# MICROBIAL CHARACTERIZATION OF GROUNDWATER FROM BOREHOLES CR9 and CR18 AT CRL (2007-2009) – IMPLICATIONS FOR A POSSIBLE FUTURE REPOSITORY FOR RADIOACTIVE NON-FUEL WASTE

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## ABSTRACT

A microbiological characterization study was carried out on groundwater samples taken from various depths in boreholes CR9 and CR18 at Chalk River Laboratories (CRL), over a three-year period (2007 – 2009), as part of the technical feasibility study for siting a proposed Geologic Waste Management Facility (GWMF) at the CRL site. The characterization included a variety of methods to determine total, viable and culturable cell counts, as well as identifications using molecular methods. Results indicated a total population of $10^4$ to $10^5$ cells/mL of which < 1% could be cultured. However, a large percentage of this population was in fact viable and appeared to survive in a viable-but-not-culturable (VBNC) state. The microbial results combined with geochemical observations suggested that the oligotrophic biogeochemical system in CR9 may have exhausted the process of nitrate reduction but, due to a lack of electron donors (dissolved organic carbon, DOC) and acceptors (e.g., Fe(III)), is not able to lower Eh values sufficiently to allow the occurrence of sulphate reduction and methanogenesis as major processes. The results from a biofilm experiment in CR18 showed that most cells appeared to be suspended rather than attached. From a future GWMF perspective, the presence of a population of largely VBNC cells implies that, given an increased source of electron donors (DOC) and acceptors (e.g., metals) leached from the waste, microbial activity could increase significantly. This can have both positive effects, such as a lower redox potential and lower radionuclide solubility, and negative effects, such as increased radionuclide mobility, and $^{14}C$-containing gas production. It is expected that ultimately the biogeochemical system would return to its original oligotrophic conditions but the rate at which this would occur is, at present, uncertain because both waste leach rates and *in situ* microbial metabolic rates are unknown. The current results, limited to samples from CR9 and CR18, do not preclude the feasibility of a GWMF at the CRL site, but suggest that microbial effects need to be considered in the safety assessment of a GWMF.

## 1. INTRODUCTION

An assessment of the technical feasibility for siting a Geologic Waste Management Facility (GWMF) for radioactive non-fuel waste (i.e., low and intermediate level waste, L/ILW) in the fractured gneissic bedrock at Chalk River Laboratories (CRL) was initiated in 2005. The geochemical and microbiological characterization of the groundwater in the bedrock form part of this assessment and is performed through the analysis of groundwater samples from both existing and newly drilled boreholes in the CRL bedrock. Geochemical data are important for the characterization of groundwater flow and solute (radionuclide) speciation and transport in fractured rock. Research over the past 25 years has revealed the occurrence of microbes in groundwater environments to a depth of > 3 km [1]. Microbiological reactions can affect many

geochemical parameters, including Eh, pH, dissolved organic and inorganic carbon (DOC, DIC), Fe(II)/Fe(III), $NO_3^-$, $PO_4^{2-}$, and $SO_4^{2-}$ concentrations and hence have an effect on radionuclide speciation and transport, and on container corrosion in a future GWMF. Microbial gas production (e.g., $CO_2$ and $CH_4$) is also a concern because of the possible $^{14}C$ content in, and mobility of, such gases. Therefore, microbial processes must be taken into account when characterizing groundwater geochemistry.

While many studies have been conducted in Sweden and Finland [1 – 5], only a limited amount of data is available on the microbial characteristics of deep granitic groundwater in Canada. Jain *et al*. [6] studied the microbial characteristics of deep granitic nutrient-poor groundwater from two boreholes at the Underground Research Laboratory (URL) of Atomic Energy of Canada Limited (AECL). Electron microscopy of the groundwater samples revealed significant numbers of bacteria ($10^5$cells/mL) of various sizes and shapes. The viable (live) population ranged from 1 to 83% of the total cell count, while culturable cells ranged from 0.01 to 10% of the total cell count. Identification of > 160 isolates showed a predominance of *Pseudomonas* species. Phospholipid fatty acid (PLFA) analysis showed that the bacteria in the water samples faced starvation stress but could efficiently uptake and mineralize organic substrates in laboratory studies [6].

The characterization of deep subsurface microbiology requires taking samples. Water samples are usually easier to obtain than solid samples (i.e., cores). Studies of field-collected aquifer samples found that > 90% of the total cells or biomass in sedimentary aquifers was attached to the sediment in biofilms [7], suggesting that both water and core samples should be analyzed. However, several studies that analyzed the microbiology of core and groundwater samples collected from the same borehole in fractured hard rock (as opposed to sedimentary rock) showed that higher cell numbers and a greater diversity of physiological types were present in the groundwater samples compared to the cores [7]. It is, therefore, not a given that in fractured hard rock environments, such as the crystalline rock of the CRL boreholes, most biomass is attached (i.e., sessile). Core samples from fractured crystalline rock are analyzed infrequently for microbes, because of the difficulty in (and expense of) obtaining uncompromised samples. A proven alternative is the introduction of inert high-surface area materials into the borehole, on which microbes can adhere over time and be retrieved, analyzed and compared to the suspended (i.e., planktonic) microorganisms in the groundwater [e.g., 8, 9].

In this paper we report the results of the microbial analyses of groundwater samples taken over a period of three years (2007-2009) from borehole CR9 at CRL. Biofilm samplers in an open (accessible) borehole (CR18) at CRL were also employed in our study and preliminary results are included in this paper.

## 2. BOREHOLE LOCATIONS AND CHARACTERISTICS

Previous investigations of the CRL bedrock resulted in a number of boreholes, including borehole CR9, located at the northeast end of Lower Bass Lake on the CRL site. CR9 was drilled in 1979 to intersect a number of major lineaments and geophysical anomalies that pass around and through Lower Bass Lake [10]. A variety of fractured gneissic rock types and several fault zones were encountered [11]. Between 490 and 510 m the rock is heavily fractured, possibly representing observed surface lineaments. Shortly after it was drilled, CR9 was abandoned and left unsealed. In 2006 the entire length of borehole CR9 was salvaged, logged geophysically and instrumented with a Westbay multilevel casing system, with 12 intervals

between 0 and 704 m, such that hydrogeological monitoring and groundwater sampling were possible. Preliminary geochemical data were gathered during 2006 after initial flushing of all intervals. Flushing (pumping) continued over time until a stable geochemistry appeared to have been reached, indicating that most contamination from drilling and surface inputs (from 1979 to 2006) had been removed and that the water in the borehole intervals was typical of formation water.

Borehole CR18 is a shallow borehole with a depth of ~30 m. This borehole (drilled in 1995) intersects a zone of highly brecciated, carbonate-rich pegmatite on the Maskinonge-Chalk Lake fault and an upward hydraulic head indicates discharge conditions into Maskinonge Lake. Previous geochemical work in CR18 indicated older (~8000 years) water and although CR18 is not as deep as CR9, the apparent age of the groundwater is similar to that of the water in CR9. Because it does not contain packers, CR18 provided an opportunity to assess both the planktonic and sessile microbial communities in the same borehole. On two separate occasions, a biofilm sampler was placed directly in the groundwater of CR18, for periods of two and 12 months, respectively. Only the two-month data are included in this paper.

## 3. MATERIALS AND METHODS

### 3.1 Sampling Method

CR9 intervals 3 (116-151 m), 5 (242-316 m), 8 (464-515 m) and 11 (584-639) m were sampled once a year over the period 2007-2009. Intervals 2 (78-114 m) and 12 (640-704 m) were sampled in 2008 and 2009 only. Sampling followed a prescribed protocol, with emphasis on precautions to prevent microbial contamination during sampling, subsequent handing and distribution.

For the sampling of each interval, four 250 mL metal sampling tubes and their end-fittings and connections were cleaned thoroughly with detergent and rinsed with distilled water. The tubes and end-fittings were wrapped in aluminum foil and autoclaved (15 to 20 min. at 121$^{o}$C), while the other parts (connecting tubes, valves and o-rings) were cleaned with 70% ethanol and air-dried in a sterilized bio-safety cabinet. Subsequently the sampling string was assembled (with the connecting valves in the open position except for the bottom and top valves), taking every precaution to do this aseptically. The bottom and top of the assembled string were capped with a sterile plug. In the field, the sample tube string was connected to a Westbay MOSDAX sampler system, consisting of an electric reel and a probe with a motorized, surface-controlled valve that transported the string to the desired sample port, sampled the groundwater and returned it to surface. All components of the probe contacting the water sample (the valve, face seal and extraction tube) were triple rinsed with distilled deionized water and cleaned with 70% ethanol immediately before connection to the sample string. A vacuum coupling adapter was installed on the probe and the top valve of the sample string was opened. The entire string was evacuated with a vacuum pump, followed by closing the electric valve and removal of the vacuum coupling adapter. Any ethanol still present would be pumped from the string during this evacuation. The sampling string was then lowered down the casing to the desired sample port at depth. The MOSDAX sampler probe was used to locate the sample port, seal against it, open the valve and allow the water sample to enter the string. After closing the electric valve, the probe and sample string were reeled to surface, where the valves between the four sampling tubes were manually closed and the string detached from the MOSDAX sampling probe. The filled tubes were transferred immediately to the laboratory. The contents of the four 250 mL sample tubes were

mixed aseptically inside a glovebox under nitrogen gas and divided into subsamples (in sterile bottles) for analyses. The samples were either analyzed at CRL (immediately and following 24 h of storage at 4$^{o}$C) or shipped on icepacks to Whiteshell Laboratories (WL) for additional analysis, which was initiated within 24 h after sampling.

A Bio-Trap$^{TM}$ biofilm sampler (Microbial Insights (MI), Rockport, TN, USA) was placed in CR18 for two months in 2009, at a depth of ~25 m. A Bio-Trap$^{TM}$ sampler contains high surface area inert beads housed in a holder that allows for groundwater to flow through the housing and over the beads. During retrieval of the Bio-Trap$^{TM}$, water samples were also taken (pumped from a depth of ~14 m into a sterilized container) in order to study both the sessile and planktonic microbial populations in CR18. The CR18 water sample was orange-brown in colour, likely due to the presence of iron oxides/hydroxides and upon standing, some particulate formed.

## 3.2 Microbial Analyses

### 3.2.1 Total cell counts using Acridine Orange (AO) and Sybr Green I

Total cell counts in a water sample include both live and dead cells.

*Acridine Orange (AO) and Sybr Green I* dyes were used to count total cells. Both dyes can penetrate microbial cells and bind to DNA and RNA molecules in the cells (e.g., [12, 13]). The resulting fluorescence can be observed by both microscopy and flow cytometry (FC). FC was carried out for Sybr Green I-stained samples in addition to microscopy because FC allows the counting of cells without human bias. The samples were processed using a 22 mW 488 nm argon laser (blue) Beckman Coulter Lab Quanta SC instrument. The flow rate was 50 µL/min, a total volume of 100 µL sample was analyzed and the gating strategy for these measurements was taken from [14]. The water samples were counted several times during the 24 h after sample retrieval, with both dyes.

### 3.2.2 Viable or live cell counts using a number of different dyes and biomarkers

Viable (live) microorganisms have intact membranes across which they can create and maintain a potential difference through a proton gradient along which electrons are transported. This electron transport chain is coupled to the production of adenosine triphosphate (ATP), the energy "currency" in the cell. Metabolically active cells have both intact membranes and an active electron transport chain. Several dyes were used to quantify viable cells (by microscopy) because most methods have drawbacks and no single method will likely give a reliable picture of the percentage of viable cells in natural water samples containing heterogeneous populations with a high percentage of viable-but-not-culturable (VBNC) cells.

*Propidium iodide* (PI) was used to evaluate microbial viability based on cells with intact cell membranes. PI is a polar fluorescent compound that only penetrates inactive (membrane-damaged or dead) cells and is excluded by the intact membranes from live cells. Intracellular PI binds to double-stranded nucleic acids with intercalation [15]. Dead cells were counted in both as-retrieved and in formaldehyde-treated water samples and the quantity of live cells was calculated by subtraction. Due to complications during microscopy, the cells were counted using FC. The gating protocol in this case was derived from [16].

*5-Cyano-2,3-ditolyl tetrazolium chloride* (CTC) was used to evaluate microbial viability based on the reduction of CTC to CTC-formazan by respiratory enzymes in respiring cells. CTC-formazan is a water-insoluble red-fluorescent compound [15] and the use of CTC provides

information on microbes with an intact membrane and a functioning respiratory chain, indicating *in situ* active cells. A drawback is that CTC reduction kinetics may vary significantly between samples, making it difficult to establish a universal staining protocol [17]. Therefore, this method could underestimate the number of viable cells.

*Rhodamine-123* (Rh-123) was used to evaluate microbial viability based on cells maintaining a potential difference across the cell membrane. Rh-123, a lipophilic cation, accumulates in viable cells that have both an intact membrane and maintain a membrane potential [18, 19]. Under certain conditions, the extent to which cells take up Rh-123 quantitatively reflects the extent of their viability (i.e., whether they are culturable, nonculturable, or dormant) [20]. Drawbacks include the tendency of larger cells to accumulate more Rh-123 than smaller ones. Rh-123 may be pumped out of viable cells by microbial efflux pumping and the permeability of gram-negative bacteria is lower than for gram-positive bacteria unless the cells are pre-treated with EDTA, which is difficult to standardize [20]. Rh-123 also self-quenches if concentrations become too high.

*Carboxy-fluorescein diacetate* (CFDA) was used to evaluate microbial viability based on enzymatic cleavage (by non-specific esterases) of CFDA whereby carboxyfluorescein (CF), a fluorescent compound, is liberated [15]. CFDA is membrane-permeable and non-fluorescent. CF is polar and tends to stay in cells with enzyme activity, although some loss can occur. CF can be actively pumped out of viable microbes, suggesting a lack of viability, thus underestimating the number of viable cells based on esterase activity.

*ATP* was used to evaluate microbial viability, based on its concentration in cells. ATP occurs in all living cells where it plays the role of energy currency between different cellular processes. Its intracellular concentration is carefully regulated to similar levels in all types of cells and gives a good estimate of the total intracellular volume. Most bacterial cells contain approximately $2x10^{-18}$ mol ATP per cell, while most eukaryotic cells, as a result of their larger size, contain $1x 10^{-15}$ mol ATP or more. A commercial kit (ATP SL, Bio Thema, Sweden) was used to determine total ATP in the samples. After extraction, ATP was assayed with a BiotraceUni-Lite luminometer (calibrated with a known amount of ATP). The amount of ATP in a sample was converted to a cell number, based on the above assumption. A drawback is that this conversion may not be accurate for heterogeneous populations from complex samples.

*Phospholipid fatty acids* (PLFA) were used to evaluate microbial viability based on the PLFA presence in cells. PLFA are important components of all live cellular membranes. They can be used to quantitatively determine viable biomass and community structure in samples, without the need for culturing (e.g., [21]). Different types of PLFA signal the presence of different groups of microbes, which is used for community structure analysis. PLFA analysis is especially useful when comparing samples from a particular location over time, or when comparing samples from different parts of a borehole. All water samples were shipped on ice to MI by overnight courier (24 h). At MI, lipids were extracted using one-phase chloroform-methanol-buffer, recovered, dissolved in chloroform, and fractionated on disposable silicic acid columns into neutral-, glyco-, and polar-lipid fractions. The polar lipid fraction was transesterified with mild alkali to recover the PLFA as methyl esters in hexane. PLFA were analyzed by gas chromatography with peak confirmation performed by electron impact mass spectrometry. Cell equivalents can be calculated from the total amount of PLFA extracted by using an established conversion factor ($2 \times 10^4$ cells/pmol PLFA). The use of such a conversion factor can result in uncertainty because

of the potential errors inherent in the practice of applying conversion factors to heterogeneous populations from complex environments.

### 3.2.3 Culturable cell counts

Culturable cells are sub-populations of viable cells that can grow (by cell division) on specific growth media. Culturable cells have intact cell membranes, an active respiration, maintain a potential gradient across the cell membrane and have active enzymes.

*Heterotrophic aerobes and anaerobes* (HAB and HAnB) were counted by plating 1 mL of serial dilutions ($10^0$ to $10^{-4}$) of the water samples onto sterile R2A agar [22]. The plates were incubated at 30°C for 5 to 7 days for HAB and for 4 weeks for HAnB (in an anaerobic glovebox with a $N_2/CO_2/H_2$ (85/10/5 %) atmosphere) before counting of colonies. HAB 2007 colonies were identified using DNA-based methods (section 3.2.4).

*Nitrate-utilizing and nitrate-reducing bacteria* (NUB and NRB) were enumerated by inoculating most probable number (MPN) tubes, containing R2A medium [22] amended with 0.1% nitrate, with 1 mL of serial dilutions ($10^0$ to $10^{-4}$) of the water samples. The tubes were scored for gas production (visible in the inverted Durham tubes) and presence of nitrite (NRB) or absence of nitrate (NRB) after 4 weeks of incubation at 30°C.

*Sulphate-reducing bacteria* (SRB) were enumerated by inoculating $N_2$-degassed tubes containing sterile modified Postgate's B medium [23] with 1 mL of serial dilutions ($10^0$ to $10^{-3}$) of the water samples in an anaerobic glovebox ($N_2/CO_2/H_2$ (85/10/4 %) atmosphere). The sealed tubes were incubated at 30°C for about 4 weeks before they were scored for the occurrence of a black precipitate, indicative of sulphate reduction.

### 3.2.4 Molecular methods

The advantage of molecular methods for characterizing microbial communities in natural water samples is that they are not dependent on the ability to culture those organisms in the laboratory. The whole microbial communities in the groundwater samples from CR9 and CR18 were captured onto sterile 0.22 µm filters (Pall Life Science GN-6 membrane) using between 0.5 to 1L of the samples. Whole community genomic nucleic acid was extracted using either a MoBio Ultraclean® soil DNA isolation kit or a MoBio Ultraclean® Water DNA extraction kit. The nucleic acid content of the extraction solution was estimated by measuring absorbance at 260 nm and 280 nm, using a UV/VIS spectrophotometer (HACH DR2000 or Evolution 60S). The isolated nucleic acid was stored at -20°C, when not used for analysis.

*Polymerase Chain Reaction* (PCR) amplification of the 16 S rRNA gene within the isolated genomic DNA was performed using a Bio-Rad DNA Thermal Engine thermal cycler, following a protocol based on the touchdown method [24] and the booster method [25]. Amplification of the 16S rRNA gene was performed using the Eubacterial-specific universal forward and reverse primers, 357F and 518R , the Archaeal-specific forward and reverse primer pairs ArUn4F/Ar958R and Arc344F/519R and the Eukaryote-specific forward and reverse primers Euk1427 and Euk1616. The resulting PCR products were run on an agarose gel (1.5%), stained using ethidium bromide (1 µg/mL) and imaged under UV light using an Alpha Innotech imager to confirm successful amplification. The electrophoresis unit consisted of a Bio-Rad Mini-Sub GT Cell horizontal gel electrophoresis system and a Bio-Rad PowerPac Basic Power Supply. Gels were run using a DNA size marker in one well to confirm the size of the PCR product.

*Denaturing gradient gel electrophoresis* (DGGE) was used to detect base pair differences in DNA sequences obtained from the PCR amplifications. The electrophoresis was performed using the Universal Mutation Detection system (Bio-Rad). Gradient gels were run using 8% acrylamide and a gradient of the denaturants formamide and urea. DGGE gels were visualized by staining with ethidium bromide and imaged under UV light using the Alpha Innotech imager. The resulting DGGE pattern provides a qualitative measure of the population diversity in the samples. Sequencing of DNA bands excised from the DGGE gels was performed by MI or the Ottawa Health Sciences Centre. Identification of bacteria was based on comparing partial rRNA gene sequence information with known sequences in the database SeqMatch (Ribosome Database Project (RDP)).

*Real Time Quantitative PCR* (qPCR) was performed by MI. This method enables both detection and quantification of the amplification reaction during PCR. For the water and biofilm samples collected from CR18 in 2009, a number of oligonucleotide probes were used, directed to both phylogenetic and functional genes. These were 16S rRNA gene probes for ammonia-oxidizing bacteria (AOB), methane-oxidizing bacteria (MOB), methanogens (MGM), iron- and sulphate-reducing bacteria (IRB/SRB) and functional gene probes for dissimilatory sulphate reductase (DSR) for dissimilatory sulphate-reducing prokaryotes, and nitrate reductase genes (nir S, nir K) for denitrifying bacteria.

### 3.2.5 Geochemistry

Extensive geochemical analyses of CR9 (and CR18) samples, taken during several sampling events each year in 2007, 2008 and 2009 (CR18), were carried out at CRL and elsewhere. For this paper, only selected geochemical data were obtained from samples taken just before (or during) the microbial sampling campaigns, in order to compare geochemical data with microbial data. The data reported in this paper include: DOC, Eh, pH, $Fe_T$, $HCO_3^-$, Conductivity, $Ca^{2+}$, $Na^+$, $Cl^-$ and $SO_4^{2-}$.

## 4. RESULTS

Results for total cell counts are shown in Figure 1 (i.e., averages of immediate counts and after 24 h counts), while culturable cell numbers (after 24 h) are shown in Figure 2.

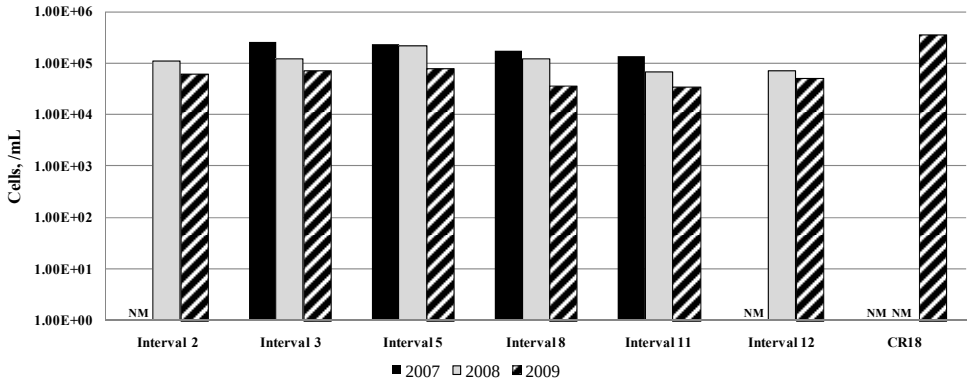


**Figure 1. Average total cell numbers in CR9 and CR18 groundwater samples.**

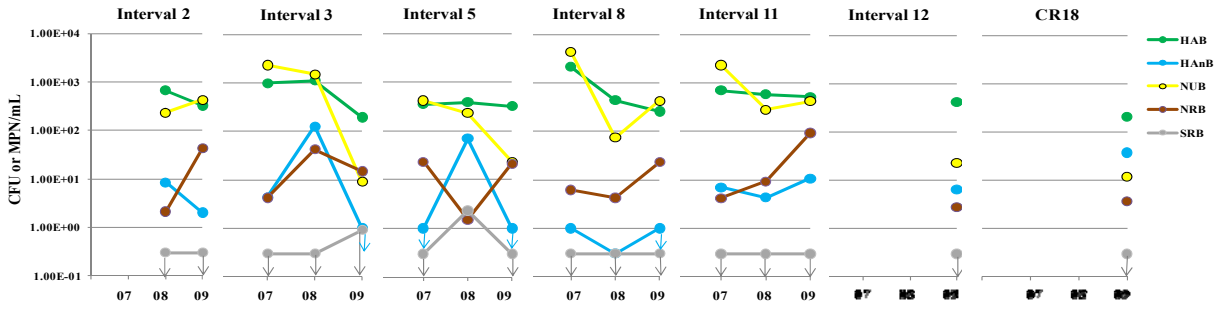


**Figure 2. Culturable cells numbers in CR9 and CR18 groundwater samples (↓ = below detection limit shown)**

Results for viable (live) cell counts (immediately after sampling) are shown in Figure 3, including a comparison with culturable HAB.

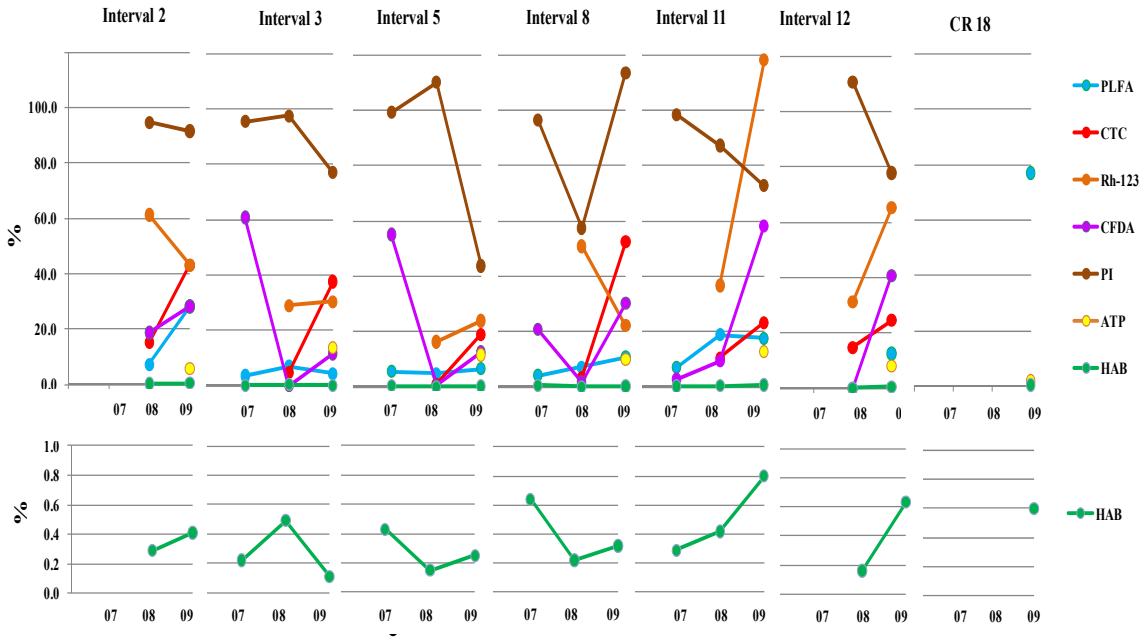


**Figure 3. Viable cells as a percent of total cells, obtained by a number of different methods (see text for explanation of methods and abbreviations).**

Figure 4 shows the community profiles according to the PLFA composition (after 24 h).

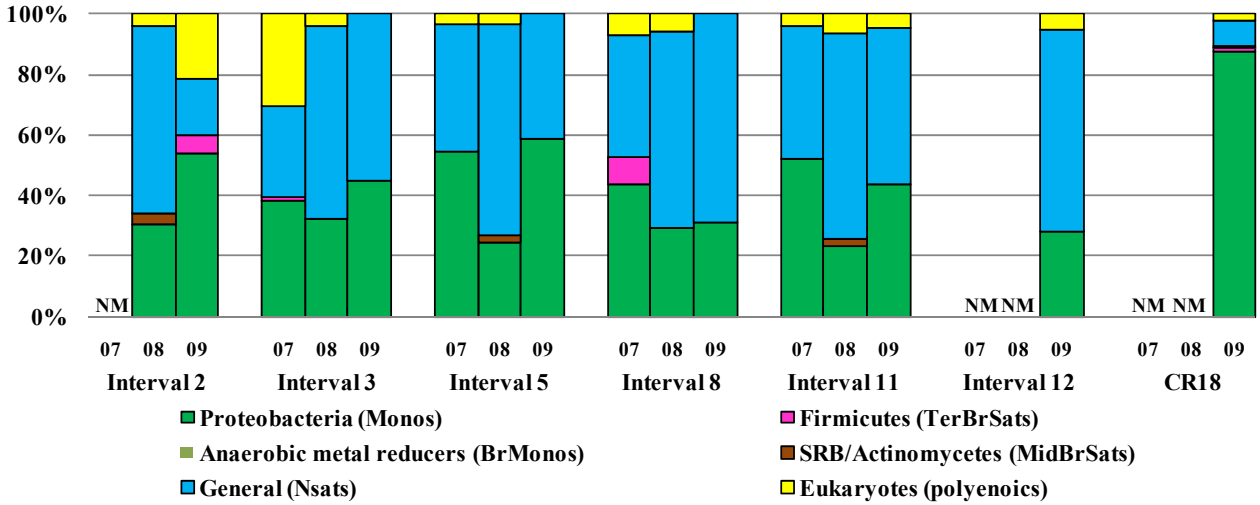


**Figure 4. PLFA-based community structure in CR9 and CR18 groundwater samples.**

Figure 5 shows the most pertinent geochemical data over the period 2007-2009 for CR9 and CR18.

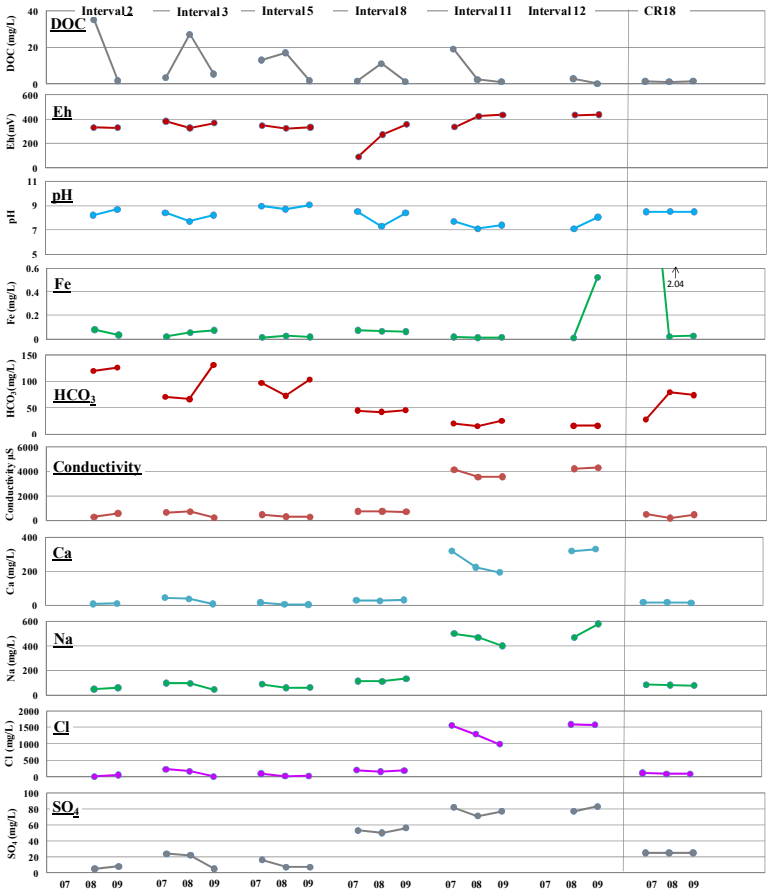


**Figure 5. Pertinent geochemical parameters for CR9 and CR18 groundwaters.**

Table 1 gives the PCR/DGGE/Sequencing results for the CR9 whole water samples (2007 and 2008), the isolates from the 2007 CR9 water samples and the 2009 CR18 whole water and biofilm samples. Although probes for Archaea and Eukaryotes were also used, only Eubacteria were found.

**Table 1. PCR/DGGE/Sequencing Results for CR9 and CR18 samples**

| Interval | Similar Genus (RDP) | RDP SI | Taxonomic Class |
|---|---|---|---|
| **Whole water samples (2007 and 2008) from CR9** | | | |
| 2007-3 | No ID | | |
| 2007-6 | *Sterolibacterium* spp. | 0.949 | Beta-Proteobacteria |
| 2007-8 | *Sterolibacterium* spp. | 0.824 | Beta-Proteobacteria |
| 2007-11 | No ID | | |
| 2008-2 | Unclassified Bacterium | 0.825 | |
| | *Roseomonas* spp. | 0.628 | Alpha-Proteobacteria |
| 2008-3 | *Clostridium* spp. | 0.701 | Firmicutes |
| 2008-5 | *Pseudomonas* spp. | 1.00 | Gamma-Proteobacteria |
| | *Comamonas* spp. | 0.733 | Beta-Proteobacteria |
| | *Pseudomonas* spp. | 1.00 | Gamma-Proteobacteria |
| 2008-8 | *Pseudomonas* spp. | 1.00 | Gamma-Proteobacteria |
| 2008-11 | *Comamonas* spp. | 0.733 | Beta-Proteobacteria |
| 2008-12 | Unclassified "Ruminococcacaea" | 0.685 | Firmicutes |
| | Unclassified Bacterium | 0.843 | |
| | *Magnetobacterium* spp. | 0.895 | Nitrospira |
| **Isolated colonies from CR9 2007 samples** | | | |
| 2007-3 | *Pseudomonas* spp. | 1.00 | Gamma-Proteobacteria |
| | *Acidovorax* spp. | 0.954 | Beta-Proteobacteria |
| | *Acidovorax* spp. | 1.00 | Beta-Proteobacteria |
| 2007-5 | *Roseomonas* spp. | 0.946 | Alpha-Proteobacteria |
| | *Pseudomonas* spp. | 1.00 | Gamma-Proteobacteria |
| | *Pseudomonas* spp. | 0.985 | Gamma-Proteobacteria |
| 2007-8 | *Pseudomonas* spp. | 0.966 | Gamma-Proteobacteria |
| | *Pseudomonas* spp. | 0.908 | Gamma-Proteobacteria |
| | *Brevundimonas* spp. | 1.00 | Alpha-Proteobacteria |
| | *Brevundimonas* spp. | 1.00 | Alpha-Proteobacteria |
| | *Brevundimonas* spp. | 0.948 | Alpha-Proteobacteria |
| 2007-11 | *Ralstonia* spp. | 0.888 | Beta-Proteobacteria |
| | *Ralstonia* spp. | 1.00 | Beta-Proteobacteria |
| | *Pseudomonas* spp. | 0.962 | Gamma-Proteobacteria |
| | *Ralstonia* spp. | 0.992 | Beta-Proteobacteria |
| **Whole water sample and Biofilm sample from CR18 (2009)** | | | |
| 2009-1W | Uncultured *Chlorobium* | 0.788 | |
| 2009-2W | Uncultured bacterium | 0.930 | |
| 2009-3W | *Gallionella* spp. | 0.990 | Beta-Proteobacteria |
| 2009-1BF | Uncultured SRB | 0.880 | |

RDP = Ribosome Database Project; SI = Similarity Index; ID = Identity; W = water sample; BF = Biofilm sample; SRB = sulphate-reducing bacterium.

## 5. DISCUSSION

The total cell counts in Figure 1 showed a consistent decline over time for each CR9 interval, as these were flushed repeatedly prior to each sampling event. Total cell numbers in 2007, 2008 and 2009 were in the range of 1.4 to 2.5 x $10^5$ cells/mL, 0.7 to 2.1 x $10^5$ cells/mL and 0.3 to 0.6 x $10^5$ cells/mL, respectively. This probably indicates a progression towards *in situ* biogeochemical conditions after installation of the packer system in 2006. Total cell counts (2009) were up to a factor of 10 higher in CR18 water.

Culturability (Figure 2) for all physiological groups attempted (HAB, HAnB, NUB, NRB and SRB) was two to five orders of magnitude lower than total cell counts in all CR9 and CR18 samples, with HAB culturability ranging from 0.2 to 0.8% of the total cell counts. This is commonly observed for oligotrophic groundwater samples. Figure 2 further shows that the culturable NUB population often overlapped with the HAB population. This suggests the presence of mainly facultative aerobes that use nitrate as an electron acceptor when oxygen is depleted. Both populations appeared to decline over time in CR9. The fact that nitrate was below the detection limit in all samples (data not shown) may corroborate the activity of NUB in CR9. Culturability of HAnB and NRB was much lower in CR9 and CR18, and NRB appeared to increase over time in CR9, especially in the deeper intervals. SRB could be detected only in the 2008 CR9 interval 5 sample.

Figure 3 shows that the percentage of viable cells found depends strongly on the method used, generally with PI > Rh-123 > CTC > CFDA > PLFA > ATP > HAB. It should be noted that both PLFA- and ATP-based cell counts are not direct counts but are calculated using a conversion factor and as such may be subject to error. It has been proposed that viability in microbial cells may be a continuum rather than a discrete cut-off point and that the ability to form colonies on plates is lost first, followed by substrate responsiveness, membrane potential, respiratory activity and lastly membrane integrity, as the continuum progresses from live to dead bacteria [26]. Most organisms lose culturability on appropriate media under environmental stress conditions but these viable-but-not-culturable (VBNC) cells can maintain certain characteristics of viable cells such as the potential for some signs of metabolic activity and respiration, as well as cellular integrity for long (but unknown) times until death occurs [27]. In that context, it is logical that in naturally occurring oligotrophic populations, methods based on culturing, CFDA cleavage (esterase activity) and ATP (energy currency) content would tend to indicate lower percentages of viable cells than methods based on membrane potential (Rh-123 retention), respiratory activity (CTC) and membrane and cell wall integrity (PI exclusion and PLFA content). Accordingly, for the CR9 samples, the highest number of viable cells was obtained with the PI method and the lowest by culturing, while the viable cell numbers found with the other methods fell between these two extremes. Together these results allow the conclusion that, although very few cells could be cultured (0.2. to 0.8% of the total cell count), a large percentage of the cells in CR9 and CR18 groundwater are in fact alive and exist in the VBNC state.

Although DNA extraction failed to show Eukaryotes or Archaea in CR9 and CR18, the PLFA-based community structure (Figure 4) showed the presence of some Eukaryotes in almost all samples. In CR9, the mostly Eubacterial population consisted for a large part of Proteobacteria, i.e., gram-negative, typically fast growing aerobic and anaerobic bacteria that use many carbon sources and adapt quickly to a variety of environments. Large percentages of normal fatty acids, common in all cells, were also present in all CR9 samples and may indicate a less-diverse population. Firmicutes (anaerobic fermenters) and SRB were present in only a few samples,

while metal reducers appeared to be absent. The percentage of Proteobacteria in CR18 is much larger than in CR9, with a concurrent lower presence of general PLFA and small percentages of SRB (0.3%), Firmicutes (1.0%) and anaerobic metal reducers (0.4%) present. These results may suggest a more diverse population in CR18 compared to CR9.

The Eh values for all CR9 intervals were in the (mildly) oxidizing range of about +100 to +440 mV (Figure 5). DOC concentrations were generally low and nitrate concentrations were below 0.02 mg/L in all intervals (data not shown), the latter possibly in accordance with the abundant presence of NUB. Dissolved Fe (II) concentrations were low and stable in almost all CR9 intervals, which could suggest that a lack of Fe(III) is inhibiting iron-reduction. Sulphate concentrations were low (5-7 mg/L) in the upper part of CR9 (Intervals 2, 3 and 5) but ten times higher (55-80 mg/L) and presumably adequate for supporting SRB in the lower part (Intervals 8, 11 and 12). However, culturable SRB occurred in only one sample and PLFA analysis showed SRB in very low quantities in only a few CR9 samples. These observations could suggest that the biogeochemical system in CR9 has exhausted the process of nitrate reduction but, due to a lack of electron donors (DOC) and perhaps acceptors (Fe(III)), is not able to move beyond the iron-reduction process to lower Eh values sufficiently to allow sulphate reduction as a major process. The higher salt concentrations in Intervals 11 and 12 have no discernable effects on the microbial communities compared to the lower-salinity intervals.

The PCR/DGGE/Sequencing method was applied to both the whole CR9 water samples from 2007 and 2008 and to the isolates (aerobic heterotrophs) from the 2007 samples (Table 1). A comparison shows that the 2007 isolates did not reflect the 2007 whole water community but the more extensive 2008 whole community analysis showed that at least some species identified from the 2007 isolates were also present in the 2008 whole water sample, specifically *Pseudomonas* spp. (with a very high similarity index (SI)) and *Roseomonas* spp. Many *Pseudomonas* species were found previously in granitic groundwaters from the URL [6, 8]. Nevertheless, these results show that results from whole community analysis and from culturing can differ substantially. The PCR/DGGE/Sequencing results for CR18 water and biofilm samples (Table 1) showed *Gallionella* spp. as one of three planktonic species identified. *Gallionella* are gram-negative micro-aerophilic iron-oxidizing, chemolithotrophic bacteria that derive energy from the oxidation of ferrous iron and prefer to live in a redox gradient in cool, iron-containing natural waters, and in soil. The cells produce fibrous 'stalks', which commonly contain ferric hydroxide. CR18 water had very high iron content in 2007 and was orange in colour when sampled in 2009, likely from oxidized iron particles, which would concur with the presence of *Gallionella* spp. Also found was a species related to an uncultured *Chlorobium* spp., an obligate anaerobic photoautotroph green sulphur bacterium that uses sulphide, hydrogen or ferrous iron as electron donors, which would also concur with the observed iron precipitates in CR18. The one species identified in the biofilm sample was an uncultured SRB.

The qPCR results for the CR18 biofilm and corresponding water sample (Table 2) show that the total cell count for the BioTrap$^{TM}$ surface was $1.5 \times 10^2$ cells/cm$^2$, which is low compared to other biofilms grown on surfaces in granitic groundwaters [8, 9].

**Table 2. qPCR Results for the CR18 water and biofilm samples (2009)**

| Biofilm sample | Water sample | |
|---|---|---|
| Total cells (qPCR) | 1.5 x $10^6$ cells/$m^2$ | 4.5 x $10^6$ cells/mL |
| Community Profile (qPCR) | | |
| Denitrifying bacteria (nir S) | 10.0 % | 8.8 % |
| Denitrifying bacteria (nir K) | 0.17 % | 0.33 % |
| Ammonia oxidizing bacteria | 0.55 % | 2.02 % |
| Methane oxidizing bacteria | 7.4 % | 10.2 % |
| Methanogens | 0.46 % | 0.35% |
| Iron- and sulphate reducing bacteria | 1.3 % | 1.2 % |
| Dissimilatory sulphate-reducing prokaryotes | 0 % | 0.06 % |

The qPCR total cell count in the CR18 water sample was about ten times higher than the total cell counts obtained with AO and Subr Green I, a discrepancy possibly caused by the particulate precipitate in CR18 water. Only about 20% of the total population was captured with the qPCR probes used. In this fraction, denitrifying bacteria were dominant both in the water and biofilm samples, followed by methane-oxidizing bacteria. The qPCR probes also indicated the presence of IRB or SRB and small percentages of ammonia-oxidizing bacteria, methanogens and dissimilatory sulphate-reducing prokaryotes. These results appear to confirm the presence of a diverse population in CR18 as indicated by the PLFA results, and also suggest that most bacteria are in fact planktonic rather than sessile in CR18. Because the microbial and geochemical profile of CR18 appears to differ from CR9, further study to assess the importance of biofilms in CRL groundwater is needed.

## 6. POSSIBLE IMPLICATIONS FOR A FUTURE REPOSITORY

The results from this microbial characterization study have shown that the cells in deep CRL groundwater (i.e., in CR9 and CR18) occur in a mostly VBNC state and that a large percentage belongs to the Proteobacteria. These VBNC bacteria may be limited *in situ* in some of their nutritional or energy requirements and may be surviving in a "dormant' state, sustaining only a very low maintenance metabolism. However, they are alive and could be revived to increase their metabolism substantially if their requirements were met.

In a future L/ILW repository, a considerable portion of the waste would consist of organic materials, stored in metal containers. Upon saturation of the repository with groundwater, which will probably occur within the first few decades after closure, organics may start to leach from the waste and become available as carbon source (electron donors) for the *in situ* microorganisms. It is not known how much or how fast the DOC will increase in the groundwater in and surrounding a repository but it is highly likely that DOC will increase as a result of the presence of the L/ILW. There may also be an increased source of electron acceptors in the form of oxidized metals from corroding waste containers. The depletion of $O_2$ by microbial (and chemical) reactions and the creation of progressively more reducing conditions in a future L/ILW repository would constitute favourable conditions with respect to radionuclide speciation and mobility. Many radionuclides are considerably less soluble and, therefore, less mobile in a reducing environment. However, the presence of a large and *in situ* very active

microbial population may also have negative effects in and around a L/ILW repository. The increased DOC and resulting enhanced microbial metabolism will result in increased microbial gas production, especially $CO_2$ and $CH_4$ (the latter depending on the redox conditions), which could contain $^{14}C$. Because gases are mobile, they present the possibility of fast transport of radioactivity in the form of $^{14}C$ from the repository via the fractured geosphere to the biosphere. Archaea ($CH_4$-producing species) were thus far not found in the CR9 and CR18 water but would probably emerge in a repository when conditions are suitably redox-negative. Methane-oxidizing species (present in CR18) may reduce the amount of $CH_4$ in a repository, but would concurrently produce $CO_2$.

Enhanced microbial activity and increased cell numbers may also enhance radionuclide mobility. Increased DOC and increased microbial production of chelating molecules (for instance siderophores) could lead to increased complexation of radionuclides, which would enhance their solubility and mobility. In addition, microbes are known to sorb radionuclides either on or in the cells and many are motile in aqueous environments, which would present the possibility that radionuclide mobility is enhanced. SRB appear present only in low quantities but could increase in activity and numbers by the increasingly reducing conditions created by enhanced microbial activity. Sulphate reduction may, therefore, also occur. It is expected that there is sufficient Fe from the corroding waste containers to precipitate the sulphide in the form of FeS products.

Therefore, both the positive and negative effects of microbial metabolism need to be taken into account in the hydrogeochemical safety assessment model of a L/ILW repository. Since there are many reactions to take into account, the microbial effects could be dealt with in a separate model, with the results providing geochemical input into the overall hydrogeochemical model. The biogeochemistry of gas production from L/ILW has been modelled by Small *et al*. [28]. An example of modelling a large number of interdependent microbial reactions is given by King *et al*. [29, 30].

## 7. CONCLUSIONS

This study has shown that after more than three years of extensive flushing and sampling, the geochemistry of CR9 suggests a return to "pristine" conditions. The microbial characterization has shown that the biomass in CR9 water samples (in the range of $10^4$ to $10^5$ cells/mL) is indicative of fairly oligotrophic conditions and consists largely of Proteobacteria. Although the culturable population is very low, most cells are in fact viable and appear to survive in a VBNC state. The Eh values for all CR9 intervals were in the (mildly) oxidizing range of about +100 to +440 mV. DOC concentrations were generally low and nitrate concentrations were below the detection limit in all intervals, the latter possibly in accordance with the abundant presence of nitrate-utilizing bacteria. Dissolved Fe (II) concentrations were low and stable, possibly suggesting that a lack of Fe(III) is inhibiting iron-reduction. Sulphate concentrations were low in the upper part of the borehole but ten times higher and adequate for supporting sulphate reduction in the lower part of the borehole. However, SRB and Archaea were mostly below the detection limit. These observations suggest that the biogeochemical system in CR9 may have exhausted the process of nitrate reduction but, due to a lack of electron donors (DOC) and perhaps acceptors (e.g., Fe(III)), is not able to lower Eh values sufficiently to allow the occurrence of sulphate reduction and methanogenesis as major processes.

From a future repository perspective for L/ILW, the presence of a population of largely VBNC cells implies that, given an increased source of electron donors in the form of DOC supplied by

the L/ILW, these viable cells will likely become more numerous with increased respiration and fermentation, concurrently lowering the redox potential in the system. This would be a favourable effect with respect to radionuclide speciation and migration. However, a large and *in situ* very active microbial population may also have negative effects on radionuclide migration due to cellular sorption and mobility. In addition, enhanced microbial activity will increase gas production, with the possibility of releasing $^{14}C$ in the form of $^{14}CO_2$ and $^{14}CH_4$ and may also enhance corrosion effects. The effects of microbial metabolism would need to be taken into account when modelling the hydrogeochemical environment of a future repository for L/ILW at the CRL site. It is expected that ultimately the DOC from the waste would be exhausted as a result of microbial metabolism and that the biogeochemical system would return to its original oligotrophic conditions but the rate at which this would occur is at present uncertain because both DOC leach rates and *in situ* microbial metabolic rates are unknown.

The current results, limited to samples from boreholes CR9 and CR18, do not preclude the feasibility of a GWMF at the CRL site. They do, however, suggest that microbial effects need to be considered in the safety assessment of such a future GWMF. This study is continuing with sampling from the new GWMF boreholes (CRG-1 to CRG-7) to obtain a more complete understanding of the subsurface microbial communities in the CRL bedrock. Future results may, therefore, change or modify the microbial characterization results for deep CRL groundwater reported in this paper.

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