It is generally agreed that the reaction specific to the sought after species is due to only one cell in the last positive dilution. It is necessary to observe:

The final dilution where both series show a positive result.

The number of positive results in the following two dilutions. These three values can then be used to produce a characteristic three figure number.

Dilution	Series 1	Series 2	Number of Positive Results
- 1	+	+	
- 2	+	+	2
- 3	+	-	1
- 4	-	-	0

Here, our three figure number is 210.

The MAC GRADY table provides the number of bacteria in the tube of the first considered dilution. It is sufficient to multiply by the dilution factor to produce the number of micro-organisms/ml or g of the sample.

4.4 DETECTION LIMITS

First, it is important to note that the given results are minimum. Indeed, it is not certain that ultra-sound dislodges all the bacteria from the clay particles. Nevertheless, it is the best method that we have at the moment and the margin of error could be negligible.

Moreover, another limiting factor is that most of the samples are solid and therefore it is impossible to test them in their raw state.

A preliminary dilution step is necessary which increases the detection limit. Finally, the detection level is related to the volume of the sample tested, and thus dependent on the method of analysis used:

Method Used	**Detection Limit**
* Colony Forming Unit (Heterotrophic aerobic bacteria, fungi)	5/ml or 50/g wet weight
* Most Probable Number (*Thiobacillus*, methanogenic, sulphate- reducing and anaerobic bacteria)	0.5/ml or 5/g wet weight

For solid samples the number of microorganisms is calculated per dry gram.

For liquid samples the number of microorganisms is calculated per milliliter.

Note that the two different types of samples have different detection limits and that a result that has a value of zero means that the number of microorganisms present is in fact less than the detection limit.

4.5 ENUMERATION OF MICROORGANISMS

4.5.1 HETEROTROPHIC STRICT AEROBIC AND FACULTATIVE ANAEROBIC BACTERIA

The standard solid medium is plate count agar (PCA).

Tryptone	5.0 g
Yeast Extract	2.5 g
Glucose	1.0 g
Bacterial Agar	15.0 g
Distilled Water pH = 7.0 ± 0.2	1000.0 ml

After stirring and autoclaving (30 min, 110°C), the medium is sterilely distributed to 90 mm Petri dishes (20 ml/dish).

A sample is taken from the mother suspension and diluted in cascades by a factor of ten in sterile physiological serum. Each dilution is homogenized before a 0.2 ml sample is taken to inoculate the culture by "spreading".

4.5.2 Heterotrophic strict and facultative anaerobic bacteria (AnHB)

Widdel and Pfennig (1977) developed this liquid anaerobic medium in 1977 which was modified by Widdel (1980). This medium was modified by GRAM for the growth of AnHB.

- Biotrypcase	5.0 g
- Yeast Extract	2.5 g
- Glucose	1.0 g
- Resazurin	100.0 mg
- Widdel Trace Elements Solution	2.0 ml
- MOPS	3.0 g
- 30% NaOH Solution	1.0 ml
- Distilled Water	1.0 l

After autoclaving (30 min, 110°C), the flask atmosphere is replaced by a sterile N_2/H_2 (80 to 20%) gas mixture while cooling and the medium is subsequently introduced into the anaerobic glovebox.

The following two solutions are sterilized by filtration before adding to the medium:

- 20 ml of a titanium citrate (reductor) solution = solution composed of 10 ml titanium (III) chloride 15% solution and 100 ml 0,2 M Tri Sodium citrate.
- 1 ml of vitamins solution.
- pH is adjusted to 7.2 to 7.4.

The medium is then sterilely and anaerobically distributed (9 ml/flask).

Two series of cascade dilutions by factors of ten are made, anaerobically, from the mother suspensions.

Growth of AnHB is detected by the medium turning cloudy. Since the medium of the first dilutions are already cloudy from the clay particles, a microscopic study is carried out to confirm the presence of microorganisms.

4.5.3 Fungi

This culture medium provides favourable conditions for the growth of microscopic fungi (molds and yeasts). An antibiotic is added to the medium to inhibit the growth of bacteria.

The standard solid medium for the selective isolation of fungi is Sabouraud Chloramphenicol.

- Bio-thione	3.0 g
- Bio-trypcase	3.0 g
- Bio-soyase	3.0 g
- Yeast Extract	2.0 g
- Malt Extract	1.0 g
- Dextrose	19.0 g
- Monopotassium Phosphate	0.5 g
- Disodium Phosphate	0.5 g
- Agar	13.0 g
- Chloramphenicol	0.5 g
- Distilled Water	1.0 l
- pH = 6.4	

After stirring and autoclaving (30 min, 110°C), it is sterilely distributed to 90 mm Petri dishes (20 ml/dish).

A sample is taken from the mother suspension and diluted in cascade by factors of ten in sterile physiological serum. Each dilution is homogenized before a 0.2 ml sample is taken to inoculate the culture by "spreading".

4.5.4 Specialized groups

4.5.4.1 Sulphate-reducing bacteria (SRB)

Enumeration of all known sulphate reducing bacteria (SRB) using either lactic acid or acetic acid as an electron donor.

The Labège test-kits, manufactured by us in a controlled atmosphere glove box (90% Nitrogen, 10% Hydrogen), contain a strictly anaerobic liquid medium composed of:

- NH_4Cl	1.0 g
- $CaCl_2 2H_2O$	1.0 g
- $MgSO_4$, 7 H_2O	1.0 g
- NaCl	1.0 g
- Sodium Acetate, 3 H_2O	2.0 g
- MOPS	3.0 g
- KHCO3	0.2 g
- Sodium Lactate 60% Aqueous Solution	6.0 ml
- Yeast Extract	1.0 g
- Resazurin (0.2%)	1.0 ml
- Na2SO4	2.0 g
- NaOH 30%	1.0 ml
Distilled water make up to	1.0 l

After autoclaving (30 min, 110°C), the flask atmosphere is replaced by a sterile N_2/H_2 (80 to 20%) gas mixture while cooling and the medium is subsequently introduced into the anaerobic glovebox.

The following two solutions are sterilized by filtration before adding to the medium:

- 20 ml of 25 g/l $FeSO_4$, 7 H_2O solution.
- 20 ml of a titanium citrate (reductor) solution = solution composed of 10 ml Titanium (III) chloride 15% solution and 100 ml 0,2 M Tri Sodium citrate.
- pH is adjusted to 7.2 to 7.4.

The medium is then sterily and anaerobically distributed (9 ml/flask).

Two series of cascade dilutions by factors of ten are made, anaerobically, from the mother suspensions.

Growth of SRBs is detected by a black precipitate of FeS which reveals the presence of H_2S produced during the reduction of sulphates.

4.5.4.2 Methanogens

Widdel and Pfennig developed this liquid anaerobic medium in 1997 which was modified by Widdel in 1980. This medium is adapted by GRAM for the growth of methanogenic bacteria (MB):

- Sodium Formate	2.5 g
- Acetate	5.0 g
- MOPS	3.0 g
- Yeast Extract	1.0 g
- Widdel Salt Solution	100.0 ml
- Widdel Trace Elements Solution	2.0 ml
- Distilled Water	1.0 l

After autoclaving (30 min, 110°C), the flask atmosphere is replaced by a sterile N_2-H_2 (80% to 20%) mixture while cooling and the medium is subsequently introduced into the anaerobic glovebox.

The following two solutions are sterilized by filtration before adding to the medium:

- 20 ml of a titanium citrate (reductor) solution = solution composed of 10 ml Titanium (III) chloride 15% solution and 100 ml 0,2 M Tri Sodium citrate.
- 1 ml of vitamins solution.

pH is adjusted to 7.2 to 7.4.

The medium is then sterily and anaerobically distributed (9 ml/flask).

Two series of cascade dilutions, by factors of ten, are made, anaerobically, from the mother suspensions.

Just after inoculation, the atmosphere in the flask is replaced by a mixture of hydrogen and carbon dioxide (H_2 80% - CO_2 20%). H_2 is used as the electron donor and is kept at a pressure of 3 bars. Growth of MB is confirmed by the presence of methane in the flask atmosphere produced by their metabolism. This gas is measured by gas phase chromatography.

4.5.4.3 Aerobic sulphur-oxidizing bacteria

Thiobacillus thiooxydans and *Thiobacillus ferrooxydans* (TT/TF) are sulphur-oxidizing bacteria that can be cultivated in a liquid aerobic medium.

The Starkey medium (1935), adapted to the growth of TF, is a strictly autotrophic medium:

- KH_2PO_4	3.0 g
- $(NH_4)_2\ SO_4$	3.0 g
- $MgSO_4$, 7 H_2O	0.5 g
- $CaCl_2$, 2 H_2O	0.5 g
- Distilled Water 1.	0 l

After autoclaving (30 min, 110°C), the pH is adjusted to 3.8 to 4.4.

Then 10 milligrams/l of $FeSO_4$, 7 H_2O are sterilized by filtration before adding to the medium, which is then distributed sterilely (9 ml/flask). Just before inoculation, 5 g/l of sterile flower of sulphur is added to each flask.

Two series of cascade dilutions are made by factors of ten from the mother suspension.

(TT/TF) growth is verified by microscopic observation: TT/TF are small motile bacilli.

4.5.4.4 Anaerobic sulphur-oxidizing bacteria

Thiobacillus denitrificans (TD) is a sulphur-oxidizing bacteria that can be cultivated in an anaerobic liquid medium:

The Taylor, Hoare and Hoare (1971) medium is a strictly autotrophic medium:

- KNO_3	2.0 g
- NH_4Cl	1.0 g
- KH_2PO_4	2.0 g
- $NaHCO_3$	2.0 g
- $MgSO_4$, 7 H_2O	0.8 g
- $Na_2S_2O_3$, 5 H_2O	5.0 g
- Widdel Trace Elements Solution	1.0 ml
- Distilled Water	1.0 l

After autoclaving (30 min, 110°C), the flask atmosphere is replaced by a sterile N_2-H_2 (80% to 20%) mixture while cooling.

pH is then adjusted to between 6.8 to 7.0.

The medium is then sterilely and anaerobically distributed (9 ml/flask).

Two series of dilutions, in cascade, by factors of ten, anaerobically from the mother suspension. Since the medium is specific for TD, their growth is verified by microscope:

TD are small motile bacilli.

4.5.4.5 Microbial mineralization of low molecular weight organic compunds

Measurement of microbial mineralization of ^{14}C-glucose and ^{14}C-amino acid mixture is used as an index of potential microbial activity.

For five metal- tube samples (G 008-B, Q 092-B, K 054-B, K 006-B and N 006-B), a slurry (1/10 wet clay weight/volume) was aseptically and anoxically prepared and then distributed into sterile polypropylene tubes (15 ml/tube) under a nitrogen atmosphere. This slurry is in fact the "mother suspension" used to inoculate the culture media.

A solution of D-(U-^{14}C)-glucose, specific activity 10.6 GBq.mmol^{-1} (Amersham Corp.), or a solution of ^{14}C-amino acid mixture, specific activity 1.92 GBq/mg atom C (Amersham Corp.) is aseptically and anoxically added to the 15 ml clay slurry samples to reach a final concentration of 7 mg C l^{-1} or 5 mg C l^{-1} respectively. Sterile blanks are killed by addition of buffered formaldehyde (3% final concentration) before label injection.

A set of labelled samples is incubated under a sterile nitrogen atmosphere, another set is incubated in oxic conditions. To distinguish between mesopilic and thermophilic microorganisms, three different incubation temperatures are used: 20, 40 and 60°C.

After 24, 48 and 72 h of incubation, bacteria are killed by addition of buffered formaldehyde (3% final concentration). Samples are decarbonated by acidification under a nitrogen flow during 30 minutes. Carbon dioxide produced by bacterial metabolism is trapped into two serial scintillation vials containing a CO_2 trapping mixture (ethanolamine 2 ml, methanol 1 ml and scintillation liquid 4 ml). $^{14}CO_2$ is counted using a Beckman LS 1800 scintillation counter. Counting efficiency and quenching corrections are determined by use of internal standards. Data are corrected against those from sterile blanks killed before label addition.

4.5.4.6 Bacterial identification

Ten strains per sample have to be identified. Five samples do not contain any aerobic bacteria: the sand sample, the hollow stem auger sample and the three metal-tube 054-B samples.

So 150 heterotrophic strictly aerobic or facultatively anaerobic strains, and some autotrophic and strictly anaerobic strains, are studied.

Ten aerobic strains are isolated from the plate count nutrient agar. These colonies grown at 25°C are chosen at random on enumeration Petri dishes containing between 30 and 300 colonies, and reseeded on a new Plate Count Agar Petri dish until they are pure. Purity is checked by macroscopic and microscopic observation.

After purification, macroscopic and microscopic description of the colony, and Gram staining, biochemical and nutritional tests are necessary to allow identification of the strain: oxidase, catalase, glucose oxidation or fermentation, reduction of nitrates.

For some strains, the identification can be made with help of the Api system; for some others a reference book (Holt et al. 1994; Starr et al. 1981) has to be used.

4.5.4.7 Main characteristics of the identified genera and species in Table 22

Of the heterotrophic aerobic bacteria, 150 isolates have been identified. Their main characteristics are discussed below.

Pseudomonas stutzeri (56 isolates identified): Most *Pseudomonas* have a strictly respiratory type of metabolism. Among them, *P. stutzeri* and a few other species are capable of growing both aerobically (with O_2 as the terminal electron acceptor) and anaerobically (with nitrate as an alternate electron acceptor). Breakdown of carbohydrates is oxidative, never fermentative. Because the nutritional demands of *Pseudomonas* are modest, they can survive a long time in moist environments. *P. stutzeri* is widely distributed in nature, it is isolated from plants, water and soil and its optimal growth temperature is 35°C, but growth is possible at 40°C.

Bacillus (49 isolates identified): This genus is endospore-forming. This is a very important property because spores are very resistant to many adverse conditions (e.g., heat and desiccation). *Bacilli* are aerobic or facultative anaerobic bacteria, with a fermentative or respiratory metabolism. They play, by virtue of their considerable powers of proteolysis and ability to break down polysaccharides, an important role in the cycling of nutrients in nature. They are most commonly found as inhabitants of soils. Species identification is difficult because of their large number (>50) of species and the often incomplete descriptions of newly reported species. The *Bacillus* isolates from the BCE samples may be composed of five to ten species, and some of them are capable of denitrification.

Cellulomonas (?) (17 isolates identified): This genus is very similar to *Oerskovia*, except for a morphological characteristic, since the latter genus forms a mycelium and none was observed. Therefore, the isolates have been identified as belonging to the genus *Cellulomonas*, although the cellulolytic power, which is not a classical test, has not been checked. *Cellulomonas* are facultative anaerobes, with a respiratory and also a fermentative metabolism producing acid from glucose both aerobically and anaerobically. They are widely distributed in soils. The isolates from the BCE samples are able to reduce nitrate to nitrogen. It should be noted here that in other studies using this buffer material, a branched organism was found that was tentatively identified as *Oerskovia*. This organism appears resistant to radiation in liquid medium, possibly because of the formation of microcolonies (i.e., clumps) (Stroes-Gascoyne and West 1995). Microcolonies consist of a number of cells, although each microcolony will produce only one colony on a solid plate. It would take proportionally more radiation to destroy a microcolony than it would to destroy a single cell.

Curtobacterium (9 isolates identified): These are obligate aerobic bacteria with a respiratory metabolism. The optimum growth temperature is 25 to 30°C and they they have been isolated from plants and soils.

Bradyrhyzobium japonicum (8 isolates identified): This aerobic genus with an optimum growth temperature of 25 to 30°C is able to invade the root-hairs of tropical-zone and some temperate-zone leguminous plants.

Actinomycetes group (9 isolates identified): Actinomycetes include organisms that are the most filamentous of all bacteria. Among the 9 Actinomycetes isolates, 4 are identified as belonging to the aerobic genus *Nocardia* which is widely distributed and abundant in soils.

Acinetobacter (lwoffi ?) (2 isolates identified): This aerobic organism has a strictly respiratory type of metabolism with O_2 as the terminal electron acceptor. All strains grow between 20 and 35°C, with most strains having temperature optima of 33 to 35°C. They occur naturally in soils and water.

Of the non-specialized heterotrophic anaerobic bacteria, the genus ***Clostridium*** has been identified. This is an obligately anaerobic organism that forms heat resistant endospores. Usually it produces mixtures of organic acids and alcohol from carbohydrates or peptones, but it does not carry out a dissimilatory sulphate reduction. The genus now contains about 100 species and is widespread in the environment.

Of the specialized bacteria, SRB and SOB were identified. SRB are strictly anaerobic bacteria that can reduce sulphate to H_2S. H_2 or organic compounds (e.g., lactate) serve as electron donors. Oxidation of organic compounds is either incomplete, leading to acetate as an end product, or complete, leading to CO_2. Typical habitats are anoxic sediments or bottom waters. The two main genera found in the samples from the BCE are ***Desulfotomaculum***, which comprises all

spore-forming SRB, and ***Desulfovibrio***, which may also reduce sulphur. For both genera, new species have been reported that are able to use acetate as carbon source. In contrast to many other culture media, such species can be enumerated in the Labège test kits used at the GRAM laboratory. Of the SOB, the genus ***Thiobacillus*** is present in almost every BCE sample analyzed at the GRAM laboratory. These are chemolithotrophic bacteria (obligate or facultative), which means that they use inorganic compounds as a source of electrons; in fact, energy is derived from the oxidation of one or more reduced sulphur compounds. Sulphate is the end product of sulphur compound oxidation. All species are capable of autotrophic growth, i.e., they can use CO_2 as their sole carbon source. Two species are present in the BCE samples, ***Thiobacillus thiooxydans***, an obligate aerobe found in small quantity in only one sample (P-008-B), and ***Thiobacillus denitrificans*** which is found in large numbers in almost all samples. This latter species is a facultative denitrifying species, that can grow anaerobically using nitrate as the terminal electron acceptor. The genus *Thiobacillus* occurs ubiquitously in marine, freshwater and soil environments, especially where oxidizable sulphur compounds are abundant.

4.6 REFERENCES

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5 APPENDIX 5 - Statistical evaluation of all colony-forming unit counts obtained from the buffer/container experiment metal tube samples using the general linear models procedure

5.1 STATISTICAL MODEL DESCRIPTION

A hypothesis for variables that may have had an effect on the number of colony forming units in each sample is expressed in the following model:

$$CFU_{fghijkl} = m + T2_f + W2_{fg}(T2_f) + lab_{fgh} * T3_{fghi} * ox_{fghij} * medium_{fghijk} + E_{fghijkl}$$

* $CFU_{fghijkl}$ is the *l*th measurement of the number of colony forming units (CFU) growing on the *k*th medium with the *j*th oxygen culturing conditions at the *i*th culturing temperature cultured by the *h*th lab sampled from a buffer mass with the *g*th % water gdw^{-1} and the *f*th buffer mass temperature.

* *m* is the overall mean.

* $T2_f$ is the effect from the *in situ* temperature of the buffer mass. This variable ranged from 19.8 up to 60.9°C and was divided in four classes, 20 to 30 (25°), 30 to 40 (35°), 40 to 50 (45°) and 50 to 60 (55°). Degrees within parenthesis are the class level names used.

* $W2_{fg}$ is the effect from the % water gdw^{-1} of the sampled buffer mass. This variable ranged from 13.8 up to 23.6 % water gdw^{-1}, and was divided in four classes, 11 to 15, (13.5% water gdw^{-1}), 15 to 18 (16.5% water gdw^{-1}), 18 to 21 (19.5 % water gdw^{-1}) and 21 to 24 (22.5 % water gdw^{-1}). Percentages within parentheses are the class level names used.

* lab_{fgh} is the effect from the different labs doing the analyses, including transportation effects. This variable had three classes, Canada (C), France (F) and Sweden (S).

* $T3_{fghi}$ is the effect from the culturing temperature used. This variable was given two classes 20°C (including the different culturing temperatures 17°C (C), 20°C (C) and 25°C (F)) and 50°C (C and S).

* ox_{fghi} is the effect from the oxygen conditions used for culturing. This variable had two classes, aerobic (C, F) and anaerobic conditions C, F and S).

* $medium_{fghijk}$ is the effect from the different media used. This variable was given four classes, medium for heterotrophic bacteria (C, F and S), methanogenic bacteria (S), sulphur reducing bacteria (F) and sulphate reducing bacteria (F and S).

* $E_{fghijkl}$ is the random sampling effect not explained by the model, also called the residual.

* It was assumed that the effect from the % water gdw^{-1} was nested within the buffer mass temperature (W2 is dependent on T2) and that the effects from the labs, culturing temperatures, oxygen conditions and the medium used were crossed (the class variables were interacting). It is assumed that the effect from each variable is random. An analysis of variance and an estimation of the variance-components were executed running the General Linear Models procedure (GLM) for unbalanced data (SAS 1989) on the colony forming number data obtained.

Tables 25 through 29 (main text) give the results from the analysis, as well as Figures 34 and 35 (main text).

5.2 REFERENCES

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Table A-5-1 All input data (all culture analyser at WL, GRAM, University of Göteborg) for statistical evaluation

Sample	Lab	Layer	Temp.	T-class	H^2_0 %	H^2_0-Class	gdw/g sample	C-temp	A/An	Dilution	count #1	count #2	count #3	Culture
G-008-B	F	G	19,84	25	19,85	19,5	0,87	20	A	5000	221	m	m	H
G-008-B	F	G	19,84	25	19,85	19,5	0,87	20	A	50000	33	m	m	H
G-008-B	F	G	19,84	25	19,85	19,5	1	20	An	1	2900	m	m	H
G-008-B	F	G	19,84	25	19,85	19,5	1	20	An	1	15	m	m	SRB
G-008-B	F	G	19,84	25	19,85	19,5	1	20	An	1	1500	m	m	SR
G-024-B	C	G	20,64	25	17,62	16,5	0,042	20	A	100	200	192	212	H
G-024-B	C	G	20,64	25	17,62	16,5	0,042	20	A	1000	23	27	25	H
G-024-B	C	G	20,64	25	17,62	16,5	0,042	50	A	10	2	2	1	H
G-024-B	C	G	20,64	25	17,62	16,5	0,042	20	An	10	20	11	20	H
G-024-B	C	G	20,64	25	17,62	16,5	0,042	50	An	1000	0	0	0	H
G-024-B	S	G	20,64	25	17,62	16,5	0,042	50	An	1	12	1	m	SRB
G-024-B	S	G	20,64	25	17,62	16,5	0,042	50	An	1	0	0	m	H
G-024-B	S	G	20,64	25	17,62	16,5	0,042	50	An	1	3	9	m	M
G-092-B	F	G	19,91	25	18,88	19,5	0,835	20	A	5000	71	m	m	H
G-092-B	F	G	19,91	25	18,88	19,5	0,835	20	A	50000	14	m	m	H
G-092-B	F	G	19,91	25	18,88	19,5	1	20	An	1	150000	m	m	H
G-092-B	F	G	19,91	25	18,88	19,5	1	20	An	1	0	m	m	SRB
G-092-B	F	G	19,91	25	18,88	19,5	1	20	An	1	300000	m	m	SR
G-100-B	C	G	20,67	25	18,79	19,5	0,045	20	A	10	37	34	34	H
G-100-B	C	G	20,67	25	18,79	19,5	0,045	50	A	10	0	1	0	H
G-100-B	C	G	20,67	25	18,79	19,5	0,045	20	An	10	7	5	6	H
G-100-B	C	G	20,67	25	18,79	19,5	0,045	50	An	100	0	0	0	H
H-008-B	F	H	23,41	25	19,56	19,5	0,8547	20	A	5000	56	m	m	H
H-008-B	F	H	23,41	25	19,56	19,5	0,8547	20	A	50000	6	m	m	H
H-008-B	F	H	23,41	25	19,56	19,5	1	20	An	1	70000	m	m	H
H-008-B	F	H	23,41	25	19,56	19,5	1	20	An	1	70	m	m	SRB
H-008-B	F	H	23,41	25	19,56	19,5	1	20	An	1	70000	m	m	SR
H-024-B	C	H	24,31	25	20,52	19,5	0,03	20	A	10	31	30	38	H
H-024-B	C	H	24,31	25	20,52	19,5	0,03	50	A	10	2	2	1	H
H-024-B	C	H	24,31	25	20,52	19,5	0,03	20	An	10	4	0	0	H
H-024-B	C	H	24,31	25	20,52	19,5	0,03	50	An	10	1	0	0	H
H-092-B	F	H	24,22	25	18,52	19,5	0,8628	20	A	5000	17	m	m	H
H-092-B	F	H	24,22	25	18,52	19,5	0,8628	20	A	50000	5	m	m	H
H-092-B	F	H	24,22	25	18,52	19,5	1	20	An	1	15000	m	m	H
H-092-B	F	H	24,22	25	18,52	19,5	1	20	An	1	290	m	m	SRB
H-092-B	F	H	24,22	25	18,52	19,5	1	20	An	1	295000	m	m	SR
H-100-B	S	H	24,58	25	17,36	16,5	0,03	50	An	1	0	8	m	SRB
H-100-B	S	H	24,58	25	17,36	16,5	0,03	50	An	1	30	5	m	H
H-100-B	S	H	24,58	25	17,36	16,5	0,03	50	An	1	4	8	m	M
H-100-B	C	H	24,68	25	17,36	16,5	0,05	20	A	10	32	24	23	H
H-100-B	C	H	24,68	25	17,36	16,5	0,05	50	A	10	6	3	2	H
H-100-B	C	H	24,68	25	17,36	16,5	0,05	20	A	10	2	10	4	H
H-100-B	C	H	24,68	25	17,36	16,5	0,05	50	A	10	1	0	0	H
I-008-B	F	I	28,99	25	20,65	19,5	0,8532	20	A	5000	35	m	m	H
I-008-B	F	I	28,99	25	20,65	19,5	0,8532	20	A	50000	0	m	m	H
I-008-B	F	I	28,99	25	20,65	19,5	1	20	An	1	29000	m	m	H
I-008-B	F	I	28,99	25	20,65	19,5	1	20	An	1	0	m	m	SRB
I-008-B	F	I	28,99	25	20,65	19,5	1	20	An	1	2800	m	m	SR
I-024-B	C	I	28,71	25	20,41	19,5	0,045	20	A	10	26	100	62	H
I-024-B	C	I	28,71	25	20,41	19,5	0,045	50	A	10	0	0	3	H
I-024-B	C	I	28,71	25	20,41	19,5	0,045	20	An	10	4	0	0	H
I-024-B	C	I	28,71	25	20,41	19,5	0,045	50	An	10	0	1	1	H
I-092-B	F	I	33,46	35	16,75	16,5	0,8857	20	A	50	71	m	m	H
I-092-B	F	I	33,46	35	16,75	16,5	0,8857	20	A	500	11	m	m	H
I-092-B	F	I	33,46	35	16,75	16,5	1	20	An	1	280	m	m	H
I-092-B	F	I	33,46	35	16,75	16,5	1	20	An	1	0	m	m	SRB

Sample	Lab	Layer	Temp.	T-class	H^2_0 %	H^2_0-Class	gdw/g sample	C-temp	A/An	Dilution	count #1	count #2	count #3	Culture
I-092-B	F	I	33,46	35	16,75	16,5	1	20	An	1	28000	m	m	SR
I-100-B	S	I	32,92	35	15,4	16,5	0,043	50	An	1	1	3	m	SRB
I-100-B	S	I	32,92	35	15,4	16,5	0,043	50	An	1	6	14	m	H
I-100-B	S	I	32,92	35	15,4	16,5	0,043	50	An	1	0	0	m	M
I-100-B	C	I	32,92	35	15,4	16,5	0,043	20	A	10	11	9	17	H
I-100-B	C	I	32,92	35	15,4	16,5	0,043	50	A	10	1	2	2	H
I-100-B	C	I	32,92	35	15,4	16,5	0,043	20	An	10	0	1	1	H
I-100-B	C	I	32,92	35	15,4	16,5	0,043	50	An	10	0	0	0	H
J-024-B	C	J	38,9	35	22,51	22,5	0,039	20	A	10	20	25	15	H
J-024-B	C	J	38,9	35	22,51	22,5	0,039	50	A	10	128	76	54	H
J-024-B	C	J	38,9	35	22,51	22,5	0,039	20	An	10	2	1	0	H
J-024-B	C	J	38,9	35	22,51	22,5	0,039	20	An	100	1	1	1	H
J-024-B	C	J	38,9	35	22,51	22,5	0,039	50	An	10	1	0	1	H
J-072-B	C	J	54,64	55	18,36	19,5	0,044	20	A	10	15	10	14	H
J-072-B	C	J	54,64	55	18,36	19,5	0,044	50	A	10	310	310	277	H
J-072-B	C	J	54,64	55	18,36	19,5	0,044	50	A	100	25	15	23	H
J-072-B	C	J	54,64	55	18,36	19,5	0,044	20	An	10	1	1	4	H
J-072-B	C	J	54,64	55	18,36	19,5	0,044	50	An	10	0	0	0	H
J-072-B	S	J	54,64	55	18,36	19,5	0,044	50	An	1	0	0	m	SRB
J-072-B	S	J	54,64	55	18,36	19,5	0,044	50	An	1	130	23	m	H
J-072-B	S	J	54,64	55	18,36	19,5	0,044	50	An	1	4	1	m	M
K-006-B	F	K	39,67	35	20,55	19,5	0,8446	20	A	50	65	m	m	H
K-006-B	F	K	39,67	35	20,55	19,5	0,8446	20	A	500	4	m	m	H
K-006-B	F	K	39,67	35	20,55	19,5	1	20	An	1	300	m	m	H
K-006-B	F	K	39,67	35	20,55	19,5	1	20	An	1	7,1	m	m	SRB
K-006-B	F	K	39,67	35	20,55	19,5	1	20	An	1	30	m	m	SR
K-024-B	C	K	38,9	35	21,41	22,5	0,039	20	A	10	6	6	4	H
K-024-B	C	K	38,9	35	21,41	22,5	0,039	50	A	10	119	211	172	H
K-024-B	C	K	38,9	35	21,41	22,5	0,039	20	An	10	0	0	0	H
K-024-B	C	K	38,9	35	21,41	22,5	0,039	50	An	10	0	m	m	H
K-054-B	F	K	55,74	55	12,74	13,5	0,9166	20	A	50	0	m	m	H
K-054-B	F	K	55,74	55	12,74	13,5	0,9166	20	A	500	0	m	m	H
K-054-B	F	K	55,74	55	12,74	13,5	1	20	An	1	0	m	m	H
K-054-B	F	K	55,74	55	12,74	13,5	1	20	An	1	0	m	m	SRB
K-054-B	F	K	55,74	55	12,74	13,5	1	20	An	1	0	m	m	SR
K-072-B	S	K	54,6	55	13,08	13,5	0,041	50	An	1	0	0	m	SRB
K-072-B	S	K	54,6	55	13,08	13,5	0,041	50	An	1	0	0	m	H
K-072-B	S	K	54,6	55	13,08	13,5	0,041	50	An	1	0	0	m	M
K-072-B	C	K	54,6	55	13,08	13,5	0,041	50	A	10	0	1	1	H
K-072-B	C	K	54,6	55	13,08	13,5	0,041	20	A	10	0	0	0	H
K-072-B	C	K	54,6	55	13,08	13,5	0,041	20	An	10	0	0	0	H
K-072-B	C	K	54,6	55	13,08	13,5	0,041	50	An	10	0	0	0	H
L-006-B	F	L	45	45	21,14	22,5	0,8432	20	A	50	18	m	m	H
L-006-B	F	L	45	45	21,14	22,5	0,8432	20	A	500	2	m	m	H
L-006-B	F	L	45	45	21,14	22,5	1	20	An	1	710	m	m	H
L-006-B	F	L	45	45	21,14	22,5	1	20	An	1	7,1	m	m	SRB
L-006-B	F	L	45	45	21,14	22,5	1	20	An	1	30	m	m	SR
L-024-B	C	L	42,62	45	23,04	22,5	0,041	20	A	10	1	4	2	H
L-024-B	C	L	42,62	45	23,04	22,5	0,041	50	A	10	0	0	0	H
L-024-B	C	L	42,62	45	23,04	22,5	0,041	20	An	10	0	0	0	H
L-024-B	C	L	42,62	45	23,04	22,5	0,041	50	An	10	0	0	0	H
L-024-B	S	L	42,62	45	23,04	22,5	0,041	50	An	1	1	1	m	SRB
L-024-B	S	L	42,62	45	23,04	22,5	0,041	50	An	1	10	4	m	H
L-024-B	S	L	42,62	45	23,04	22,5	0,041	50	An	1	0	0	0	M
L-072-B	C	L	59,17	55	11,4	13,5	0,04	20	A	10	0	0	0	H
L-072-B	C	L	59,17	55	11,4	13,5	0,04	50	A	10	0	0	0	H
L-072-B	C	L	59,17	55	11,4	13,5	0,04	20	An	10	0	0	0	H
L-072-B	C	L	59,17	55	11,4	13,5	0,04	50	An	10	0	0	0	H
M-024-B	C	M	43,6	45	23,62	22,5	0,043	20	A	10	11	8	12	H
M-024-B	C	M	43,6	45	23,62	22,5	0,043	50	A	10	13	49	25	H
M-024-B	C	M	43,6	45	23,62	22,5	0,043	20	An	10	0	0	0	H

Sample	Lab	Layer	Temp.	T-class	H^2_0 %	H^2_0-Class	gdw/g sample	C-temp	A/An	Dilution	count #1	count #2	count #3	Culture
M-024-B	C	M	43,6	45	23,62	22,5	0,043	50	An	10	0	0	0	H
M-054-B	F	M	60,87	55	13,27	13,5	1	20	A	50	0	m	m	H
M-054-B	F	M	60,87	55	13,27	13,5	1	20	A	500	0	m	m	H
M-054-B	F	M	60,87	55	13,27	13,5	1	20	An	1	0	m	m	H
M-054-B	F	M	60,87	55	13,27	13,5	1	20	An	1	0	m	m	SRB
M-054-B	F	M	60,87	55	13,27	13,5	1	20	An	1	0	m	m	SR
M-072-B	C	M	60,36	55	13,41	13,5	0,051	20	A	10	0	0	0	H
M-072-B	C	M	60,36	55	13,41	13,5	0,051	50	A	10	0	0	0	H
M-072-B	C	M	60,36	55	13,41	13,5	0,051	20	An	10	0	0	0	H
M-072-B	C	M	60,36	55	13,41	13,5	0,051	50	An	10	0	0	0	H
M-072-B	S	M	60,36	55	13,41	13,5	0,051	50	An	1	6	1	m	SRB
M-072-B	S	M	60,36	55	13,41	13,5	0,051	50	An	1	0	0	m	H
M-072-B	S	M	60,36	55	13,41	13,5	0,051	50	An	1	0	3	m	M
N-006-B	F	N	45	45	22,21	22,5	0,8375	20	A	50	9	m	m	H
N-006-B	F	N	45	45	22,21	22,5	0,8375	20	A	500	2	m	m	H
N-006-B	F	N	45	45	22,21	22,5	1	20	An	1	3000	m	m	H
N-006-B	F	N	45	45	22,21	22,5	1	20	An	1	7,2	m	m	SRB
N-006-B	F	N	45	45	22,21	22,5	1	20	An	1	30	m	m	SR
N-024-B	C	N	42,68	45	22,41	22,5	0,039	20	A	10	14	19	16	H
N-024-B	C	N	42,68	45	22,41	22,5	0,039	50	A	10	1	1	0	H
N-024-B	C	N	42,68	45	22,41	22,5	0,039	20	An	10	0	0	0	H
N-024-B	C	N	42,68	45	22,41	22,5	0,039	50	An	10	m	0	0	H
N-024-B	S	N	42,68	45	22,41	22,5	0,039	50	An	1	11	19	m	SRB
N-024-B	S	N	42,68	45	22,41	22,5	0,039	50	An	1	2	14	m	H
N-024-B	S	N	42,68	45	22,41	22,5	0,039	50	An	1	4	0	m	M
N-054-B	F	N	58	55	13,8	13,5	0,8905	20	A	50	0	m	m	H
N-054-B	F	N	58	55	13,8	13,5	0,8905	20	A	500	0	m	m	H
N-054-B	F	N	58	55	13,8	13,5	1	20	An	1	1500	m	m	H
N-054-B	F	N	58	55	13,8	13,5	1	20	An	1	0	m	m	SRB
N-054-B	F	N	58	55	13,8	13,5	1	20	An	1	0	m	m	SR
N-072-B	C	N	58,84	55	13,91	13,5	0,044	20	A	10	0	0	0	H
N-072-B	C	N	58,84	55	13,91	13,5	0,044	50	A	10	0	0	0	H
N-072-B	C	N	58,84	55	13,91	13,5	0,044	20	An	10	0	0	0	H
N-072-B	C	N	58,84	55	13,91	13,5	0,044	50	An	10	0	0	0	H
O-006-B	F	O	37,86	35	22,87	22,5	0,8271	20	A	500	132	m	m	H
O-006-B	F	O	37,86	35	22,87	22,5	0,8271	20	A	5000	10	m	m	H
O-006-B	F	O	37,86	35	22,87	22,5	1	20	An	1	30000	m	m	H
O-006-B	F	O	37,86	35	22,87	22,5	1	20	An	1	6	m	m	SRB
O-006-B	F	O	37,86	35	22,87	22,5	1	20	An	1	30	m	m	SR
O-024-B	C	O	38,17	35	22,43	22,5	0,041	20	A	10	32	28	27	H
O-024-B	C	O	38,17	35	22,43	22,5	0,041	50	A	10	0	0	0	H
O-024-B	C	O	38,17	35	22,43	22,5	0,041	20	An	10	1	3	1	H
O-024-B	C	O	38,17	35	22,43	22,5	0,041	50	An	10	0	m	m	H
O-072-B	C	O	48,55	45	21,89	22,5	0,043	20	A	10	15	12	13	H
O-072-B	C	O	48,55	45	21,89	22,5	0,043	50	A	10	9	14	11	H
O-072-B	C	O	48,55	45	21,89	22,5	0,043	20	An	10	0	0	0	H
O-072-B	S	O	48,55	45	21,89	22,5	0,043	50	An	10	2	0	m	SRB
O-072-B	S	O	48,55	45	21,89	22,5	0,043	50	An	1	110	14	m	H
O-072-B	S	O	48,55	45	21,89	22,5	0,043	50	An	1	3	1	m	M
P-008-B	F	P	37,86	35	21,45	22,5	0,8306	20	A	500	137	m	m	H
P-008-B	F	P	37,86	35	21,45	22,5	0,8306	20	A	5000	4	m	m	H
P-008-B	F	P	37,86	35	21,45	22,5	1	20	An	1	7200	m	m	H
P-008-B	F	P	37,86	35	21,45	22,5	1	20	An	1	7,2	m	m	SRB
P-008-B	F	P	37,86	35	21,45	22,5	1	20	An	1	72000	m	m	SR
P-024-B	C	P	37,86	35	20,04	19,5	0,048	20	A	10	22	20	25	H
P-024-B	C	P	37,86	35	20,04	19,5	0,048	50	A	10	1	0	2	H
P-024-B	C	P	37,86	35	20,04	19,5	0,048	20	An	10	1	1	0	H
P-024-B	C	P	37,86	35	20,04	19,5	0,048	50	An	10	0	0	m	H
P-092-B	F	P	53,96	55	17,49	16,5	0,8576	20	A	50	50	m	m	H
P-092-B	F	P	53,96	55	17,49	16,5	0,8576	20	A	500	5	m	m	H
P-092-B	F	P	53,96	55	17,49	16,5	1	20	An	1	2900	m	m	H

Sample	Lab	Layer	Temp.	T-class	H^2_0 %	H^2_0-Class	gdw/g sample	C-temp	A/An	Dilution	count #1	count #2	count #3	Culture
P-092-B	F	P	53,96	55	17,49	16,5	1	20	An	1	0	m	m	SRB
P-092-B	F	P	53,96	55	17,49	16,5	1	20	An	1	290	m	m	SR
P-100-B	C	P	53,96	55	16,77	16,5	0,047	20	A	10	17	17	12	H
P-100-B	C	P	53,96	55	16,77	16,5	0,047	50	A	10	0	1	2	H
P-100-B	C	P	53,96	55	16,77	16,5	0,047	20	An	10	0	0	0	H
P-100-B	C	P	53,96	55	16,77	16,5	0,047	50	An	10	m	1	m	H
P-100-B	S	P	53,96	55	16,77	16,5	0,047	50	An	1	1	1	m	SRB
P-100-B	S	P	53,96	55	16,77	16,5	0,047	50	An	1	5	34	m	H
P-100-B	S	P	53,96	55	16,77	16,5	0,047	50	An	1	7	10	m	M
Q-092-B	F	Q	32,01	35	19,86	19,5	0,8842	20	A	50	69	m	m	H
Q-092-B	F	Q	32,01	35	19,86	19,5	0,8842	20	A	500	8	m	m	H
Q-092-B	F	Q	32,01	35	19,86	19,5	1	20	An	1	6800	m	m	H
Q-092-B	F	Q	32,01	35	19,86	19,5	1	20	An	1	0	m	m	SRB
Q-092-B	F	Q	32,01	35	19,86	19,5	1	20	An	1	680	m	m	SR
Q-100-B	C	Q	32,92	35	19,83	19,5	0,042	20	A	10	19	16	17	H
Q-100-B	C	Q	32,92	35	19,83	19,5	0,042	50	A	10	8	5	9	H
Q-100-B	C	Q	32,92	35	19,83	19,5	0,042	20	An	10	3	0	0	H
Q-100-B	C	Q	32,92	35	19,83	19,5	0,042	50	An	10	m	m	m	H
Q-001-B	C	Q	28,54	25	17,82	16,5	0,045	20	A	10	11	12	20	H
Q-001-B	C	Q	28,54	25	17,82	16,5	0,045	50	A	10	3	5	3	H
Q-001-B	C	Q	28,54	25	17,82	16,5	0,045	20	An	10	2	0	1	H
Q-001-B	C	Q	28,54	25	17,82	16,5	0,045	50	An	10	m	m	m	H

1	G-008-B	F	G	19,84	25	19,85	19,5	0,87	20	A	5000	H	6,10384
2	G-008-B	F	G	19,84	25	19,85	19,5	0,87	20	A	50000	H	6,27796
3	G-008-B	F	G	19,84	25	19,85	19,5	1	20	An	1	H	3,4624
4	G-008-B	F	G	19,84	25	19,85	19,5	1	20	An	1	SRB	1,17609
5	G-008-B	F	G	19,84	25	19,85	19,5	1	20	An	1	SR	3,17609
6	G-024-B	C	G	20,64	25	17,62	16,5	0,042	20	A	100	H	5,67778
7	G-024-B	C	G	20,64	25	17,62	16,5	0,042	20	A	100	H	5,66005
8	G-024-B	C	G	20,64	25	17,62	16,5	0,042	20	A	100	H	5,70309
9	G-024-B	C	G	20,64	25	17,62	16,5	0,042	20	A	1000	H	5,73848
10	G-024-B	C	G	20,64	25	17,62	16,5	0,042	20	A	1000	H	5,80811
11	G-024-B	C	G	20,64	25	17,62	16,5	0,042	20	A	1000	H	5,77469
12	G-024-B	C	G	20,64	25	17,62	16,5	0,042	50	A	10	H	2,67778
13	G-024-B	C	G	20,64	25	17,62	16,5	0,042	50	A	10	H	2,67778
14	G-024-B	C	G	20,64	25	17,62	16,5	0,042	50	A	10	H	2,37675
15	G-024-B	C	G	20,64	25	17,62	16,5	0,042	20	An	10	H	3,67778
16	G-024-B	C	G	20,64	25	17,62	16,5	0,042	20	An	10	H	3,41814
17	G-024-B	C	G	20,64	25	17,62	16,5	0,042	20	An	10	H	3,67778
18	G-024-B	C	G	20,64	25	17,62	16,5	0,042	50	An	1000	H	0
19	G-024-B	C	G	20,64	25	17,62	16,5	0,042	50	An	1000	H	0
20	G-024-B	C	G	20,64	25	17,62	16,5	0,042	50	An	1000	H	0
21	G-024-B	S	G	20,64	25	17,62	16,5	0,042	50	An	1	SRB	2,45593
22	G-024-B	S	G	20,64	25	17,62	16,5	0,042	50	An	1	SRB	1,37675
23	G-024-B	S	G	20,64	25	17,62	16,5	0,042	50	An	1	H	0
24	G-024-B	S	G	20,64	25	17,62	16,5	0,042	50	An	1	H	0
25	G-024-B	S	G	20,64	25	17,62	16,5	0,042	50	An	1	M	1,85387
26	G-024-B	S	G	20,64	25	17,62	16,5	0,042	50	An	1	M	2,33099
27	G-092-B	F	G	19,91	25	18,88	19,5	0,835	20	A	5000	H	5,62854
28	G-092-B	F	G	19,91	25	18,88	19,5	0,835	20	A	50000	H	5,92341
29	G-092-B	F	G	19,91	25	18,88	19,5	1	20	An	1	H	5,17609
30	G-092-B	F	G	19,91	25	18,88	19,5	1	20	An	1	SRB	0
31	G-092-B	F	G	19,91	25	18,88	19,5	1	20	An	1	SR	5,47712
32	G-100-B	C	G	20,67	25	18,79	19,5	0,045	20	A	10	H	3,91499
33	G-100-B	C	G	20,67	25	18,79	19,5	0,045	20	A	10	H	3,87827
34	G-100-B	C	G	20,67	25	18,79	19,5	0,045	20	A	10	H	3,87827
35	G-100-B	C	G	20,67	25	18,79	19,5	0,045	50	A	10	H	0
36	G-100-B	C	G	20,67	25	18,79	19,5	0,045	50	A	10	H	2,34679
37	G-100-B	C	G	20,67	25	18,79	19,5	0,045	50	A	10	H	0
38	G-100-B	C	G	20,67	25	18,79	19,5	0,045	20	An	10	H	3,19189
39	G-100-B	C	G	20,67	25	18,79	19,5	0,045	20	An	10	H	3,04576
40	G-100-B	C	G	20,67	25	18,79	19,5	0,045	20	An	10	H	3,12494

41	G-100-B	C	G	20,67	25	18,79	19,5	0,045	50	An	100	H	0
42	G-100-B	C	G	20,67	25	18,79	19,5	0,045	50	An	100	H	0
43	G-100-B	C	G	20,67	25	18,79	19,5	0,045	50	An	100	H	0
44	H-008-B	F	H	23,41	25	19,56	19,5	0,8547	20	A	5000	H	5,51534
45	H-008-B	F	H	23,41	25	19,56	19,5	0,8547	20	A	50000	H	5,54531
46	H-008-B	F	H	23,41	25	19,56	19,5	1	20	An	1	H	4,8451
47	H-008-B	F	H	23,41	25	19,56	19,5	1	20	An	1	SRB	1,8451
48	H-008-B	F	H	23,41	25	19,56	19,5	1	20	An	1	SR	4,8451
49	H-024-B	C	H	24,31	25	20,52	19,5	0,03	20	A	10	H	4,01424
50	H-024-B	C	H	24,31	25	20,52	19,5	0,03	20	A	10	H	4
51	H-024-B	C	H	24,31	25	20,52	19,5	0,03	20	A	10	H	4,10266
52	H-024-B	C	H	24,31	25	20,52	19,5	0,03	50	A	10	H	2,82391
53	H-024-B	C	H	24,31	25	20,52	19,5	0,03	50	A	10	H	2,82391
54	H-024-B	C	H	24,31	25	20,52	19,5	0,03	50	A	10	H	2,52288
55	H-024-B	C	H	24,31	25	20,52	19,5	0,03	20	An	10	H	3,12494
56	H-024-B	C	H	24,31	25	20,52	19,5	0,03	20	An	10	H	0
57	H-024-B	C	H	24,31	25	20,52	19,5	0,03	20	An	10	H	0
58	H-024-B	C	H	24,31	25	20,52	19,5	0,03	50	An	10	H	2,52288
59	H-024-B	C	H	24,31	25	20,52	19,5	0,03	50	An	10	H	0
60	H-024-B	C	H	24,31	25	20,52	19,5	0,03	50	An	10	H	0
61	H-092-B	F	H	24,22	25	18,52	19,5	0,8628	20	A	5000	H	4,99351
62	H-092-B	F	H	24,22	25	18,52	19,5	0,8628	20	A	50000	H	5,46203
63	H-092-B	F	H	24,22	25	18,52	19,5	1	20	An	1	H	4,17609
64	H-092-B	F	H	24,22	25	18,52	19,5	1	20	An	1	SRB	2,4624
65	H-092-B	F	H	24,22	25	18,52	19,5	1	20	An	1	SR	5,46982
66	H-100-B	S	H	24,58	25	17,36	16,5	0,03	50	An	1	SRB	0
67	H-100-B	S	H	24,58	25	17,36	16,5	0,03	50	An	1	SRB	2,42597
68	H-100-B	S	H	24,58	25	17,36	16,5	0,03	50	An	1	H	3
69	H-100-B	S	H	24,58	25	17,36	16,5	0,03	50	An	1	H	2,22185
70	H-100-B	S	H	24,58	25	17,36	16,5	0,03	50	An	1	M	2,12494
71	H-100-B	S	H	24,58	25	17,36	16,5	0,03	50	An	1	M	2,42597
72	H-100-B	C	H	24,68	25	17,36	16,5	0,05	20	A	10	H	3,80618
73	H-100-B	C	H	24,68	25	17,36	16,5	0,05	20	A	10	H	3,68124
74	H-100-B	C	H	24,68	25	17,36	16,5	0,05	20	A	10	H	3,66276
75	H-100-B	C	H	24,68	25	17,36	16,5	0,05	50	A	10	H	3,07918
76	H-100-B	C	H	24,68	25	17,36	16,5	0,05	50	A	10	H	2,77815
77	H-100-B	C	H	24,68	25	17,36	16,5	0,05	50	A	10	H	2,60206
78	H-100-B	C	H	24,68	25	17,36	16,5	0,05	20	A	10	H	2,60206
79	H-100-B	C	H	24,68	25	17,36	16,5	0,05	20	A	10	H	3,30103
80	H-100-B	C	H	24,68	25	17,36	16,5	0,05	20	A	10	H	2,90309
81	H-100-B	C	H	24,68	25	17,36	16,5	0,05	50	A	10	H	2,30103
82	H-100-B	C	H	24,68	25	17,36	16,5	0,05	50	A	10	H	0
83	H-100-B	C	H	24,68	25	17,36	16,5	0,05	50	A	10	H	0
84	I-008-B	F	I	28,99	25	20,65	19,5	0,8532	20	A	5000	H	5,31199
85	I-008-B	F	I	28,99	25	20,65	19,5	0,8532	20	A	50000	H	0
86	I-008-B	F	I	28,99	25	20,65	19,5	1	20	An	1	H	4,4624
87	I-008-B	F	I	28,99	25	20,65	19,5	1	20	An	1	SRB	0
88	I-008-B	F	I	28,99	25	20,65	19,5	1	20	An	1	SR	3,44716
89	I-024-B	C	I	28,71	25	20,41	19,5	0,045	20	A	10	H	3,76176
90	I-024-B	C	I	28,71	25	20,41	19,5	0,045	20	A	10	H	4,34679
91	I-024-B	C	I	28,71	25	20,41	19,5	0,045	20	A	10	H	4,13918
92	I-024-B	C	I	28,71	25	20,41	19,5	0,045	50	A	10	H	0
93	I-024-B	C	I	28,71	25	20,41	19,5	0,045	50	A	10	H	0
94	I-024-B	C	I	28,71	25	20,41	19,5	0,045	50	A	10	H	2,82391
95	I-024-B	C	I	28,71	25	20,41	19,5	0,045	20	An	10	H	2,94885
96	I-024-B	C	I	28,71	25	20,41	19,5	0,045	20	An	10	H	0
97	I-024-B	C	I	28,71	25	20,41	19,5	0,045	20	An	10	H	0
98	I-024-B	C	I	28,71	25	20,41	19,5	0,045	50	An	10	H	0
99	I-024-B	C	I	28,71	25	20,41	19,5	0,045	50	An	10	H	2,34679
100	I-024-B	C	I	28,71	25	20,41	19,5	0,045	50	An	10	H	2,34679
101	I-092-B	F	I	33,46	35	16,75	16,5	0,8857	20	A	50	H	3,60294
102	I-092-B	F	I	33,46	35	16,75	16,5	0,8857	20	A	500	H	3,79308
103	I-092-B	F	I	33,46	35	16,75	16,5	1	20	An	1	H	2,44716
104	I-092-B	F	I	33,46	35	16,75	16,5	1	20	An	1	SRB	0
105	I-092-B	F	I	33,46	35	16,75	16,5	1	20	An	1	SR	4,44716
106	I-100-B	S	I	32,92	35	15,4	16,5	0,043	50	An	1	SRB	1,36653
107	I-100-B	S	I	32,92	35	15,4	16,5	0,043	50	An	1	SRB	1,84365
108	I-100-B	S	I	32,92	35	15,4	16,5	0,043	50	An	1	H	2,14468
109	I-100-B	S	I	32,92	35	15,4	16,5	0,043	50	An	1	H	2,51266
110	I-100-B	S	I	32,92	35	15,4	16,5	0,043	50	An	1	M	0
111	I-100-B	S	I	32,92	35	15,4	16,5	0,043	50	An	1	M	0
112	I-100-B	C	I	32,92	35	15,4	16,5	0,043	20	A	10	H	3,40792
113	I-100-B	C	I	32,92	35	15,4	16,5	0,043	20	A	10	H	3,32077

114	I-100-B	C	I	32,92	35	15,4	16,5	0,043	20	A	10	H	3,59698
115	I-100-B	C	I	32,92	35	15,4	16,5	0,043	50	A	10	H	2,36653
116	I-100-B	C	I	32,92	35	15,4	16,5	0,043	50	A	10	H	2,66756
117	I-100-B	C	I	32,92	35	15,4	16,5	0,043	50	A	10	H	2,66756
118	I-100-B	C	I	32,92	35	15,4	16,5	0,043	20	An	10	H	0
119	I-100-B	C	I	32,92	35	15,4	16,5	0,043	20	An	10	H	2,36653
120	I-100-B	C	I	32,92	35	15,4	16,5	0,043	20	An	10	H	2,36653
121	I-100-B	C	I	32,92	35	15,4	16,5	0,043	50	An	10	H	0
122	I-100-B	C	I	32,92	35	15,4	16,5	0,043	50	An	10	H	0
123	I-100-B	C	I	32,92	35	15,4	16,5	0,043	50	An	10	H	0
124	J-024-B	C	J	38,9	35	22,51	22,5	0,039	20	A	10	H	3,70997
125	J-024-B	C	J	38,9	35	22,51	22,5	0,039	20	A	10	H	3,80688
126	J-024-B	C	J	38,9	35	22,51	22,5	0,039	20	A	10	H	3,58503
127	J-024-B	C	J	38,9	35	22,51	22,5	0,039	50	A	10	H	4,51615
128	J-024-B	C	J	38,9	35	22,51	22,5	0,039	50	A	10	H	4,28975
129	J-024-B	C	J	38,9	35	22,51	22,5	0,039	50	A	10	H	4,14133
130	J-024-B	C	J	38,9	35	22,51	22,5	0,039	20	An	10	H	2,70997
131	J-024-B	C	J	38,9	35	22,51	22,5	0,039	20	An	10	H	2,40894
132	J-024-B	C	J	38,9	35	22,51	22,5	0,039	20	An	10	H	0
133	J-024-B	C	J	38,9	35	22,51	22,5	0,039	20	An	100	H	3,40894
134	J-024-B	C	J	38,9	35	22,51	22,5	0,039	20	An	100	H	3,40894
135	J-024-B	C	J	38,9	35	22,51	22,5	0,039	20	An	100	H	3,40894
136	J-024-B	C	J	38,9	35	22,51	22,5	0,039	50	An	10	H	2,40894
137	J-024-B	C	J	38,9	35	22,51	22,5	0,039	50	An	10	H	0
138	J-024-B	C	J	38,9	35	22,51	22,5	0,039	50	An	10	H	2,40894
139	J-072-B	C	J	54,64	55	18,36	19,5	0,044	20	A	10	H	3,53264
140	J-072-B	C	J	54,64	55	18,36	19,5	0,044	20	A	10	H	3,35655
141	J-072-B	C	J	54,64	55	18,36	19,5	0,044	20	A	10	H	3,50268
142	J-072-B	C	J	54,64	55	18,36	19,5	0,044	50	A	10	H	4,84791
143	J-072-B	C	J	54,64	55	18,36	19,5	0,044	50	A	10	H	4,84791
144	J-072-B	C	J	54,64	55	18,36	19,5	0,044	50	A	10	H	4,79903
145	J-072-B	C	J	54,64	55	18,36	19,5	0,044	50	A	100	H	4,75449
146	J-072-B	C	J	54,64	55	18,36	19,5	0,044	50	A	100	H	4,53264
147	J-072-B	C	J	54,64	55	18,36	19,5	0,044	50	A	100	H	4,71828
148	J-072-B	C	J	54,64	55	18,36	19,5	0,044	20	An	10	H	2,35655
149	J-072-B	C	J	54,64	55	18,36	19,5	0,044	20	An	10	H	2,35655
150	J-072-B	C	J	54,64	55	18,36	19,5	0,044	20	An	10	H	2,95861
151	J-072-B	C	J	54,64	55	18,36	19,5	0,044	50	An	10	H	0
152	J-072-B	C	J	54,64	55	18,36	19,5	0,044	50	An	10	H	0
153	J-072-B	C	J	54,64	55	18,36	19,5	0,044	50	An	10	H	0
154	J-072-B	S	J	54,64	55	18,36	19,5	0,044	50	An	1	SRB	0
155	J-072-B	S	J	54,64	55	18,36	19,5	0,044	50	An	1	SRB	0
156	J-072-B	S	J	54,64	55	18,36	19,5	0,044	50	An	1	H	3,47049
157	J-072-B	S	J	54,64	55	18,36	19,5	0,044	50	An	1	H	2,71828
158	J-072-B	S	J	54,64	55	18,36	19,5	0,044	50	An	1	M	1,95861
159	J-072-B	S	J	54,64	55	18,36	19,5	0,044	50	An	1	M	1,35655
160	K-006-B	F	K	39,67	35	20,55	19,5	0,8446	20	A	50	H	3,58523
161	K-006-B	F	K	39,67	35	20,55	19,5	0,8446	20	A	500	H	3,37438
162	K-006-B	F	K	39,67	35	20,55	19,5	1	20	An	1	H	2,47712
163	K-006-B	F	K	39,67	35	20,55	19,5	1	20	An	1	SRB	0,85126
164	K-006-B	F	K	39,67	35	20,55	19,5	1	20	An	1	SR	1,47712
165	K-024-B	C	K	38,9	35	21,41	22,5	0,039	20	A	10	H	3,18709
166	K-024-B	C	K	38,9	35	21,41	22,5	0,039	20	A	10	H	3,18709
167	K-024-B	C	K	38,9	35	21,41	22,5	0,039	20	A	10	H	3,011
168	K-024-B	C	K	38,9	35	21,41	22,5	0,039	50	A	10	H	4,48448
169	K-024-B	C	K	38,9	35	21,41	22,5	0,039	50	A	10	H	4,73322
170	K-024-B	C	K	38,9	35	21,41	22,5	0,039	50	A	10	H	4,64446
171	K-024-B	C	K	38,9	35	21,41	22,5	0,039	20	An	10	H	0
172	K-024-B	C	K	38,9	35	21,41	22,5	0,039	20	An	10	H	0
173	K-024-B	C	K	38,9	35	21,41	22,5	0,039	20	An	10	H	0
174	K-024-B	C	K	38,9	35	21,41	22,5	0,039	50	An	10	H	0
175	K-054-B	F	K	55,74	55	12,74	13,5	0,9166	20	A	50	H	0
176	K-054-B	F	K	55,74	55	12,74	13,5	0,9166	20	A	500	H	0
177	K-054-B	F	K	55,74	55	12,74	13,5	1	20	An	1	H	0
178	K-054-B	F	K	55,74	55	12,74	13,5	1	20	An	1	SRB	0
179	K-054-B	F	K	55,74	55	12,74	13,5	1	20	An	1	SR	0
180	K-072-B	S	K	54,6	55	13,08	13,5	0,041	50	An	1	SRB	0
181	K-072-B	S	K	54,6	55	13,08	13,5	0,041	50	An	1	SRB	0
182	K-072-B	S	K	54,6	55	13,08	13,5	0,041	50	An	1	H	0
183	K-072-B	S	K	54,6	55	13,08	13,5	0,041	50	An	1	H	0
184	K-072-B	S	K	54,6	55	13,08	13,5	0,041	50	An	1	M	0
185	K-072-B	S	K	54,6	55	13,08	13,5	0,041	50	An	1	M	0
186	K-072-B	C	K	54,6	55	13,08	13,5	0,041	50	A	10	H	0

187	K-072-B	C	K	54,6	55	13,08	13,5	0,041	50	A	10	H	2,38722
188	K-072-B	C	K	54,6	55	13,08	13,5	0,041	50	A	10	H	2,38722
189	K-072-B	C	K	54,6	55	13,08	13,5	0,041	20	A	10	H	0
190	K-072-B	C	K	54,6	55	13,08	13,5	0,041	20	A	10	H	0
191	K-072-B	C	K	54,6	55	13,08	13,5	0,041	20	A	10	H	0
192	K-072-B	C	K	54,6	55	13,08	13,5	0,041	20	An	10	H	0
193	K-072-B	C	K	54,6	55	13,08	13,5	0,041	20	An	10	H	0
194	K-072-B	C	K	54,6	55	13,08	13,5	0,041	20	An	10	H	0
195	K-072-B	C	K	54,6	55	13,08	13,5	0,041	50	An	10	H	0
196	K-072-B	C	K	54,6	55	13,08	13,5	0,041	50	An	10	H	0
197	K-072-B	C	K	54,6	55	13,08	13,5	0,041	50	An	10	H	0
198	L-006-B	F	L	45	45	21,14	22,5	0,8432	20	A	50	H	3,02831
199	L-006-B	F	L	45	45	21,14	22,5	0,8432	20	A	500	H	3,07407
200	L-006-B	F	L	45	45	21,14	22,5	1	20	An	1	H	2,85126
201	L-006-B	F	L	45	45	21,14	22,5	1	20	An	1	SRB	0,85126
202	L-006-B	F	L	45	45	21,14	22,5	1	20	An	1	SR	1,47712
203	L-024-B	C	L	42,62	45	23,04	22,5	0,041	20	A	10	H	2,38722
204	L-024-B	C	L	42,62	45	23,04	22,5	0,041	20	A	10	H	2,98928
205	L-024-B	C	L	42,62	45	23,04	22,5	0,041	20	A	10	H	2,68825
206	L-024-B	C	L	42,62	45	23,04	22,5	0,041	50	A	10	H	0
207	L-024-B	C	L	42,62	45	23,04	22,5	0,041	50	A	10	H	0
208	L-024-B	C	L	42,62	45	23,04	22,5	0,041	50	A	10	H	0
209	L-024-B	C	L	42,62	45	23,04	22,5	0,041	20	An	10	H	0
210	L-024-B	C	L	42,62	45	23,04	22,5	0,041	20	An	10	H	0
211	L-024-B	C	L	42,62	45	23,04	22,5	0,041	20	An	10	H	0
212	L-024-B	C	L	42,62	45	23,04	22,5	0,041	50	An	10	H	0
213	L-024-B	C	L	42,62	45	23,04	22,5	0,041	50	An	10	H	0
214	L-024-B	C	L	42,62	45	23,04	22,5	0,041	50	An	10	H	0
215	L-024-B	S	L	42,62	45	23,04	22,5	0,041	50	An	1	SRB	1,38722
216	L-024-B	S	L	42,62	45	23,04	22,5	0,041	50	An	1	SRB	1,38722
217	L-024-B	S	L	42,62	45	23,04	22,5	0,041	50	An	1	H	2,38722
218	L-024-B	S	L	42,62	45	23,04	22,5	0,041	50	An	1	H	1,98928
219	L-024-B	S	L	42,62	45	23,04	22,5	0,041	50	An	1	M	0
220	L-024-B	S	L	42,62	45	23,04	22,5	0,041	50	An	1	M	0
221	L-024-B	S	L	42,62	45	23,04	22,5	0,041	50	An	1	M	0
222	L-072-B	C	L	59,17	55	11,4	13,5	0,04	20	A	10	H	0
223	L-072-B	C	L	59,17	55	11,4	13,5	0,04	20	A	10	H	0
224	L-072-B	C	L	59,17	55	11,4	13,5	0,04	20	A	10	H	0
225	L-072-B	C	L	59,17	55	11,4	13,5	0,04	50	A	10	H	0
226	L-072-B	C	L	59,17	55	11,4	13,5	0,04	50	A	10	H	0
227	L-072-B	C	L	59,17	55	11,4	13,5	0,04	50	A	10	H	0
228	L-072-B	C	L	59,17	55	11,4	13,5	0,04	20	An	10	H	0
229	L-072-B	C	L	59,17	55	11,4	13,5	0,04	20	An	10	H	0
230	L-072-B	C	L	59,17	55	11,4	13,5	0,04	20	An	10	H	0
231	L-072-B	C	L	59,17	55	11,4	13,5	0,04	50	An	10	H	0
232	L-072-B	C	L	59,17	55	11,4	13,5	0,04	50	An	10	H	0
233	L-072-B	C	L	59,17	55	11,4	13,5	0,04	50	An	10	H	0
234	M-024-B	C	M	43,6	45	23,62	22,5	0,043	20	A	10	H	3,40792
235	M-024-B	C	M	43,6	45	23,62	22,5	0,043	20	A	10	H	3,26962
236	M-024-B	C	M	43,6	45	23,62	22,5	0,043	20	A	10	H	3,44571
237	M-024-B	C	M	43,6	45	23,62	22,5	0,043	50	A	10	H	3,48047
238	M-024-B	C	M	43,6	45	23,62	22,5	0,043	50	A	10	H	4,05673
239	M-024-B	C	M	43,6	45	23,62	22,5	0,043	50	A	10	H	3,76447
240	M-024-B	C	M	43,6	45	23,62	22,5	0,043	20	An	10	H	0
241	M-024-B	C	M	43,6	45	23,62	22,5	0,043	20	An	10	H	0
242	M-024-B	C	M	43,6	45	23,62	22,5	0,043	20	An	10	H	0
243	M-024-B	C	M	43,6	45	23,62	22,5	0,043	50	An	10	H	0
244	M-024-B	C	M	43,6	45	23,62	22,5	0,043	50	An	10	H	0
245	M-024-B	C	M	43,6	45	23,62	22,5	0,043	50	An	10	H	0
246	M-054-B	F	M	60,87	55	13,27	13,5	1	20	A	50	H	0
247	M-054-B	F	M	60,87	55	13,27	13,5	1	20	A	500	H	0
248	M-054-B	F	M	60,87	55	13,27	13,5	1	20	An	1	H	0
249	M-054-B	F	M	60,87	55	13,27	13,5	1	20	An	1	SRB	0
250	M-054-B	F	M	60,87	55	13,27	13,5	1	20	An	1	SR	0
251	M-072-B	C	M	60,36	55	13,41	13,5	0,051	20	A	10	H	0
252	M-072-B	C	M	60,36	55	13,41	13,5	0,051	20	A	10	H	0
253	M-072-B	C	M	60,36	55	13,41	13,5	0,051	20	A	10	H	0
254	M-072-B	C	M	60,36	55	13,41	13,5	0,051	50	A	10	H	0
255	M-072-B	C	M	60,36	55	13,41	13,5	0,051	50	A	10	H	0
256	M-072-B	C	M	60,36	55	13,41	13,5	0,051	50	A	10	H	0
257	M-072-B	C	M	60,36	55	13,41	13,5	0,051	20	An	10	H	0
258	M-072-B	C	M	60,36	55	13,41	13,5	0,051	20	An	10	H	0
259	M-072-B	C	M	60,36	55	13,41	13,5	0,051	20	An	10	H	0

260	M-072-B	C	M	60,36	55	13,41	13,5	0,051	50	An	10	H	0
261	M-072-B	C	M	60,36	55	13,41	13,5	0,051	50	An	10	H	0
262	M-072-B	C	M	60,36	55	13,41	13,5	0,051	50	An	10	H	0
263	M-072-B	S	M	60,36	55	13,41	13,5	0,051	50	An	1	SRB	2,07058
264	M-072-B	S	M	60,36	55	13,41	13,5	0,051	50	An	1	SRB	1,29243
265	M-072-B	S	M	60,36	55	13,41	13,5	0,051	50	An	1	H	0
266	M-072-B	S	M	60,36	55	13,41	13,5	0,051	50	An	1	H	0
267	M-072-B	S	M	60,36	55	13,41	13,5	0,051	50	An	1	M	0
268	M-072-B	S	M	60,36	55	13,41	13,5	0,051	50	An	1	M	1,76955
269	N-006-B	F	N	45	45	22,21	22,5	0,8375	20	A	50	H	2,73023
270	N-006-B	F	N	45	45	22,21	22,5	0,8375	20	A	500	H	3,07702
271	N-006-B	F	N	45	45	22,21	22,5	1	20	An	1	H	3,47712
272	N-006-B	F	N	45	45	22,21	22,5	1	20	An	1	SRB	0,85733
273	N-006-B	F	N	45	45	22,21	22,5	1	20	An	1	SR	1,47712
274	N-024-B	C	N	42,68	45	22,41	22,5	0,039	20	A	10	H	3,55506
275	N-024-B	C	N	42,68	45	22,41	22,5	0,039	20	A	10	H	3,68769
276	N-024-B	C	N	42,68	45	22,41	22,5	0,039	20	A	10	H	3,61306
277	N-024-B	C	N	42,68	45	22,41	22,5	0,039	50	A	10	H	2,40894
278	N-024-B	C	N	42,68	45	22,41	22,5	0,039	50	A	10	H	2,40894
279	N-024-B	C	N	42,68	45	22,41	22,5	0,039	50	A	10	H	0
280	N-024-B	C	N	42,68	45	22,41	22,5	0,039	20	An	10	H	0
281	N-024-B	C	N	42,68	45	22,41	22,5	0,039	20	An	10	H	0
282	N-024-B	C	N	42,68	45	22,41	22,5	0,039	20	An	10	H	0
283	N-024-B	S	N	42,68	45	22,41	22,5	0,039	50	An	1	SRB	2,45033
284	N-024-B	S	N	42,68	45	22,41	22,5	0,039	50	An	1	SRB	2,68769
285	N-024-B	S	N	42,68	45	22,41	22,5	0,039	50	An	1	H	1,70997
286	N-024-B	S	N	42,68	45	22,41	22,5	0,039	50	An	1	H	2,55506
287	N-024-B	S	N	42,68	45	22,41	22,5	0,039	50	An	1	M	2,011
288	N-024-B	S	N	42,68	45	22,41	22,5	0,039	50	An	1	M	0
289	N-054-B	F	N	58	55	13,8	13,5	0,8905	20	A	50	H	0
290	N-054-B	F	N	58	55	13,8	13,5	0,8905	20	A	500	H	0
291	N-054-B	F	N	58	55	13,8	13,5	1	20	An	1	H	3,17609
292	N-054-B	F	N	58	55	13,8	13,5	1	20	An	1	SRB	0
293	N-054-B	F	N	58	55	13,8	13,5	1	20	An	1	SR	0
294	N-072-B	C	N	58,84	55	13,91	13,5	0,044	20	A	10	H	0
295	N-072-B	C	N	58,84	55	13,91	13,5	0,044	20	A	10	H	0
296	N-072-B	C	N	58,84	55	13,91	13,5	0,044	20	A	10	H	0
297	N-072-B	C	N	58,84	55	13,91	13,5	0,044	50	A	10	H	0
298	N-072-B	C	N	58,84	55	13,91	13,5	0,044	50	A	10	H	0
299	N-072-B	C	N	58,84	55	13,91	13,5	0,044	50	A	10	H	0
300	N-072-B	C	N	58,84	55	13,91	13,5	0,044	20	An	10	H	0
301	N-072-B	C	N	58,84	55	13,91	13,5	0,044	20	An	10	H	0
302	N-072-B	C	N	58,84	55	13,91	13,5	0,044	20	An	10	H	0
303	N-072-B	C	N	58,84	55	13,91	13,5	0,044	50	An	10	H	0
304	N-072-B	C	N	58,84	55	13,91	13,5	0,044	50	An	10	H	0
305	N-072-B	C	N	58,84	55	13,91	13,5	0,044	50	An	10	H	0
306	O-006-B	F	O	37,86	35	22,87	22,5	0,8271	20	A	500	H	4,90199
307	O-006-B	F	O	37,86	35	22,87	22,5	0,8271	20	A	5000	H	4,78141
308	O-006-B	F	O	37,86	35	22,87	22,5	1	20	An	1	H	4,47712
309	O-006-B	F	O	37,86	35	22,87	22,5	1	20	An	1	SRB	0,77815
310	O-006-B	F	O	37,86	35	22,87	22,5	1	20	An	1	SR	1,47712
311	O-024-B	C	O	38,17	35	22,43	22,5	0,041	20	A	10	H	3,89237
312	O-024-B	C	O	38,17	35	22,43	22,5	0,041	20	A	10	H	3,83437
313	O-024-B	C	O	38,17	35	22,43	22,5	0,041	20	A	10	H	3,81858
314	O-024-B	C	O	38,17	35	22,43	22,5	0,041	50	A	10	H	0
315	O-024-B	C	O	38,17	35	22,43	22,5	0,041	50	A	10	H	0
316	O-024-B	C	O	38,17	35	22,43	22,5	0,041	50	A	10	H	0
317	O-024-B	C	O	38,17	35	22,43	22,5	0,041	20	An	10	H	2,38722
318	O-024-B	C	O	38,17	35	22,43	22,5	0,041	20	An	10	H	2,86434
319	O-024-B	C	O	38,17	35	22,43	22,5	0,041	20	An	10	H	2,38722
320	O-024-B	C	O	38,17	35	22,43	22,5	0,041	50	An	10	H	0
321	O-072-B	C	O	48,55	45	21,89	22,5	0,043	20	A	10	H	3,54262
322	O-072-B	C	O	48,55	45	21,89	22,5	0,043	20	A	10	H	3,44571
323	O-072-B	C	O	48,55	45	21,89	22,5	0,043	20	A	10	H	3,48047
324	O-072-B	C	O	48,55	45	21,89	22,5	0,043	50	A	10	H	3,32077
325	O-072-B	C	O	48,55	45	21,89	22,5	0,043	50	A	10	H	3,51266
326	O-072-B	C	O	48,55	45	21,89	22,5	0,043	50	A	10	H	3,40792
327	O-072-B	C	O	48,55	45	21,89	22,5	0,043	20	An	10	H	0
328	O-072-B	C	O	48,55	45	21,89	22,5	0,043	20	An	10	H	0
329	O-072-B	C	O	48,55	45	21,89	22,5	0,043	20	An	10	H	0
330	O-072-B	S	O	48,55	45	21,89	22,5	0,043	50	An	10	SRB	2,66756
331	O-072-B	S	O	48,55	45	21,89	22,5	0,043	50	An	10	SRB	0
332	O-072-B	S	O	48,55	45	21,89	22,5	0,043	50	An	1	H	3,40792

333	O-072-B	S	O	48,55	45	21,89	22,5	0,043	50	An	1	H	2,51266
334	O-072-B	S	O	48,55	45	21,89	22,5	0,043	50	An	1	M	1,84365
335	O-072-B	S	O	48,55	45	21,89	22,5	0,043	50	An	1	M	1,36653
336	P-008-B	F	P	37,86	35	21,45	22,5	0,8306	20	A	500	H	4,9163
337	P-008-B	F	P	37,86	35	21,45	22,5	0,8306	20	A	5000	H	4,38164
338	P-008-B	F	P	37,86	35	21,45	22,5	1	20	An	1	H	3,85733
339	P-008-B	F	P	37,86	35	21,45	22,5	1	20	An	1	SRB	0,85733
340	P-008-B	F	P	37,86	35	21,45	22,5	1	20	An	1	SR	4,85733
341	P-024-B	C	P	37,86	35	20,04	19,5	0,048	20	A	10	H	3,66118
342	P-024-B	C	P	37,86	35	20,04	19,5	0,048	20	A	10	H	3,61979
343	P-024-B	C	P	37,86	35	20,04	19,5	0,048	20	A	10	H	3,7167
344	P-024-B	C	P	37,86	35	20,04	19,5	0,048	50	A	10	H	2,31876
345	P-024-B	C	P	37,86	35	20,04	19,5	0,048	50	A	10	H	0
346	P-024-B	C	P	37,86	35	20,04	19,5	0,048	50	A	10	H	2,61979
347	P-024-B	C	P	37,86	35	20,04	19,5	0,048	20	An	10	H	2,31876
348	P-024-B	C	P	37,86	35	20,04	19,5	0,048	20	An	10	H	2,31876
349	P-024-B	C	P	37,86	35	20,04	19,5	0,048	20	An	10	H	0
350	P-024-B	C	P	37,86	35	20,04	19,5	0,048	50	An	10	H	0
351	P-024-B	C	P	37,86	35	20,04	19,5	0,048	50	An	10	H	0
352	P-092-B	F	P	53,96	55	17,49	16,5	0,8576	20	A	50	H	3,46466
353	P-092-B	F	P	53,96	55	17,49	16,5	0,8576	20	A	500	H	3,46466
354	P-092-B	F	P	53,96	55	17,49	16,5	1	20	An	1	H	3,4624
355	P-092-B	F	P	53,96	55	17,49	16,5	1	20	An	1	SRB	0
356	P-092-B	F	P	53,96	55	17,49	16,5	1	20	An	1	SR	2,4624
357	P-100-B	C	P	53,96	55	16,77	16,5	0,047	20	A	10	H	3,55835
358	P-100-B	C	P	53,96	55	16,77	16,5	0,047	20	A	10	H	3,55835
359	P-100-B	C	P	53,96	55	16,77	16,5	0,047	20	A	10	H	3,40708
360	P-100-B	C	P	53,96	55	16,77	16,5	0,047	50	A	10	H	0
361	P-100-B	C	P	53,96	55	16,77	16,5	0,047	50	A	10	H	2,3279
362	P-100-B	C	P	53,96	55	16,77	16,5	0,047	50	A	10	H	2,62893
363	P-100-B	C	P	53,96	55	16,77	16,5	0,047	20	An	10	H	0
364	P-100-B	C	P	53,96	55	16,77	16,5	0,047	20	An	10	H	0
365	P-100-B	C	P	53,96	55	16,77	16,5	0,047	20	An	10	H	0
366	P-100-B	S	P	53,96	55	16,77	16,5	0,047	50	An	1	SRB	1,3279
367	P-100-B	S	P	53,96	55	16,77	16,5	0,047	50	An	1	SRB	1,3279
368	P-100-B	S	P	53,96	55	16,77	16,5	0,047	50	An	1	H	2,02687
369	P-100-B	S	P	53,96	55	16,77	16,5	0,047	50	An	1	H	2,85938
370	P-100-B	S	P	53,96	55	16,77	16,5	0,047	50	An	1	M	2,173
371	P-100-B	S	P	53,96	55	16,77	16,5	0,047	50	An	1	M	2,3279
372	Q-092-B	F	Q	32,01	35	19,86	19,5	0,8842	20	A	50	H	3,59127
373	Q-092-B	F	Q	32,01	35	19,86	19,5	0,8842	20	A	500	H	3,65551
374	Q-092-B	F	Q	32,01	35	19,86	19,5	1	20	An	1	H	3,83251
375	Q-092-B	F	Q	32,01	35	19,86	19,5	1	20	An	1	SRB	0
376	Q-092-B	F	Q	32,01	35	19,86	19,5	1	20	An	1	SR	2,83251
377	Q-100-B	C	Q	32,92	35	19,83	19,5	0,042	20	A	10	H	3,6555
378	Q-100-B	C	Q	32,92	35	19,83	19,5	0,042	20	A	10	H	3,58087
379	Q-100-B	C	Q	32,92	35	19,83	19,5	0,042	20	A	10	H	3,6072
380	Q-100-B	C	Q	32,92	35	19,83	19,5	0,042	50	A	10	H	3,27984
381	Q-100-B	C	Q	32,92	35	19,83	19,5	0,042	50	A	10	H	3,07572
382	Q-100-B	C	Q	32,92	35	19,83	19,5	0,042	50	A	10	H	3,33099
383	Q-100-B	C	Q	32,92	35	19,83	19,5	0,042	20	An	10	H	2,85387
384	Q-100-B	C	Q	32,92	35	19,83	19,5	0,042	20	An	10	H	0
385	Q-100-B	C	Q	32,92	35	19,83	19,5	0,042	20	An	10	H	0
386	Q-001-B	C	Q	28,54	25	17,82	16,5	0,045	20	A	10	H	3,38818
387	Q-001-B	C	Q	28,54	25	17,82	16,5	0,045	20	A	10	H	3,42597
388	Q-001-B	C	Q	28,54	25	17,82	16,5	0,045	20	A	10	H	3,64782
389	Q-001-B	C	Q	28,54	25	17,82	16,5	0,045	50	A	10	H	2,82391
390	Q-001-B	C	Q	28,54	25	17,82	16,5	0,045	50	A	10	H	3,04576
391	Q-001-B	C	Q	28,54	25	17,82	16,5	0,045	50	A	10	H	2,82391
392	Q-001-B	C	Q	28,54	25	17,82	16,5	0,045	20	An	10	H	2,64782
393	Q-001-B	C	Q	28,54	25	17,82	16,5	0,045	20	An	10	H	0
394	Q-001-B	C	Q	28,54	25	17,82	16,5	0,045	20	An	10	H	2,34679

6 APPENDIX 6 - Buffer/container experiment decommissioning additional analyses

6.1 PHOSPHOLIPID FATTY ACID ANALYSIS

Phospholipid Fatty Acid (PLFA) Analysis was performed on three hollow-stem Auger samples, H-001-AB, M-001-AB and P-001-AB (Table 24, main text). This analysis was performed by Microbial Insights Inc., Knoxville, Tennessee, USA. The data reference sheet for PLFA analysis given by Microbial Insights is given here.

6.1.1 Data reference sheet

BIOMASS

This is the quantity of Phospholipid Fatty Acids (PLFA) detected in the sample. Phospholipids are part of the intact cell membranes, thus the biomass is a measure of viable or potentially viable cells. As the cell ruptures, the phosphate groups are hydrolyzed and are no longer PLFA.

The cell equivalent value is calculated by using an established PLFA content of *E. coli*. This cell equivalent is based on 1.0×10^8 picomols of PLFA per g/dry weight of cells and 5.9×10^{12} cells per g/dry weight of cells. This gives 5.9×10^4 cells per picomol of PLFA. The number of cells/g dry weight could vary by an order of magnitude depending on their environmental condition.

6.1.2 Nutritional status

Old Age or Slowed Growth

cy/w7c	The monoenoics (#1w7c) change to cyclopropyl fatty acids (cy#:0) as microbes move from a log to a stationary phase of growth (i.e., stop growing). This ratio varies from organism to organism or environment to environment, but usually will fall within the range of 0.5 (log phase) to 2.5 (stationary phase).

Starvation or Toxicity

w7t/w7c	Bacteria begin making trans fatty acids (w7t) under varying environmental stresses. For example, bacteria made w7t fatty acids in the presence of toxic pollutants like phenol. Ratios greater than 0.1 have been shown to indicate the effects of starvation on bacterial isolates. The range is generally between 0.05 (healthy) to 0.3 (starved).

Unbalanced Growth (PHA/PLFA):

This is a ratio of storage lipid (PHA) to membrane lipid (PLFA) from the bacterial cell. Ratios range from ND to 40.00. Ratios greater than 0.2 usually indicate the beginnings of unbalanced growth in at least part of the microbial community. Unbalanced growth often occurs in bacterial communities when a necessary nutrient is missing from the environment.

6.1.3 Community structure

These estimates are based on the percent of the specific PLFA indicative of the different types, or classes of fatty acid. The analysis is broken down into several different classes of fatty acids.

Terminally Branch Saturates(TerBrSats)

These are common to Gram positive bacteria and can be synthesized by some sulfate reducing bacteria.

Monoenoics(Monos)

Monos are common in many Gram negative microorganisms and some microeukaryotes.

Polyenoics(Polys)

Polys indicate the presence of eukaryotes.

Branched Monoenoics(BrMonos)

BrMonos are common to sulfate reducing bacteria.

Mid -chain Branched Saturates(MidBrSats)

MidBrSats are common in *Actinomycetes* and other Gram positive microorganisms. These have also been recovered in some sulfate reducing bacteria.

Normal Branched Saturates(NSats)

These fatty acids are used in the total biomass determinations but not for differentiating between classes of microorganisms.

Gram negative bacteria generally are faster growing, utilize many carbon sources, and adapt quickly to a variety of environments.

Gram positive bacteria are generally slower growing than gram negative bacteria, more resilient, and are capable of degrading more complex compounds.

Eukaryotes include organisms like protozoa, algae, and plants. Protozoa in many instances are capable of feeding on bacteria.

Respiratory Quinones

Benzoquinones are produced by aerobic bacteria with oxygen or nitrate as the terminal electron acceptor.

Napthoquinones are produced with anaerobic respiration. The proportion of benzoquinones to mapthoquinones gives an indication of the proportion of aerobic to anaerobic microbial activity.

Specific PLFA Biomarkers:

Sulfate Reducing Bacteria	i17:lw7c, 10me16:0, 17:1w6
Bacillus-type Bacteria	i15:0, a15:0, i17:0, a17:0
Clostridia:	cy15:0
Barophilic, physhrophilic bacteria	20:5, 22:6
Actinomycetes	10me18:0
Fungi	16:0, 18:lw9, 18:2w6, 18:3w6, 18:3we
Protozoa	20:3w6, 20:4w6
Diatoms	16:1w3t, 20:5w5, 20:5w3
Green Algae	16:1w13t, 18:3w3, 18:1w9
Micro Algae	16:3w6
Higher Plants	18:1w11, 18:3w3, 20:5w3, 26:0

6.1.4 Definitions

PLFA	Phospholipid Fatty Acids. These are a vital part of the cell membrane of microorganisms.
PHA	Poly β-hydroxy alkanoate acid is formed by bacteria accumulating carbon.
NC	Not calculated. Because of division by zero or not applicable.
ND	Not detected
NA	Not applicable.
LB	Found in the Laboratory Blank.
FB	Found in the Field Blank.
BLB	Sample has similar or lower biomass as the Laboratory Blank
BLF	Sample has similar or lower biomass as the Field Blank.
pmoles	10^{-12} moles
nmoles	10^{-9} moles

6.2 ENVIRONMENTAL SCANNING ELECTRON MICROSCOPY (ESEM)

6.2.1 Samples examined

Four samples were examined with the Environmental Scanning Electron Microscopy (ESEM) coupled with Energy-Dispersive x-ray Spectroscopy (EDS), for the presence of individual microorganisms and biofilm formation. The samples were submitted to Dr. Brenda Little, Research chemist and consultant, Diamondhead, MS, USA. These samples were:

HE-003-B(SEM), Teflon cloth with adhering sand grains, shipped in cacodylate buffered glutaraldehyde

HE-005-B(SEM), a piece of black adhesive tape with adhering sand grains, shipped in cacodylate buffered glutaraldehyde

SEM-001-N, a piece of corroded thermocouple from layer N, shipped in cacodylate buffer

SEM-002-N, a sample of buffer in which the thermocouple piece had been embedded.

6.2.2 Sample treatment

The Teflon cloth, black tape and thermocouple samples were rinsed in distilled water before imaging in the ESEM. The clay sample was examined directly without any manipulation.
Specimens were attached to a Peltier stage maintained at 4°C and imagined in an environment of water vapour at 2 to 5 torr to mainatin samples in a hydrated state. An Electroscan (Wilmington, MA) Type II microscope was used. The ESEM was operated at 20 keV using the environmental secondary detector. EDS data were collected with a Tracor Northern (Middleton, WI) System II analyzer. Samples were held at 33.3° tilt during spectrum acquisition. A program for correcting atomic number, absorption and fluorescence was used for semiquantitative analysis during data acquisition.

6.3 TRANSMISSION ELECTRON MICROSCOPY

6.3.1 Samples examined

Four samples were submitted to Drs. T Beveridge and R. Harris, Department of Microbiology, University of Guelph, for transmission electron microscopy. These samples were K-072-B, L-072-B, M-072-B and N-024-B. Specific details of these samples are given in Tables 3 and 4 (main text).

6.3.2 Sample treatment

Initially, sample N-024-B was chosen to work with because of its moist character (Tables 3 and 4) (the other samples were drier). Sample N-024-B was suspended in 0.05M HEPES buffer at pH 6.8. It was viewed under the Light Microscope (LM) and the Philips EM 300 (TEM). Under phase LM one could see that microorganisms were present but in very small numbers. Whole mounts viewed in the TEM showed that the field was totally obscured by both large (>5 μm) and extremely fine (often less than 50 nm) grain material. The suspension was washed further until the field of view was clearer. The bacteria that were seen were adhered to larger particles and very difficult to see clearly, as can be seen from Figure 30 (main text). The rest were badly lysed and not photographed.

The molarity of the HEPES buffer was increased to 0.15 M, and, although this helped, definitive pictures could still not be taken (Figure 31, main text).

The suspension of the four samples were then washed 8 times with 0.15M HEPES buffer and embedded in two types of resin:

Nanoplast is a water miscible resin which does not require the use of dehydrating solvents such as ethanol and acteone, but does require prolonged drying and polymerizing times.

LRwhite is a resin that does require ethanol dehydration but can be polymerised at 60°C in one hour, not the days that other plastic resins require.

Extremely thin sections of the embedded material were examined by TEM and proved again that the bacteria were indeed in very low number and very difficult to preserve. The Nanoplast embeddings did contain a few cells (Figure 32, main text) and seem to indicate that the cells prefer to reside in groups rather than singly. They also show a great deal of associated material, indicating a reactive surface component, probably capsule or slime.

Since the embedding and sectioning procedures are very time consuming it was decided to attempt to increase the numbers of cells present to increase the chances of viewing greater numbers and types of organisms.

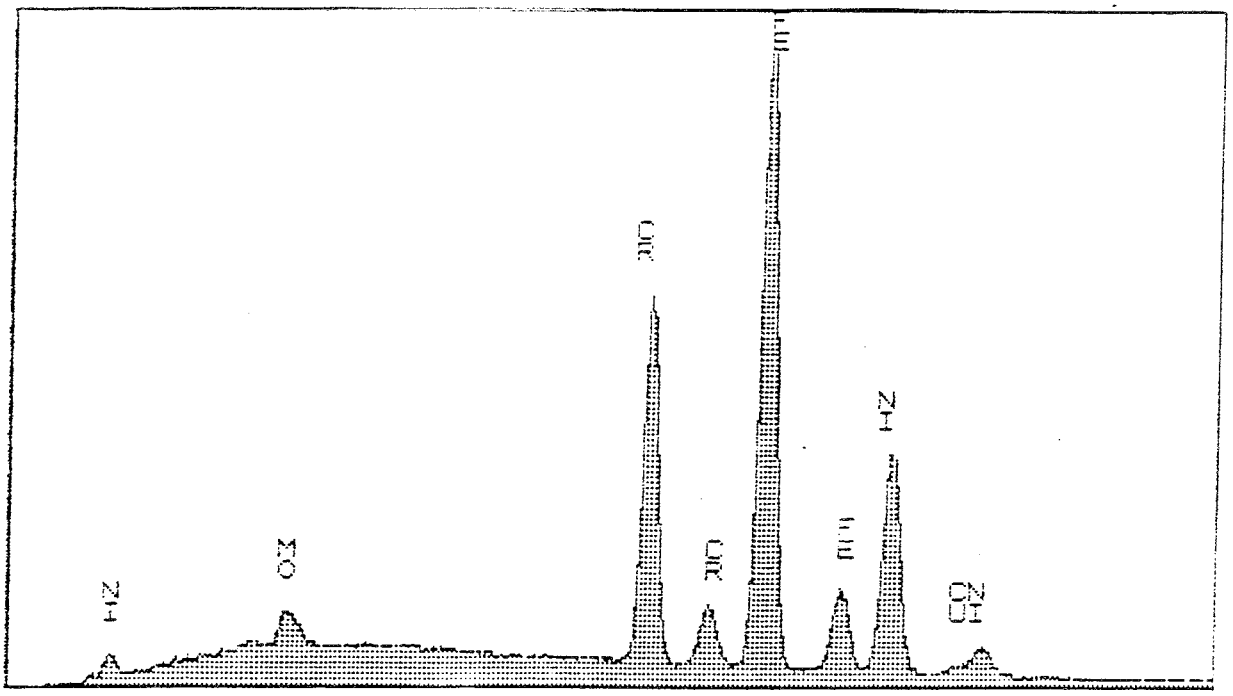
One gram of each of the 4 samples was suspended in 250 mL of M-9 salts broth (composition see below). Subsequent Phase contrast LM observation showed increaesd cell numbers but still too few to ensure successful viewing by TEM. Subsequently, a medium consisting of equal parts of Nutrient Broth (see below) and M-9 salts was inoculated from the M-9 growth experiment. Cell numbers increased and could be viewed successfully with TEM, as shown in Figure 33 (main text).

	Component	**amount (g/L)**
M-9 salts medium:	Na_2HPO_4	6
	KH_2PO_4	3
	NH_4Cl	1
	NaCl	0.5
	Glucose	100 mL
	$MgSO_4$	1
	Thiamine-HCl	1
	$CaCl_2$	1

	Component	**amount (g/L)**
Nutrient broth medium:	Peptone	5
	NaCl	5
	Yeast extract	2
	Beef extract	1

Table A-6-1 Complete PLFA analysis results

Sample	**P-001-AB**	**M-001-AB**	**H-001-AB**	**Lab. Blanc**
Sample Weight	**75.37 g**	**75.39 g**	**75.49 g**	**NA**
Water Content	**15.84%**	**11.08%**	**14.64%**	**NC**
Dry Weight	**63.43 g**	**67.04 g**	**64.44 g**	**NC**
Total pmoles of PLFA	**1,861**	**2,211**	**405**	**35**
Percent of Total PLFA Detected Equivalent Chain Length				
Terminally Branched Saturates (TerBr Sats)				
i15:0 14610	1.2	1.0		7.0
a15:0 14700	1.7	0.7		
i16:0 15622	3.1	2.4	3.8	6.5
i17:0 16620	1.8	1.0		
a17:0 16720	4.9	2.2	5.0	
Monoenoics (Monos)				
16:1w7c 15770	4.6	2.7	5.7	
16:1w5c 15790	2.1	1.6		
cy17:0 16853	5.9	1.2	5.1	
18:1w7c 17790	7.1	4.9	9.6	11.5
18:1w7t 17845	1.5			
cy19:0 18860	17.5	1.3	10.5	
Polyenoics (Polys)				
18:2w6 17670	2.1	2.7		
18:1w9c 17730	7.6	18.2	15.4	11.3
Branched Monoenoics (Br Monos)				
i17:1w7c 16380	1.3			
Mid-Chain Branched Saturates (MidBrSats)				
10me16:0 16440	4.9	1.3	4.7	
10me18:0 18372	3.0			
12me16:0 18380	1.3	0.9		
Normal Saturates (NSats)				
14:00 14000				12.9
15:00 15000	0.6	1.0		8.0
16:00 16000	16.0	30.7	21.5	15.7
17:00 17000				17.0
18:00 18000	8.0	22.1	13.0	10.2
22:00 22000	2.2	3.2	5.6	
24:00 24000	1.9	1.1		



0.000 VFS = 4096 10.240

200 94-62:UNCORRODED THERMOCOUPLE TIP:20 KEV:20°

SQ: QUANTIFY

94-62:UNCORRODED THERMOCOUPLE TIP:20 KEV:20'
Standardless Analysis
20.0 kV 33.3 Degrees

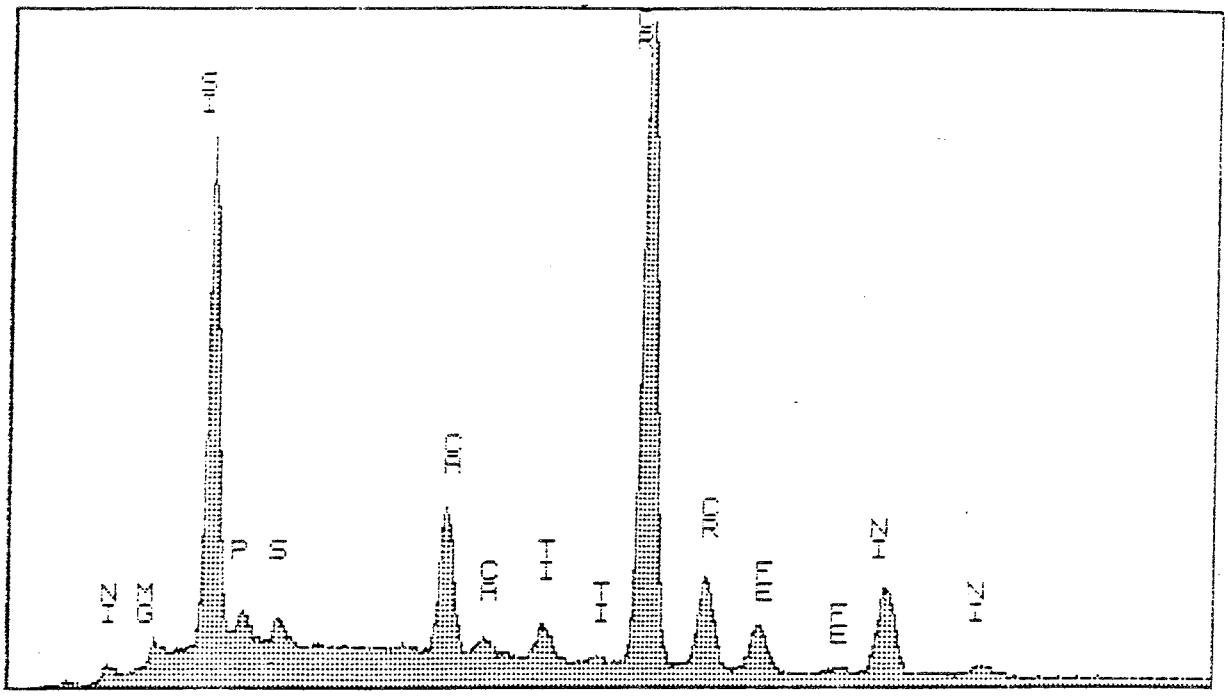
Chi-sqd = 2.78

Element	Rel. K-ratio	Net Counts
Ni-L	0.09255 +/- 0.00643	1842 +/- 128
Mo-L	0.02207 +/- 0.00181	5415 +/- 443
Cr-K	0.19813 +/- 0.00203	38656 +/- 397
Fe-K	0.45091 +/- 0.00324	71226 +/- 512
Cu-K	0.01178 +/- 0.00255	1140 +/- 247
Ni-K	0.22455 +/- 0.00345	26110 +/- 401

ZAF Correction 20.00 kV 33.30 deg
No.of Iterations = 1

Element	K-ratio	Z	A	F	ZAF	Atom%	Wt%
Mo-L	-0.024	1.082	1.257	0.996	1.355	1.91	3.24
Cr-K	0.215	1.006	1.021	0.901	0.925	21.60	19.85
Fe-K	0.488	1.001	1.044	0.967	1.010	50.00	49.33
Cu-K	0.013	1.030	1.075	1.000	1.107	1.26	1.41
Ni-K	0.243	0.983	1.095	1.000	1.076	25.23	26.17
						Total=	100.00%

Figure A-6-1 EDS Spectrum of Uncorroded Thermocouple Tip



213 94-62:CORRODED THERMOCOUPLE (SCALE):20 KEV&°
SQ: QUANTIFY

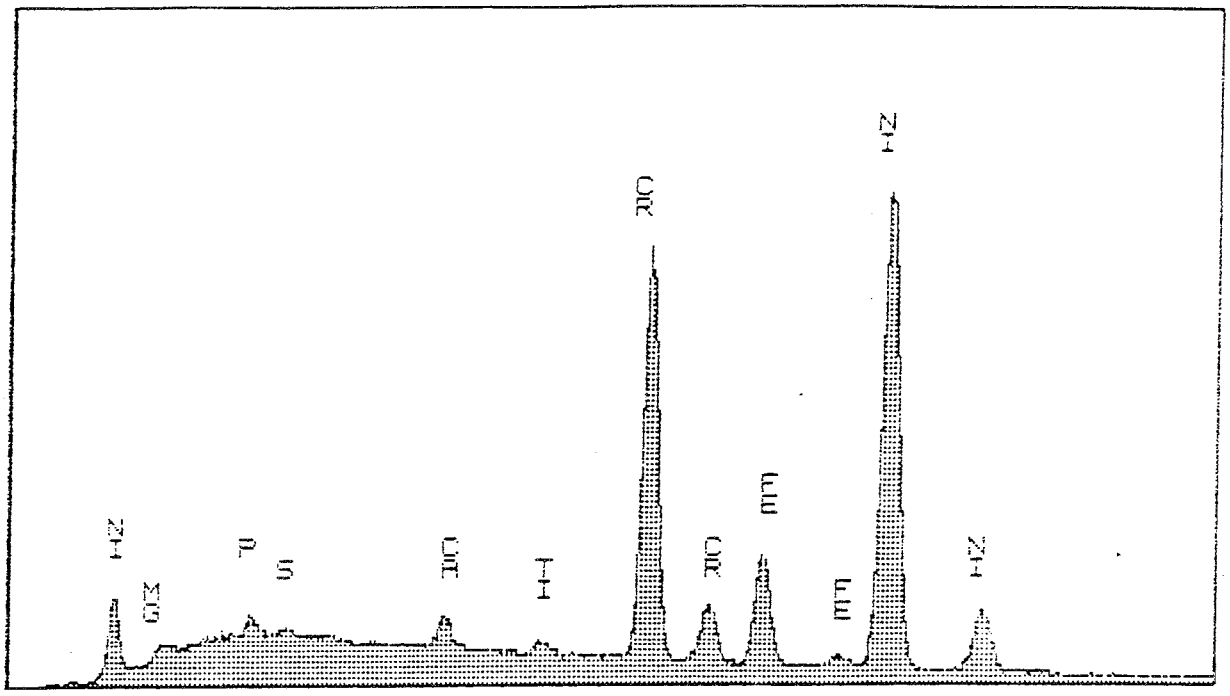
94-62:CORRODED THERMOCOUPLE (SCALE):20 KEV&'
Standardless Analysis
20.0 kV 33.3 Degrees
Chi-sqd = 1.56

Element	Rel. K-ratio	Net Counts
Ni-L	0.08989 +/- 0.00832	1264 +/- 117
Mg-K	0.00656 +/- 0.00074	1372 +/- 154
Si-K	0.12111 +/- 0.00148	36324 +/- 443
P -K	0.01480 +/- 0.00242	3629 +/- 593
S -K	0.01052 +/- 0.00148	2888 +/- 405
Ca-K	0.06903 +/- 0.00161	14131 +/- 330
Ti-K	0.01922 +/- 0.00140	3358 +/- 244
Cr-K	0.50233 +/- 0.00350	69236 +/- 483
Fe-K	0.04752 +/- 0.00227	5303 +/- 253
Ni-K	0.11902 +/- 0.00345	9777 +/- 283

ZAF Correction 20.00 kV 33.30 deg
No.of Iterations = 1

Element	K-ratio	Z	A	F	ZAF	Atom%	Wt%
Mg-K	0.006	0.934	2.886	0.997	2.687	2.94	1.66
Si-K	0.114	0.934	1.745	0.997	1.625	28.44	18.51
P -K	0.014	0.965	1.760	0.996	1.692	3.28	2.35
S -K	0.010	0.940	1.525	0.994	1.425	1.90	1.41
Ca-K	0.065	0.951	1.110	0.965	1.018	7.12	6.61
Ti-K	0.018	1.039	1.076	0.923	1.031	1.68	1.86
Cr-K	0.472	1.039	1.039	0.985	1.064	41.69	50.24
Fe-K	0.045	1.037	1.117	0.986	1.141	3.94	5.10
Ni-K	0.112	1.019	1.074	1.000	1.095	9.01	12.26
						Total=	100.00%

Figure A-6-2 EDS Spectrum of Corroded Thermocouple Segment



0.000 VFS = 4096 10.240

200 94-62:CORRODED THERMOCOUPLE (PIT):20 KEV 20°
SQ: QUANTIFY

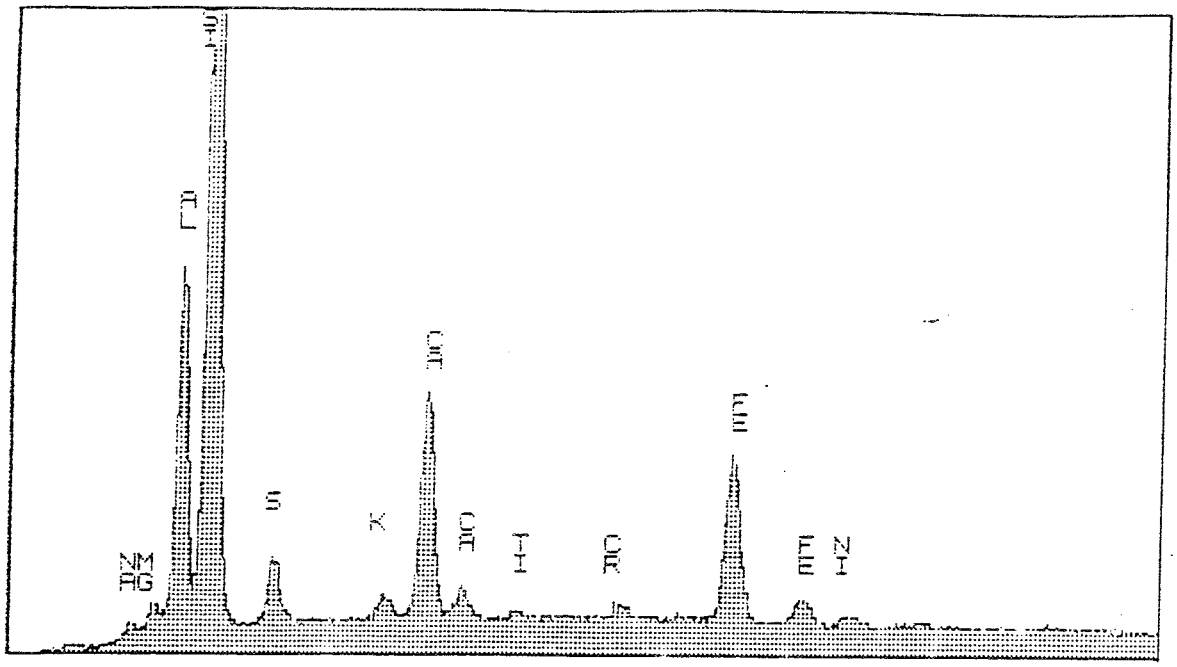
94-62:CORRODED THERMOCOUPLE (PIT):20 KEV 20°
Standardless Analysis
20.0 kV 33.3 Degrees
Chi-sqd = 2.47

Element	Rel. K-ratio	Net Counts
Ni-L	0.29264 +/- 0.00748	6613 +/- 169
Mg-K	0.00249 +/- 0.00048	838 +/- 161
P -K	0.00295 +/- 0.00086	1163 +/- 338
S -K	0.00252 +/- 0.00083	1114 +/- 364
Ca-K	0.00857 +/- 0.00083	2820 +/- 272
Ti-K	0.00407 +/- 0.00083	1143 +/- 234
Cr-K	0.19226 +/- 0.00186	42579 +/- 412
Fe-K	0.06580 +/- 0.00171	11799 +/- 307
Ni-K	0.42869 +/- 0.00363	56586 +/- 479

ZAF Correction 20.00 kV 33.30 deg
No.of Iterations = 1

Element	K-ratio	Z	A	F	ZAF	Atom%	Wt%
Mg-K	0.003	0.921	4.182	1.000	3.851	2.97	1.32
P -K	0.004	0.951	1.847	0.998	1.752	1.25	0.71
S -K	0.003	0.925	1.566	0.997	1.444	0.85	0.50
Ca-K	0.012	0.935	1.117	0.971	1.014	1.63	1.19
Ti-K	0.006	1.020	1.059	0.930	1.005	0.64	0.56
Cr-K	0.264	1.019	1.025	0.933	0.975	27.06	25.73
Fe-K	0.090	1.015	1.058	0.907	0.974	8.62	8.80
Ni-K	0.589	0.996	1.043	1.000	1.039	56.98	61.18
						Total=	100.00%

Figure A-6-3 EDS Spectrum of Corrosion Pit on Thermocouple (Figure 28, main text)



200 94-64:DRY SEDIMENT:20 KEV:20 DEG
SQ: QUANTIFY

94-64:DRY SEDIMENT:20 KEV:20 DEG
Standardless Analysis
20.0 kV 33.3 Degrees
Chi-sqd = 0.64

Element	Rel. K-ratio	Net Counts
Na-K	0.01017 +/- 0.00283	424 +/- 118
Mg-K	0.01011 +/- 0.00318	814 +/- 256
Al-K	0.13014 +/- 0.00464	12811 +/- 457
Si-K	0.41865 +/- 0.00401	48392 +/- 463
S -K	0.03074 +/- 0.00182	3253 +/- 193
K -K	0.01220 +/- 0.00213	1127 +/- 197
Ca-K	0.13813 +/- 0.00354	10898 +/- 279
Ti-K	0.00322 +/- 0.00269	217 +/- 181
Cr-K	0.00915 +/- 0.00376	486 +/- 200
Fe-K	0.21463 +/- 0.00649	9231 +/- 279
Ni-K	0.02286 +/- 0.00682	724 +/- 216

ZAF Correction 20.00 kV 33.30 deg
No.of Iterations = 2

Element	K-ratio	Z	A	F	ZAF	Atom%	Wt%
Na-K	0.007	1.000	2.481	0.994	2.468	2.52	1.79
Mg-K	0.007	0.975	1.892	0.989	1.824	1.75	1.32
Al-K	0.093	1.004	1.562	0.985	1.545	17.19	14.36
Si-K	0.299	0.975	1.583	0.998	1.540	52.91	46.02
S -K	0.022	0.982	1.739	0.997	1.703	3.76	3.74
K -K	0.009	1.020	1.261	0.985	1.267	0.91	1.10
Ca-K	0.099	0.996	1.185	0.994	1.173	9.33	11.57
Ti-K	0.002	1.090	1.139	0.986	1.224	0.19	0.28
Cr-K	0.007	1.092	1.071	0.970	1.134	0.46	0.74
Fe-K	0.153	1.091	1.036	0.997	1.126	9.98	17.25
Ni-K	0.016	1.075	1.042	1.000	1.120	1.01	1.83
						Total=	100.00%

Figure A-6-4 EDS Spectrum of Clay Fragment in Which Thermocouple Segment was Embedded

List of SKB reports

Annual Reports

1977-78
TR 121
KBS Technical Reports 1 – 120
Summaries
Stockholm, May 1979

1979
TR 79-28
The KBS Annual Report 1979
KBS Technical Reports 79-01 – 79-27
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Stockholm, March 1980

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Summaries
Stockholm, June 1984

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TR 85-01
Annual Research and Development Report 1984
Including Summaries of Technical Reports Issued during 1984. (Technical Reports 84-01 – 84-19)
Stockholm, June 1985

1985
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Annual Research and Development Report 1985
Including Summaries of Technical Reports Issued during 1985. (Technical Reports 85-01 – 85-19)
Stockholm, May 1986

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TR 86-31
SKB Annual Report 1986
Including Summaries of Technical Reports Issued during 1986
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SKB Annual Report 1987
Including Summaries of Technical Reports Issued during 1987
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SKB Annual Report 1988
Including Summaries of Technical Reports Issued during 1988
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SKB Annual Report 1991
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Stockholm, April 1992

1992
TR 92-46
SKB Annual Report 1992
Including Summaries of Technical Reports Issued during 1992
Stockholm, May 1993

1993
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SKB Annual Report 1993
Including Summaries of Technical Reports Issued during 1993
Stockholm, May 1994

1994
TR 94-33
SKB Annual Report 1994
Including Summaries of Technical Reports Issued during 1994.
Stockholm, May 1995

1995
TR 95-37
SKB Annual Report 1995
Including Summaries of Technical Reports Issued during 1995.
Stockholm, May 1996

List of SKB Technical Reports 1996

TR 96-01
Bacteria, colloids and organic carbon in groundwater at the Bangombé site in the Oklo area
Karsten Pedersen (editor)
Department of General and Marine Microbiology,
The Lundberg Institute, Göteborg University,
Göteborg, Sweden
February 1996