Preliminary Microbial Analysis of Limestone and Shale Rock Samples

NWMO TR-2008-09 **July 2008**

S. Stroes-Gascoyne and C.J. Hamon

Atomic Energy of Canada Limited

nwmo
NUCLEAR WASTE MANAGEMENT ORGANIZATION
SOCIÉTÉ DE GESTION DES DÉCHETS NUCLÉAIRES

Nuclear Waste Management Organization
22 St. Clair Avenue East, 6th Floor
Toronto, Ontario
M4T 2S3
Canada

Tel: 416-934-9814
Web: www.nwmo.ca

Preliminary Microbial Analysis of Limestone and Shale Rock Samples

NWMO TR-2008-09

July 2008

S. Stroes-Gascoyne and C.J. Hamon

Atomic Energy of Canada Limited

ABSTRACT

Title: **Preliminary Microbial Analysis of Limestone and Shale Rock Samples**
Report No.: **NWMO TR-2008-09**
Author(s): S. Stroes-Gascoyne and C.J. Hamon
Company: Atomic Energy of Canada Limited
Date: July 2008

Abstract
Samples of Ordovician sediments (Queenston shale and Cobourg limestone) were obtained from Ontario Power Generation Inc. for preliminary microbial characterization in support of the Nuclear Waste Management Organization's technical research and development program. The samples were analyzed with culture techniques using both dilute growth media and media based on the saline porewater compositions in these sediments, to account for the possible presence of indigenous halotolerant or halophilic microorganisms. The samples were also examined for phospholipid fatty acids (PLFA), neutral lipid fatty acids (NLFA) and glyco-lipids (diglyceride fatty acids, GLFA), three biomarkers from which the presence of viable (PLFA) and dead cells (NLFA and GLFA) can be derived. Results suggested strongly that in accordance with expectations, based on measured water content (0%) and very low water activity (<0.2), no viable (halophilic or other) bacteria were present in the limestone rock but that some contamination of the limestone sample with common (facultative) aerobic cells occurred, likely during drilling or sample handling. The results for the shale sample indicated the presence of viable microorganisms, based on PLFA measurements. However, because of the very low water activity in this shale sample (0.34) (1% water content), this result may not be valid, because it is currently somewhat uncertain whether the PLFA, NLFA and GLFA biomarkers are reliable in a very low water activity environment. If the method is valid, the results for the shale sample would indicate a biologically mostly inactive environment because of the presence of relatively large quantities of dead cells, compared to viable (live) cells. Bacterial species indicated in the aerobic and anaerobic shale enrichment cultures were common non-halophilic, mostly facultative anaerobic sporeformers (including a pathogen). Because of the presence of PLFA, it cannot be ruled out that some of the microorganisms could be indigenous to the shale and survived for a long time in situ as spores in the shale formation. However, this is expected to be very unlikely because of the high similarity with common aerobic surface bacteria (including a human pathogen).

TABLE OF CONTENTS

Page

ABSTRACT ..... **v**

1. INTRODUCTION **1**

2. HALOTOLERANT, HALOPHILIC AND OTHER SALT-RESISTANT FORMS OF MICROBIAL CELLS **2**

3. THE USE AND INTERPRETATION OF PHOSPHO-, NEUTRAL- AND GLYCO-LIPID FATTY ACID BIOMARKERS **3**

4. MATERIALS AND METHODS **6**

4.1 SUBSAMPLING OF COBOURG LIMESTONE CORE SAMPLE **6**
4.2 SUBSAMPLING OF QUEENSTON SHALE CORE SAMPLE **6**
4.3 SYNTHETIC POREWATER COMPOSITION **7**
4.4 ANALYSES **7**
4.4.1 Water Activity 7
4.4.2 Water Content 7
4.4.3 Heterotrophic Aerobes and Anaerobes 7
4.4.4 Sulphate-reducing Bacteria 8
4.4.5 Nitrate-utilizing and Nitrate-reducing Bacteria 8
4.4.6 Iron-reducing Bacteria 9
4.4.7 Enrichment Cultures 9
4.4.8 Phospholipid Fatty Acid Analysis, 16S rDNA Extraction and Denaturing Graduated Gel Electrophoresis 10

5. RESULTS **11**

6. DISCUSSION **13**

6.1 PHOSPHOLIPID FATTY ACIDS **13**
6.2 LIMESTONE SAMPLE **14**
6.3 SHALE SAMPLE **15**

7. CONCLUSIONS **16**

ACKNOWLEDGMENTS **17**

REFERENCES **17**

LIST OF TABLES

Page

Table 1: Composition of Synthetic Porewaters 21
Table 2: Culture Results for Cobourg Limestone and Queenston Shale Samples 22
Table 3: Enrichment Culture Results (Visible Growth) for Limestone and Shale Samples 23
Table 4: Water Content and Water Activity in Cobourg Limestone and Queenston Shale Samples 24
Table 5: Results of PLFA, NLFA and GLFA Analyses 25
Table 6: Sequence Results from DDGE Profile for Aerobic R2A 10-day Old Enrichment Culture of Powdered Cobourg Limestone Sample (Figure 1) 26
Table 7: Sequence Results from DGGE Profile for Aerobic R2A 6 Week Old Enrichment ..... Culture of Crushed Queenston Shale Sample (Figure 2 1804 AER) 27
Table 8: Sequence Result from DGGE Profile for Anaerobic R2A 6 Week Old Enrichment ..... Culture of Crushed Queenston Shale Sample (Figure 2, 1804 AW) 28

LIST OF FIGURES

Page

Figure 1: DGGE Gel Band Pattern for 10-day Old Aerobic R2A Enrichment Culture of Powdered Limestone Sample (1805 R) 29
Figure 2: DGGE Gel Band Pattern for 6 Week Old Aerobic (1804 AER) and Anaerobic (1804 AW) R2A Enrichment Cultures of Crushed Shale Core Sample (1804) 30

1. INTRODUCTION

Samples of Ordovician sediments (Queenston shale and Cobourg limestone) were obtained from Ontario Power Generation Inc. and preserved at 4^{o}C (in PE aluminum bags) for preliminary microbial characterization in support of the Nuclear Waste Management Organization's (NWMO's) technical Research and Development (R&D) program.

One of the issues in the technical R&D program is the longevity of used fuel containers over many thousands of years in the potential presence of microbial activity in the host rock and especially in the engineered clay-based barriers directly adjacent to such containers. Studies at Atomic Energy of Canada Limited's Whiteshell Laboratories have shown that a barrier of highly compacted 100% bentonite (with a minimum dry density of 1.6 Mg/m^3) will suppress indigenous microbial activity in the as-bought bentonite (Stroes-Gascoyne et al. 2006, 2007a, b). However, although a high degree of compaction can be guaranteed in the bulk of such a barrier, dry densities may be lower at interface locations and in places where the compacted bentonite barrier would have opportunity to swell into placement gaps or compress less dense backfill materials.

Studies at WL have shown further that another factor that appears to suppress microbial activity in a bentonite barrier is a minimum pore water salinity of 100 g NaCl/L (Stroes-Gascoyne et al. 2006, 2007a, b). In these studies, sterilized distilled deionized water with a variety of added salinities (as NaCl) was used to saturate the compacted bentonite. It appears, therefore, that the highly saline pore water in Ordovician sediments (Koroleva and Mazurek 2006) could potentially be beneficial in suppressing microbial activity in bentonite barrier materials, although this has to be investigated further with saline solutions other than NaCl (e.g., artificial Ordovician sediment porewater, $CaCl_2$). In addition, during construction of a deep geological repository, bentonite barriers may be partially saturated with host rock formation water, thereby introducing microbes indigenous to such host rock water. The very saline pore water in the Ordovician sediments could potentially contain indigenous halotolerant (salt-tolerant) or halophilic (salt-requiring) microorganisms (Section 2). Such microorganisms, if introduced into a compacted bentonite barrier, could potentially thrive in compacted bentonite with a less than optimal dry density (i.e., less than 1.6 Mg/m^3) despite high pore water salinity. Also, contamination of other sealing (backfill) materials with (halotolerant or halophilic) microorganisms indigenous to host rock could occur through mixing of sealing materials (bentonite) with crushed host rock.

Adaptive Phase Management (APM) (NWMO 2005) considers both crystalline rock and sedimentary rock as potential host rock formations for a deep geological repository for Canada's used nuclear fuel. Over the past decade, microbial characterization of granitic host rock has been conducted, both in Canada (Jain et al. 1997, Haveman et al. 1995, Stroes-Gascoyne and Hamon 2005) and in Sweden and Finland (e.g., Pedersen 2001, Haveman et al. 1998). Although microbial characterization of sedimentary host rock (clay) has been carried out in countries such as Switzerland, France and Belgium (e.g., Stroes-Gascoyne 2005, Stroes-Gascoyne et al. 2007c), such analysis has not yet been carried out in Canada. Therefore, the availability of preserved Ordovician cores from Ontario Power Generation Inc. presented an opportunity to carry out preliminary microbial characterization of sedimentary host rock types in Canada.

Although the preserved Ordovician cores were not drilled with sterile techniques, it is likely that, due to the highly impermeable character of these sediments, only the outside of the core would be contaminated with microbes from drill fluids. This was concluded from a study in Opalinus clay where micro-spheres placed near the drill bit did not enter the interior of core that was drilled with sterile technique and air, but were found only in a 5 mm layer around the core. This layer likely consisted of clay stone powder that was re-compacted during drilling (Stroes-Gascoyne et al. 2005, 2007c). Several additional samples of freshly drilled cores from the Opalinus clay at Mont Terri (Switzerland) were analyzed recently for microbial presence. These cores were not drilled with sterile techniques (air was used for drilling) and were preserved in plastic and shipped by courier on ice to Canada, a process that took up to a week. From the undisturbed dense Opalinus clay of these samples, virtually no microbes could be cultured on standard and saline artificial porewater-based growth media (corroborating the results found previously by other laboratories, using sterily drilled Opalinus clay core, Stroes-Gascoyne et al. 2005, 2007c). This confirmed both the uncompromised and uncontaminated nature of the cores and the lack of contamination in the WL laboratory.

However, the Opalinus cores were drilled with air whereas the Ordovician cores were drilled using drilling muds. Nevertheless, due to the impermeable nature of these sediments it is possible that the center part of the Ordovician cores could be free of contamination (from drilling muds) and suitable for a preliminary microbial characterization. If preliminary analysis as reported here fails to find significant culturability and viability of contaminating microorganisms, any future characterization of these deposits may not require strictly sterile drilling methods.

2. HALOTOLERANT, HALOPHILIC AND OTHER SALT-RESISTANT FORMS OF MICROBIAL CELLS

The Ordovician sediments contain highly saline porewater (Koroleva and Mazurek 2006). It is, therefore, possible that any indigenous microorganisms may be adapted to those conditions and in fact require high salinity for survival. Microorganisms that can withstand high salinity or require it for optimal functioning are halotolerant or halophilic, respectively. Spores are also resistant to high salinity.

Because a selectively permeable plasma membrane separates microorganism from their environment, they can be affected by changes in the osmotic concentration of their surroundings. Placed in a hypotonic solution, water will enter a microbial cell and cause it to burst, unless osmotic concentrations are adjusted intracellularly to prevent the influx of water. Placed in a hypertonic solution, water will flow out of the cell, dehydrating the cell, which may cause cell membrane damage and cause the cell to become metabolically inactive. Many microorganisms keep the osmotic concentration of their protoplasm somewhat above that of their external environment through the synthesis or uptake of so-called compatible solutes, such that the plasma membrane is always pressed firmly against the cell wall. Compatible solutes (e.g., choline, betaine, proline, glutamic acid and other amino acids; elevated levels of K^+) are solutes that do not interfere with metabolism and growth when at high intracellular concentrations.

Most microorganisms are nonhalophilic and require water activities of 0.98 and above. This is why drying or adding large quantities of salt or sugar are so effective in preventing food spoilage. Halotolerant or osmotolerant microorganisms grow over a wide range of salt

concentrations (up to about 3 M NaCl or ~175 g NaCl/L) and water activities. Many fungi are osmotolerant, and the water activity range at which a variety of fungi grows is 0.60 – 0.95. Halophilic microorganisms require high salt concentrations, usually above about 0.2 M NaCl. Moderate halophiles have their optimum around 2 M NaCl (~115 g/L), while extreme halophiles require salt concentrations between 2 M and saturation (about 6.2 M, or ~ 360 g NaCl/L). Most extreme halophiles accumulate enormous quantities of K^+ in order to remain hypertonic to their environment. Below a water activity of about 0.55 – 0.60, DNA disorders and cells do not survive (Willey et al. 2008).

Spores are special resistant dormant structures that are formed by a number of gram-positive bacteria when growth ceases due to lack of nutrients. Spores are extraordinarily resistant to environmental stresses such as heat, UV radiation, gamma radiation, chemical disinfectants and desiccation (salts, sugars). Spores often survive boiling for an hour or more, hence the need to autoclave in order to sterilize materials. Spores contain some DNA repair enzymes but DNA is repaired only once the spore germinates and the cell becomes active again. The transformation of dormant spores into active vegetative cells is complex and occurs in three stages, activation (often by certain treatments such as heat), germination (spore swelling and rupture), and outgrowth (development into an active bacterium).

3. THE USE AND INTERPRETATION OF PHOSPHO-, NEUTRAL- AND GLYCO-LIPID FATTY ACID BIOMARKERS

The number of cells that is culturable from a soil or sediment sample is often orders of magnitude lower that the total amount of live cells present. This is due to a variety of factors, including different nutritional and physiological needs that cannot all be captured by using a limited number of growth media and conditions. Therefore, one cannot rely on culturable cell counts as an indication of the number of live cells in such samples. In addition, total cell counts using staining and microscopy often cannot distinguish between live and dead cells, although better methods are now emerging. However, microscopic cell counting if often unsuccessful for sediment samples because of mineral particle interference.

Viable microbial cells have intact membranes containing phospholipid fatty acids (PLFA). Upon cell death or cell lysis, cellular enzymes (phospholipases) hydrolyse PLFA, releasing the polar head group (dephosporolation) and leaving diglycerides. The hydrolysis can occur within minutes or hours of cell lysis (White and Ringelberg 1997). The use of PLFAs as markers of live cells is, therefore, linked to the assumption that these molecules have a rapid turn-over in sediments, ranging from hours to days (White et al. 1979, Harvey et al. 1986. Kieft et al. 1997a, b). Besides PLFAs, two other lipid fractions can be extracted from samples containing microorganisms, i.e., neutral lipids (neutral lipid fatty acids, NLFAs) and glycolipid (diglyceride) fatty acids (GLFA). These types of lipids are thought to be indicative of dead biomass while PLFA is indicative of viable biomass. Furthermore, the composition and various ratios of PLFAs can be used as biomarkers for various physiological groups and nutritional status of microbial populations in environmental samples.

Kieft et al. (1994) investigated the changes in PLFA profiles of subsurface bacteria during starvation and desiccation in a porous silica sand medium. The subsurface bacteria examined were a gram-negative *Pseudomonas aureofaciens* strain and a gram-positive *Arthrobacter protophormiae* strain, both isolated from a subsurface environment. Washed cells were added to sand microcosms (such treatment and environment combine to ensure nutrient starvation)

and were either maintained under saturated conditions or subjected to desiccation by slow drying over a period of 16 days to final water activities of about 0.957 for *Pseudomonas aureofaciens* and 0.897 for *Arthrobacter protophormiae*. In a third experiment, cells of both strains were added to saturated microcosms along with organic nutrients and maintained at saturated conditions.

Kieft et al. (1994) found that the number of culturable cells of both bacterial strains declined to below detection limit in both the moist and dried nutrient-deprived conditions while direct (total) cell counts and total PLFA concentrations remained relatively constant. The dried gram-negative *Pseudomonas aureofaciens* cells showed changes in PLFA profiles that are typically associated with stressed, gram-negative cells, i.e., increased ratios of saturated to unsaturated fatty acids, increased ratios of *trans*- to *cis*-monoenoic fatty acids and increased ratios of cyclopropyl fatty acids to their monoenoic precursors. *Pseudomonas aureofaciens* starved under moist conditions showed few PLFA profile changes, but cells of this strain incubated under moist conditions in the presence of nutrients showed decreases in the ratios of both saturated to unsaturated fatty acids and cyclopropyl fatty acids to their monenoic precursors. The PLFA profiles of the gram-positive *Arthrobacter protophormiae* changed very little in response to either nutrient deprivation or desiccation or both.

Glycolipid fatty acids (GLFAs), proposed as indicators of dead or lysed cells, remained relatively constant throughout the experiments. Only the starved desiccated *Arthrobacter protophormiae* cells showed an increase in the ratio of GLFAs to PLFAs.

In a later study, Kieft et al. (1997a) carried out similar starvation experiments with surface and sub-surface strains of *Pseudomonas fluorescens* and *Arthrobacter* sp., this time in microcosms of natural sediments (a silty sand paleosol (buried soil) and a sandy silt nonpaleosol), and similar results were obtained, i.e., typical PLFA stress signatures in the gram-negative microorganisms but few PLFA changes in the gram-positive microorganism. The latter survived generally somewhat better than the gram-negative species, but there were no significant differences in survival between surface and subsurface strains.

Kieft et al. (1997b) also reported results of a microbial characterization of volcanic tuff samples at Yucca Mountain, using total cell counts (3.2×10^4 to 2.0×10^5 cells/g), culturable cell plate counts 1.0×10^1 to 3.2×10^3 CFU/g) and PLFA concentrations (0.1 to 3.7 pmol/g), all indicating low biomass. Glycolipid (diglyceride) fatty acid concentrations, indicative of dead cells, were in a similar range (0.2 to 2.3 pmol/g), suggesting that close to half of the cells present in the volcanic tuff environment were dead. According to Kieft et al. (1997b), finding evidence for dead cells (GLFAs) in nearly as high amounts as live cells (PLFAs) is not surprising in a deep subsurface environment where cells may have undergone long-term sequestration and nutrient deprivation.

Mauclaire et al. (2007) and Stroes-Gascoyne et al. (2005, 2007c) reported results from a study that examined a sterile-drilled core of Opalinus clay for the presence of indigenous microbes, using PLFA extraction and culturing. The Opalinus clay contained 64 ng PLFA/g. Assuming that PLFA represents live cells and using a conversion factor of 1 pmol PLFA = 2×10^4 cells (Balkwill et al. 1988) and an average mol weight of 250 g for phospholipids (C-18), the Opalinus Clay contained an average of about 5×10^6 cells per g dry weight, yet very few cells could be cultured. Based on the PLFA composition, the microbial community in Opalinus Clay consisted mainly of anaerobic gram-negative bacteria. There were also biomarkers for SRB,

corroborating the (limited) culture results. Generally, these results are consistent with an anaerobic environment in the Opalinus Clay.

The lipid extraction results from Opalinus Clay (Stroes-Gascoyne et al. 2005, 2007c) showed further that this formation appeared to contain almost 14 times more dead than viable biomass. This result could possibly suggest that dead biomass is not being recycled rapidly in Opalinus Clay, which would be indicative of a biologically quite inactive environment, in which a large portion of lipids is preserved. However, according to Mauclaire et al. (2007), equating a high concentration of GLFA with a biologically inactive environment is still somewhat tentative and has to be confirmed further. For example, significant differences in the degree of degradation of different types of biomarkers have been observed for different diagenetic conditions (oxic vs. anoxic) in marine sediments (Hoefs et al. 2002).

Additional laboratory experiments (reported in Stroes-Gascoyne et al. 2005) demonstrated that the organic matter trapped in the Opalinus Clay Formation was sensitive to microbial degradation after disturbance and incubation of the mineral matrix in enrichment cultures. Therefore, it is possible that potentially labile organic materials, such as PLFAs, were preserved on a geological time scale, due to association with minerals, (e.g., in black shales, Salmon et al. (2000)). Various experiments have demonstrated that adsorption on clay matrix preserves DNA from microbial and chemical degradation (Franchi et al. 2003, Gallori et al. 1994, Garet and Moriarty 1996). One might argue that similar interaction will occur with PLFAs. However, the recovery of low numbers of viable SRB in enrichment cultures from Opalinus Clay (Mauclaire et al. 2007, Stroes-Gascoyne et al. 2005, 2007c) could suggest that the PLFAs found in Opalinus Clay were not merely preserved PLFAs from dead cells. The rate of PLFA turnover in this particular sediment is unknown, so further experiments should be conducted to assess the extent of the interaction between PLFAs and clay and to confirm their use as valid biomarkers of live cells in clay-rich environments.

It is not unusual to find a large discrepancy between culturable and PLFA-based viable bacteria in subsurface microbiological studies (Fredrickson et al. 1997, Onstott et al. 1998; Kieft et al. 1994, 1997a, b, Pfiffner et al. 2006) although its cause remains elusive. Onstott et al. (1998) discuss the possibility that PLFA degradation may be inhibited in certain geological environments, especially in unsaturated pores in a rock matrix. The rates of dephosphorylation of PLFA and of depurination and depyrimidation of DNA of a cell that expires or lyses in pores in which only a thin film of water is present or in which the water is bound to the rock matrix are probably less than in water-filled pores, but how much less is not known. It is also possible that PLFA molecules are protected from degradation by association with certain minerals (i.e., black shales, Salmon et al. 2000, Mauclaire et al. 2007).

Therefore, while interpreting PLFA results from geologically very old rocks or sediments, the possibility of preserved PLFA from now actually dead cells must be kept in mind, although no solid indication has been found so far that this is indeed the case.

4. MATERIALS AND METHODS

Two core segments, one Queenston shale sample and one Cobourg limestone sample, were chosen for preliminary microbial characterization. This report presents the results of these analyses. The core samples shipped to WL were weighed at the time of preservation, vacuum-sealed in PE aluminum bags, and stored at 4^{o}C. The core samples arrived at WL on November 1, 2007. They were shipped from Ontario by courier and arrived within two days. However, the icepacks in the box in which the core samples were shipped had melted completely during shipping and the sample temperature was 18^{o}C upon arrival. The PE aluminum bags were intact. Upon arrival, the core segments were stored at 4^{o}C.

4.1 SUBSAMPLING OF COBOURG LIMESTONE CORE SAMPLE

The Cobourg limestone core sample (12 cm length, 7.6 cm diameter) was analyzed for microbial characteristics between Nov. 26 and December 7, 2007.

The Cobourg limestone core sample was unwrapped, wiped with a cloth soaked in ethanol, and placed on a clean (sterilized foil) surface. A sterile hammer was used to break open the core. Approximately half of the core was wrapped in sterile foil and refrigerated prior to powdering for phospholipid fatty acid (PLFA) analysis (section 4.4.8). From the other half, subsamples were taken from the center region of the segment. Each piece of core was examined for outside core surfaces, which, if found, were removed with a chisel (at least 5 mm). The pieces thus collected were crushed by hand in a sterilized mortar and pestle. This proved to be very difficult and the material could not be ground up to a fine powder by hand (i.e., in the mortar and pestle) but consisted of coarsely ground material. A portion of this was weighed and analyzed as described below. This subsample is called the crushed limestone sample

After this initial set of analyses, the remainder of the core was washed again with ethanol, the outside sterilized further by burning the ethanol away in a flame, and about 1-2 mm of the outside surface of the core chiseled away. The sample was then broken into smaller chunks with a sterile hammer (on a sterile surface). The chunks were collected and crushed to a fine powder in a shatter box, which had been cleaned thoroughly with ethanol for this purpose. This subsample is called the powdered limestone sample and this was again put through some of the microbial analyses, including analysis for PLFA (see section 4.4.8). Throughout the whole crushing and sampling procedure (both the first sampling (crushed core) and the second sampling (powdered core)), great care was taken not to contaminate the sample. However, because of the difficulty in crushing the limestone and the many steps involved, contamination at some point during the procedure cannot be ruled out fully. In addition, the ethanol and flame treatments may not have been sufficient to sterilize the outside of the core segment deep enough into the core to eliminate potential contamination from the drilling muds, although during the first sampling at least 5 mm was removed from the outside surfaces of core chunks.

4.2 SUBSAMPLING OF QUEENSTON SHALE CORE SAMPLE

The Queenston shale core sample (20 cm length, 7.6 cm diameter) was analyzed for microbial characteristics between March 18 and March 28 2008.

The Queenston shale core sample was unwrapped, wiped with a cloth soaked in ethanol, and placed on a clean (sterilized foil) surface. The end pieces of the core sample were removed to leave a clean sample. The core was placed in a sterile tray and split into two chunks with a sterile mallet and chisel. One chunk was reserved for PLFA analysis (section 4.4.8). The other chunk was broken into small pieces with a sterile mallet and chisel. These pieces were then placed in a sterile manual grinder to render even smaller pieces. Subsequently these were further ground up in a sterilized mortal and pestle to predominantly a powder with a few small pieces. This subsample is called the crushed shale sample.

4.3 SYNTHETIC POREWATER COMPOSITION

Because the porewater in Ordovician Sediments is extremely saline (Koroleva and Mazurek 2006), growth media for microbial analysis were prepared on a distilled deionized water basis (as is usual), but also on a synthetic porewater solution basis. Table 1 shows the compositions of both shale synthetic porewater (SSPW) and limestone synthetic porewater (LSPW), as developed by the University of Bern (M. Hobbs, NWMO, Pers. Comm. 2007). LSPW and SSPW were used in the microbial analyses of the limestone and shale samples, respectively.

4.4 ANALYSES

The following analyses were performed at WL on the Cobourg limestone and Queenston shale samples.

4.4.1 Water Activity

Water activity was measured on a crushed core subsample using a Decagon^{TM} WP4 Dewpoint PotentiaMeter (Decagon Devices, Pullman, WA).

4.4.2 Water Content

Water content was determined by drying the (above) water activity subsample at 105^{o}C to constant weight.

4.4.3 Heterotrophic Aerobes and Anaerobes

Limestone core sample

About 20g (carefully weighed) of crushed limestone core was added to 100 ml of Phosphate-Buffered Saline solution (PBS, i.e., 0.01M NaCl buffered to pH 7.6 with 9 mM Na_2HPO_4 and 1 mM $NaH_2PO_4.H_2O$). This suspension was stirred or shaken for 60 min. after which serial dilutions were made to 10^{-3} in PBS. The dilutions were plated onto regular R2A agar (Reasoner and Geldreich 1985) and the plates were incubated at 30^{o}C for 5-7 days (heterotrophic aerobes) and up to 4 weeks (heterotrophic anaerobes) before they were counted.

The above procedure was repeated by adding about 20 g of crushed limestone core to 100 ml of sterilized LSPW. This LSPW suspension was stirred or shaken for 60 min. after which serial

dilutions were made to 10^{-3} in LSPW, in order to plate these dilutions onto LSPW-based R2A agar. Unfortunately the saline LSPW prevented the agar plates from setting properly and the plates could not be counted accurately. Therefore, additional PBS and LSPW suspensions were made as above, this time with the powdered limestone core sample (about 10 g powder in 100 ml). The PBS suspension was serially diluted to 10^{-3} in PBS and the dilutions were plated for aerobes and anaerobes on regular R2A agar plates. The saline LSPW suspension was serially diluted to 10^{-3} in LSPW solution, which was also plated on regular R2A. The plates were incubated at 30^{o}C for 5-7 days (heterotrophic aerobes) and up to 4 weeks (heterotrophic anaerobes) before they were counted.

Shale core sample

About 20g (carefully weighed) of crushed shale core was added to 100 ml of Phosphate-Buffered Saline solution (PBS, i.e., 0.01M NaCl buffered to pH 7.6 with 9 mM Na_2HPO_4 and 1 mM $NaH_2PO_4.H_2O$). This suspension was stirred or shaken for 60 min. after which serial dilutions were made to 10^{-3} in PBS. The dilutions were plated onto regular R2A agar (Reasoner and Geldreich 1985) and the plates were incubated at 30^{o}C for 5-7 days (heterotrophic aerobes) and up to 4 weeks at room temperature in an anaerobic glove box for heterotrophic anaerobes for four weeks.

The above procedure was repeated by adding about 20 g of crushed shale to 100 mL of sterilized SSPW. This SSPW suspension was stirred or shaken for 60 min. after which serial dilutions were made to 10^{-3} in SSPW solution, which was then plated on regular R2A (because saline-based R2A plates would not set solid). The plates were incubated at 30^{o}C for 5-7 days (heterotrophic aerobes) and up to 4 weeks (heterotrophic anaerobes) before they were counted.

4.4.4 Sulphate-reducing Bacteria

Sulphate-reducing bacteria (SRB) were enumerated with the MPN method. Degassed tubes containing sterile modified Postgate's B medium (Atlas 1993) were inoculated in an anaerobic glovebox (in triplicate) with the PBS limestone or shale crushed core suspension and serially diluted to 10^{-3}. The tubes were incubated at room temperature for about 4 weeks before they were scored.

This procedure was repeated with the LSPW limestone crushed core suspension and LSPW-based modified Postgate's B medium, and with SSPW shale crushed core suspension and SSWP-based modified Postgate's B medium.

4.4.5 Nitrate-utilizing and Nitrate-reducing Bacteria

Nitrate-utilizing bacteria (NUB, that convert nitrate to nitrite) and nitrate-reducing bacteria (NRB, that convert nitrate to N_2) were enumerated. Sterile MPN tubes with degassed R2A medium (Reasoner and Geldreich 1985), amended with 0.1% nitrate, were inoculated with the PBS limestone or shale crushed core suspension in an anaerobic glovebox (in triplicate) and serially diluted to 10^{-3}. The tubes were scored for gas production (in inverted Durham tubes) or the presence of nitrite after about 4 weeks of incubation at 30^{o}C.

This procedure was repeated with the LSPW limestone crushed core suspension and LSPW-based R2A medium amended with 0.1% nitrate and with the SSPW shale crushed core suspension and SSWP-based R2A medium amended with 0.1% nitrate.

4.4.6 Iron-reducing Bacteria

Iron-reducing bacteria (IRB) were enumerated by MPN method using the method and medium of Gould et al. (2003). MPN tubes filled with sterile IRB medium were inoculated in an anaerobic glovebox (in triplicate) with the PBS limestone or shale crushed core suspension and serially diluted to 10^{-3}. The tubes were incubated at room temperature for about 4 weeks before they were scored.

This procedure was repeated with the LSPW limestone crushed core suspension and LSPW-based IRB medium and with the SSWP shale crushed core suspension and SSWP-based IRB medium.

4.4.7 Enrichment Cultures

Enrichment cultures are a qualitative method to determine the presence (or absence) of viable, growing cells in a sample. Note that in enrichment cultures the presence of one viable cell of a species can, in principle, be enough to multiply manifold such that DNA can be extracted. Enrichment cultures are not quantitative and may not represent the distribution of species present in the original sample, because one or more species may have nutritional advantages over others present in the enrichment growth medium chosen.

Enrichment cultures usually are given a long incubation time, such that difficult-to-grow microorganisms, and/or low numbers of microorganisms in the sample have time to adapt to, and multiply in, the growth medium inoculated with the sample. About 10 g of crushed limestone or crushed shale were added to 100 mL of five different sterile solutions or liquid growth media. The solutions or media used were:

- Sterile distilled deionized water;
- Sterile LSPW or SSWP solution (Table 1),
- R2A medium (regular, full strength, distilled deionized water-based),
- LSPW-based or SSPW-based R2A medium, and
- R2A (1/100 strength of regular R2A, distilled deionized water-based);

In addition, about 10 g of powdered limestone core was added to 100 mL of regular aerobic R2A medium.

The bottles were incubated aerobically and anaerobically for 2-3 months at 30^{o}C and room temperature, respectively. Growth was judged by optically scoring turbidity against sterile controls and in some cases by counting cells under a light microscope (Olympus model BX40).

4.4.8 Phospholipid Fatty Acid Analysis, 16S rDNA Extraction and Denaturing Graduated Gel Electrophoresis

A powdered limestone core sample and a crushed shale core sample were submitted to Microbial Insights (MI) Inc. Rockford, TN, USA, for Phospholipid Fatty Acid (PLFA), neutral lipid fatty acids (NLFA) and glycolipid fatty acids (GLFA) analysis. PLFA is indicative of viable cells, while NLFA abd GLFA are indicative of dead cells and organic debris.

In addition, the 10-day old aerobic enrichment culture of powdered limestone sample in regular R2A medium was submitted to MI for 16S rDNA extraction and PCR amplification, followed by Denaturing Graduated Gel Electrophoresis (DGGE) and band identification. The latter analysis was attempted to determine the origin of the aerobic microorganisms in this enrichment culture (i.e., indigenous or contamination).

For the shale sample, two 6-week old enrichment cultures in regular R2A medium (one aerobic, one anaerobic) were submitted for DGGE analysis.

PLFA, NLFA and GLFA Analysis (provided by MI)

Lipids were recovered using a modified Bligh and Dyer method (White et al. 1979). Extractions were performed using one-phase chloroform-methanol-buffer extractant. Lipids were 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. The NLFA and GLFA fractions (indicative of dead microbial cells and organic debris) were also analyzed.

16S rDNA/DGGE Analysis (provided by MI)

Nucleic acid extraction was performed using a bead-beating method (Stephen et al. 1999). Sodium phosphate buffer, chaotropic reagent, glass beads, and the sample were agitated in a microcentrifuge tube using a high-speed bead beater. Chloroform was added, mixed thoroughly, and the tube was recentrifuged. The aqueous supernatant was collected and phenol/cholroform/isoamyl alcohol (24:24:1) extracted. Glycogen was added and the DNA was precipitated from the aqueous phase with an equal volume of isopropanol. DNA was pelletted by centrifugation, washed with 80% ethanol, air-dried, and re-dissolved in Tris buffer, pH 8.0. The DNA was purified by a glass-milk DNA purification protocol using a Gene Clean™ kit as described by the manufacturer. PCR amplification of 16S rRNA gene fragments was performed as described in Muyzer et al. (1993), with modifications as follows. Thermocycling consisted of 35 cycles of 92°C for 45 sec., 55°C for 30 sec., and 68°C for 45 sec. Using 0.44 units of Clontech Advantage™ 2 polymerase and 12.5 pmole each primer (forward primer contained a 40 bp GC-clamp) in a total volume of 25 μL, thermocycling was performed using a 'Robocycler™' PCR block. Two primer sets were used in a nested PCR approach. The first primer set used primers corresponding to *E. coli* bp positions 27 and 1492 of the 16S rRNA gene. The second set of primers targeted eubacterial 16S rDNA regions corresponding to *E. coli* positions 341-534. A portion (20%) of each PCR product was analyzed by agarose gel electrophoresis (1.5% agarose, 1x TAE buffer) and ethidium bromide fluorescence. The amount of DNA used for DGGE was standardized to 150 ng by comparison to molecular weight standards using Alpha Imager™ software. DGGE was performed on a D-Code 16/16 cm gel

system maintained at a constant temperature of 60°C in 6L of 0.5 x TAE buffer (20mM Tris acetate, 0.5mM EDTA, pH 8.0). Denaturing gradients were formed at 30 – 65 % denaturant (with 100% denaturant defined as 7 M urea, 40% v/v formamide). A size gradient was imposed on the denaturing gradient by forming an 8 – 10 % acrylamide gradient as described by Cremonesi et al. (1997). Gels were electrophoresed at 35V for 16 hr. Gels were stained with ethidium bromide (0.5 mg/L) and destained twice in 0.5 x TAE for 15 min. each. Gel images were captured using an Alpha Imager™ system.

The central 1 mm portion of intensely fluorescing DGGE bands were excised using a razor blade and soaked in 50 μL of purified water overnight. A portion (15 μL) was used as the template in a PCR reaction as described above. The products were purified by electrophoresis through a 1.2% agarose/TAE gel followed by glass-milk extraction (Gene-Clean™ kit). Purified DNA was sequenced with an ABI-Prism automatic sequencer model 377 with dye terminators. Sequence identifications were performed using the BLASTN facility of the National Center for Biotechnology Information (http://ncbi.nlm.nih.gov/Blast) and the 'Sequence Match' facility of the Ribosomal Database Project(http://www.cme.msu.edu/RDP/analyses.html).

5. RESULTS

Table 2 shows all enumeration (culture) results for the crushed and powdered limestone core and the crushed shale sample.

From the crushed limestone sample, low numbers of aerobes and anaerobes could be grown on the regular R2A plates, but not from the powdered limestone sample. The crushed limestone sample also contained low numbers of NUB and IRB in the regular growth media but no SRB or NRB were found. All but one attempt to grow cells in LSPW-based growth media failed, suggesting that no halotolerant or halophilic microorganisms were present in the limestone core, or that they could not be grown easily. A low number of NRB appeared in the LSPW-based NRB medium.

It should be noted that the attempts to grow colonies from the powdered limestone sample on regular R2A media failed, indicating that no contamination occurred during the plating procedure, or possibly a devastating effect from the shatter box procedure on any surviving organisms in the limestone. However, the aerobic R2A enrichment culture of powdered limestone was positive (Table 3).

Table 3 shows the data for the enrichment cultures. Enrichment cultures were positive visibly only (i.e., cloudy) in regular R2A, for both aerobes and anaerobes for both the limestone and shale sample. The non-cloudy enrichment cultures were not checked by microscope for limited (non-visible) growth.

Table 4 shows the water content and water activity in the samples. Note that the measured values in Table 4 could be on the low side, since the samples had been preserved for several months during which they could have lost some water, despite being plastic-wrapped. The water content of the limestone sample was 0%, and the water activity registered as 0.182, which is essentially zero, because this is what the Potentiameter registers if no sample is inserted. The water content of the shale sample was 1% and the water activity was 0.341. Both water activities are much lower than the water activity below which DNA denatures. This implies that no surviving cells are expected, other than perhaps sporeformers.

Table 5 shows the PLFA results for the limestone and shale samples. For comparisons the Opalinus Clay results (Stroes-Gascoyne and Hamon 2007) are included in this table.

The PLFA concentration was below detection limit (0.05 pmol/g) in the limestone sample, suggesting either the absence of viable cells or a very low quantity of viable cells (< 10^3 cells/g). Small quantities of NLFA and GLFA were found in the limestone, indicating the presence of some dead cell material. This suggests a biologically inactive environment.

The shale sample contained measurable PLFA, implying the presence of viable cells (1 x 10^6 cells/g), low amounts of NLFA but large amounts of GLFA, indicating the presence of far more dead cells than viable cells. Based on the PLFA profile shown in Table 5, most viable cells (65%) in the shale sample were proteobacteria, typically gram-negative fast-growing bacteria, which could indicate the presence of contaminating species. About 33% were general PLFA, common in all microbes. A high percentage of general PLFA indicates a less diverse population. About 1.4 % of the PLFA indicated the presence of firmicutes, which are indicative of anaerobic gram-positive microorganisms, including the sporeforming *Bacilli*. Firmicutes are hardier microorganisms than proteobacteria and would be more suited for survival in low water activity environments.

DNA extraction, followed by PCR amplification, DGGE and sequencing failed for both the limestone and shale core samples. No DNA could be extracted from the core samples despite the application of three different DNA extraction methods. It should be noted that DNA extraction from Opalinus Clay and bentonites is also unsuccessful with current methods.

The DGGE results for the aerobic R2A limestone and aerobic and anaerobic R2A shale enrichment cultures are shown in Tables 6, 7 and 8, respectively, and in Figures 1 and 2, respectively.

The limestone aerobic R2A enrichment culture contained three distinct bands. The genus with a somewhat similar DNA sequence to that found in band 2.1 in this enrichment culture included both *Lysobacter spp*. (82.6%) and *Xanthomonadaceae* (82.6%). The former is an aerobic species that contains lytic enzymes (and is able to lyse a variety of microorganisms); the latter belongs to a family of aerobic gram-negative proteobacteria, pathogenic to plants. Bands 2.3 and 2.4 in Figure 1 show similarities of 97.4% and 80.2%, respectively, with the genus *Pseudomonas* spp., i.e., facultative aerobic gram-negative gamma-proteobacteria that can grow very rapidly in the presence of O_2 or NO_3^-, and carbon. None of the species indicated by the DGGE analysis would survive well in the harsh anaerobic limestone environment, and it is likely that the species found result from contamination.

The shale aerobic R2A enrichment culture (Figure 2) contained five distinct bands. Bands 1.1 and 1.2 show similarities of 100% and 93.7%, respectively, with the genus *Staphylococcus*, which belong to the Phyllum *Firmicutes* and the Class of *Bacilli*, i.e., gram-positive aerobic or facultative aerobic sporeformers. They are known as medical pathogens, but can survive for long times in soils. Band 1.3 has a 97.4% similarity with the genus *Bacillus* spp., which is a widespread (facultative) aerobic gram-positive sporeforming heterotroph found in soils. Bands 1.4 and 1.5 have a 100% similarity with *Peanibacillus spp*., which belong to the Phyllum *Firmicutes* and the Class of *Bacilli*, i.e., gram-positive aerobic or facultative aerobic sporeformers.

The shale anaerobic R2A enrichment culture (Figure 2) contained three distinct bands. Band 2.1 has a 92.7% similarity with *Staphylococcus*, and band 2.2 a 94.8% similarity with *Bacillus spp.*, the same species indicated in the aerobic R2A enrichment culture for the shale sample. Both species are facultative aerobic, i.e., they can grow under both aerobic and anaerobic conditions, using O_2 and NO_3^- as electron acceptor, respectively. Band 2.3 in Figure 2 failed to produce a phylogenetic match. This could potentially indicate a unique species, but more work would be required to confirm this.

6. DISCUSSION

6.1 PHOSPHOLIPID FATTY ACIDS

The analyses carried out on the limestone and shale cores included both culturing and phospholipid fatty acids (PLFA) measurements to enumerate viable (live) cells. Culturing provides actual colonies of live cells, but has as important disadvantage that not all viable cells in environmental samples can be cultured on the media in use. There are often several orders (one to four) of magnitude difference between culturable cells and viable cells present. Total cell counting by microscope enumerates both live and dead cells and is, therefore, not a good measure for live cells, although some recent methods are now able to distinguish between live and dead cells. However, total cells counts generally are not feasible for samples with a high clay or mineral content because of interference of clay particles and minerals. Viable cells in an environmental sample can be accesses without culturing by extracting biomarkers of viable cells and by translating the concentration of those extracted biomarkers into viable cell numbers. The method used here involved the extraction of PLFA. Another measure of live cells is the amount of ATP (adenosine-tri-phosphate) in a sample.

PLFA are part of the cell wall of viable bacteria. The premise is that finding PLFA indicates the presence of live cells. Finding PLFA provides no information about the in situ activity of the cells (they could be active or dormant) but finding live cells indicates the potential for microbial activity. One drawback of PLFA analysis is that it provides no actually visible live cells but only an indication of the presence of live cells (and an estimate of actual live cell numbers). Another (accepted) drawback is that PLFA analysis relies on the premise that upon cell death, PLFA hydrolyse rapidly and, therefore, disappear quickly from a water or sediment sample.

The hydrolysis (dephosphorolation) of PLFA results in the formation of di-glycerides (glycolipid fatty acids, GLFA) and neutral fatty acids (NLFA). Both GLFA and NLFA are, therefore, indicative of the presence of dead cells. A further assumption is that GLFA and NLFA are used as organic material by live cells, i.e., that the components of dead cells are recycled by metabolizing cells. Therefore, if larger quantities of GLFA and NLFA (compared to PLFA) are found in a sample, this not only indicates that there are more dead cells compared to live cells, it also implies that the live cells left are not using (much of) the GLFA and NLFA. In other words, the live cells appear not to be active (otherwise they would use the available GLFA and NLFA), i.e., the environment is biologically not (very) active, for whatever reason.

For hydrolysis of PLFA from dead cells to proceed, water is required. This is not a problem in many environments (aqueous or solid), because even in deep-sea sediments, there is water available for such reactions, as well as in the water-bearing fractures in rocks. However, in severely water-limited environments (such as high-smectite clay environments and other

compacted environments with low water activity, or even in gas-filled pores in sediments or rocks), there is some doubt whether the hydrolysis of PLFA can progress. If PLFA do not hydrolyse but remain as PLFA although the actual cells are dead, PLFA can no longer be indicative of live cells. Currently, this possibility has not been addressed explicitly in the literature, although some discussion on the perseverance of PLFA in gas-filled rock pores was provided by Onstott et al. (1998).

However, considering the very dry nature of both the limestone and shale samples in this investigation, this possibility should be considered in interpreting the enumeration and PLFA results for these samples.

6.2 LIMESTONE SAMPLE

The limestone crushed core sample did not contain any measurable water (according to the standard technique used in this study) and its water activity was essentially zero. Based on these measurements it is not likely that a thriving microbial community would be present in this limestone core. This was confirmed by the negative PLFA results that indicate a presence of less than 10^3 viable cells/g. There were small quantities of NLFA and GLFA in the limestone sample, indicating the presence of dead cells. This implies that dead cell material is not (fully or rapidly) recycled, i.e., the limestone sample is biologically (largely or fully) inactive, in agreement with the absence of any extractable water in this sample.

Further confirmation of the absence of significant viability in the limestone sample came from the largely negative culture results in Table 2. Only small numbers of aerobic and anaerobic colonies (about 40-80 CFU/g) were found on regular R2A plates and a few NUB and IRB were indicated on the regular media. Only one of the saline-based media produced a very low positive result for NRB, and the saline-based enrichment cultures (Table 3) were negative for visible growth, suggesting the general absence of halotolerant and halophilic species (or alternatively the possible presence of such species, that were unable to grow on the media and solutions provided). No species identification was done on the colonies found on the aerobic and anaerobic regular R2A plates.

Enrichment cultures were done to increase the number of viable species present in the sample that can make use of the nutrients in the solutions offered (Table 3). Enrichment cultures in principle can enrich one cell into many and, therefore, such cultures are only a qualitative measure of species present and cannot be used to determine which species are dominant in the sample. The DGGE analysis of the aerobic limestone enrichment culture in R2A showed the presence of three distinct bands (Figure 1), indicating the presence of three distinct species in this enrichment culture. Sequencing results gave positive matches but with low similarity indices (similarity indices should be above 0.98 for a positive identification), including two possible species for band 2.1 (Figure 1). This suggests the presence of species related, but not exactly similar, to the species indicated. However, the four possible species identified were largely aerobic or facultative aerobic gram-negative proteobacteria that can grow using either O_2 or NO_3^- as electron acceptors. These species are widespread and most likely indicate that contamination of the limestone sample occurred, either during drilling or during sampling and analysis. It is less likely that contamination occurred during enumeration because of the generally negative culture results obtained. Therefore, it is unlikely that the identified species are indigenous species in the limestone sample. Also, the fact that plating showed 40-80 CFU/g and PLFA < 10^3 cells/g indicated that 4-8% of the cells could be grown, rather a large

percentage (normally between 0.001 and 10%). This again points to the presence of contaminating microbes (that are easy to grow) and not to the presence of indigenous more obscure (difficult to grow) species.

Overall it can be concluded, on the basis of the various analyses performed, that the limestone sample does not contain a significant viable population of indigenous bacteria (including halophiles).

6.3 SHALE SAMPLE

The shale crushed core sample contained only 1% measurable water (according to the standard technique used in this study) and the water activity was 0.341. At such a low water activity, no vegetative cells are expected since DNA denatures below a water activity of about 0.55. Even DNA in spores would probably disappear over time at such a low water activity. Based on these measurements it is not likely that a thriving microbial community would be present in the shale core. This was corroborated by the low or negative culture results in Table 2. Low numbers of aerobic and anaerobic colonies (0-600 CFU/g) were found on regular R2A plates and a few NUB and IRB were found in the regular media. None of the saline-based media produced positive culture results and the saline-based enrichment cultures (Table 3) were negative for visible growth, suggesting the general absence of halotolerant and halophilic species, or alternatively any halophilic species present were not able to grow on the media or solutions provided.

The PLFA results for the shale sample, however, suggested the presence of about 10^6 live cells/g. The amount of GLFA found indicated the presence of considerably more dead cells (about 4×10^7 cells/g) than live cells, suggesting that not much recycling of dead material occurred in this environment and implying that the live cells indicated by the presence of PLFA are not metabolizing rapidly and may, therefore, be dormant with little or no metabolic activity.

The culture results (0 - 600 CFU/g) indicated that only about 0 to 0.06% of the viable cells (as indicated by PLFA) could be cultured. This means that 99.94% of the live cells could not be cultured on the media used, if the PLFA results are valid. If all the PLFA found stemmed from contaminating microorganisms, which are generally easy to grow, many more colonies should have been found on the R2A plates, than was the case. Therefore, these results imply that there must be a significant number of indigenous viable microorganisms in the shale, unless the PLFA results are invalid, as discussed above and in section 3.

The DGGE analysis of the aerobic and anaerobic shale enrichment cultures in R2A showed the presence of five and three distinct bands, respectively (Figure 2), indicating the presence of five and three distinct species in these enrichment cultures. Sequencing results gave positive matches with high similarity indices for the aerobic enrichment culture (Figure 1), and indicated the presence of largely aerobic or facultative aerobic gram-positive sporeformers (including a medical pathogen) that can grow using either O_2 or NO_3^- as electron acceptors. These species are widespread and could suggest that some contamination of the shale sample occurred, either during drilling or during sampling and analysis. However, sporeformers are also better survivors under adverse conditions and could perhaps survive for long times in situ. However, the water activity measured in the shale is so low, that long-term geological survival of these species is unlikely. Three distinct bands were found in the DGGE analysis for the anaerobic enrichment culture, indicating the presence of three distinct species in this enrichment culture.

Sequencing results gave two positive matches but with low similarity indices (similarity indices should be above 0.98 for a positive identification) (Figure 2). This suggests the presence of species related, but not exactly similar, to the species indicated. However, the two possible species identified were an aerobic or facultative aerobic gram-negative proteobacterium that can grow using either O_2 or NO_3^- as electron acceptors, and a human pathogen. These species are widespread and most likely indicate that contamination of the shale sample occurred, either during drilling or during sampling and analysis. Band 2.3 in Figure 2 failed to produce a phylogenetic match. This could potentially indicate the presence of a unique species, but more work would be required to confirm this.

Based on the above discussion and assuming that the current premises regarding PLFA analysis are valid, the conclusion would be that the shale contains 10^6 live cells/g, and $>10^7$ dead cells/g, suggesting a sizable indigenous population that is biologically quite inactive (i.e., the PLFA-indicated live cells are likely dormant). It can also be concluded that that the species found in the enrichment cultures were likely contamination, because they are easy to grow aerobic common species, and unlikely indigenous survivors in the shale, although this cannot be ruled out entirely, because of the presence of sporeformers. Therefore, the shale results are not as easy to interpret as the limestone results.

Similar results (measurable PLFA, indicating viable cells but higher quantities of NLFA and GLFA, indicating dead cells) were also obtained for Opalinus Clay cores that were drilled with sterile techniques (Stroes-Gascoyne et al. 2005, 2007c, Mauclaire et al. 2007). The average pore size range in Opalinus Clay would preclude the presence of viable bacteria (except perhaps in some very infrequent larger pores). The discussion presented (see also Section 3) suggests that there could be some doubt about the validity of the use of PLFA as biomarkers of viable microorganisms in certain environments with very low water activity. Therefore, the validity of PLFA- and GLFA-based conclusions regarding the presence of viable cells in low water activity environments needs further investigation. Currently this falls outside the scope of this work, although some preliminary tests are being conducted to determine the (rapid) disappearance of PLFA upon cell death (by autoclaving) in a sample of dry and wet bentonite and in a liquid bentonite enrichment culture.

7. CONCLUSIONS

These preliminary results suggest strongly that in accordance with expectations (based on water activity and absence of PLFA), no viable indigenous (halophilic or other) bacteria appear to be present in the limestone rock but that some contamination with common (facultative) aerobic cells occurred during drilling, or subsequent sample handling.

The results for the shale core indicate the presence of viable microorganisms, based on PLFA measurements. However, because of the low water activity in this shale core, this result may not be valid, because it is currently somewhat uncertain whether the PLFA and GLFA biomarkers are reliable in a low water activity environment. If the PLFA method is valid, the results for the shale core would indicate an indigenous, live population that appears to be biologically mostly inactive because of the presence of relatively large quantities of dead cells, compared to viable (live) cells. Bacterial species indicated in the aerobic and anaerobic shale enrichment cultures were common non-halophilic, mostly facultative aerobic sporeformers (including a pathogen), suggesting contamination during drilling or subsequent sample handling. Because of the presence of PLFA in the shale sample and the indication of

sporeforming microorganisms in the shale enrichment cultures, it cannot be ruled out that some of these microorganisms could be indigenous to the shale and survived for a long time in situ as spores. However, this is expected to be very unlikely because of the high similarity with common aerobic surface bacteria (including a human pathogen).

ACKNOWLEDGMENTS

This work was funded by NWMO. We thank Ken Raven (Intera) for shipping the Ordovician core samples and Monique Hobbs (NWMO) and Lisa Cave (University of New Brunswick) for the limestone and shale synthetic porewater compositions.

REFERENCES

Atlas, R.M. 1993. In Handbook of Microbiological Media, (L.C. Parks, Editor). CRC Press Inc.

Balkwill, D.L., F.R. Leach, J.T. Wilson, J.F. McNabb and D.C. White. 1988. Equivalence of microbial biomass measures based on membrane lipid and cell-wall components, adenosine-triphosphate, and direct counts in subsurface aquifer sediments. Microbial Ecol. 16, 73-84.

Cremonesi, L., S. Firpo, M. Ferrari, P.G. Righetti and C. Ggelfi. 1997. Double-gradient DGGE for optimized detection of DNA point mutations. Biotechniques 22(2), 326-330.

Franchi, M., J.P. Ferris and E. Gallori. 2003. Cations as mediators of adsorption of nucleic acids on clay surfaces in perbioticenvironments. Origins of Life and Evolution of the Biosphere 33, 1-16.

Fredrickson, J.K., J.P McKinley, B.N. Bjornstad, P.E. Long, D.B. Ringelberg, D.C. White, L.R. Krumholtz, J.M. Suflita, F.S. Colwell, R.M. Lehman, T.J. Phelps and T.C. Onstott. 1997. Pore-size constraints on the activity and survival of subsurface bacteria in late Cretaceous hale-sandstone sequence, northwest New Mexico. Geomicrobiol. 14, 183-202.

Gallori, E., M. Bazzicalupo, L. Dal Canto, R. Fani, P.Nannipier, C.Vettori and G.Stotzky.1994. Transformation of *Bacillus subtilis* by DNA bound on clay in non-sterile soil. FEMS Microb. Ecol. 15, 119-126.

Garet, M.J. and D.J.W. Moriarty. 1996. Acid extraction of tritium label from bacterial DNA in clay sediment. Microbiol. Methods 25, 1-4.

Gould, W.D., M. Stichbury, M. Francis, L. Lortie and D.W. Blowes. 2003. An MPN method for the enumeration of iron-reducing bacteria. In Proceedings from The Mining and Environment Conference III, Session 2A Bacteria. Sudbury, Ontario, Canada.

Harvey, H.R., R.D. Fallon and J.S. Patton. 1986. The effect of organic matter and oxygen on the degradation of bacterial membrane lipids in marine sediments. Geochimica et Cosmochimica Acta, 50, 795-804.

Haveman, S.A., S. Stroes-Gascoyne, C.J. Hamon and T.L. Delaney. 1995. Microbial analysis of groundwaters from seven boreholes at AECL's Underground Research Laboratory. Atomic Energy of Canada Limited, Technical Record, TR-677, COG-95-017.

Haveman, S.A., K. Pedersen and P. Ruotsalainen. 1998. Geomicrobial investigations of groundwaters from Olkiluoto, Hastholmen, Kivetty and Romuvaara, Finland. Report Posiva 98-09, Posiva Oy, Finland.

Hoefs, M.J.L., I.C.Rijpstra, W.Sinninghe and J.S. Damsté. 2002. The influence of oxic degradation on the sedimentary biomarker record I: Evidence from Madeira Abyssal Plain turbidites. Geochim. Cosmochim. Acta 66, 2719-2735

Jain, D.K., S. Stroes-Gascoyne, M. Providenti, C. Tanner and I. Cord. 1997. Characterization of microbial communities in deep groundwater from granitic rock. Canadian Journal of Microbiology 43, 272-283.

Kieft, T.L., D.B. Ringelberg and D.C. White. 1994. Changes in ester-linked phospholipid fatty acid profiles of subsurface bacteria during starvation and desiccation in a porous medium. Appl. Environ. Microbiol. 60, 3292-3299.

Kieft, T.L., E. Wilch, K. O'Connor, D.B. Ringelberg and D.C. White. 1997a. Survival and phospholipid fatty acid profiles of surface and subsurface bacteria in natural sediment microcosms. Appl. Environ. Microbiol. 63, 1531-1542.

Kieft, T.L., W.P.Kovacik, D.B. Ringelberg, D.C. White, D.L. Haldeman, P.S.Amy and L.E. Hersman. 1997b. Factors limiting microbial growth and activity at a proposed high-level nuclear repository, Yucca Mountain, Nevada. Appl. Environ. Microbiol. 63, 3128-3133.

Koroleva, M. and M. Mazurek. 2006. Characterization of pore-water composition in the Queenston Shale (Niagara, Ontario): First steps based on a mineralogical and leaching study. RWI Technical Report 2006-03, Institute of Geological Sciences, Rock-Water Interaction, University of Bern, Switzerland.

Mauclaire L., A. McKenzie, B. Schwyn and P. Bossart. 2007. Detection and cultivation of indigenous microorganisms in Mesozoic claystone core samples from the Opalinus Clay Formation (Mont Terri Rock Laboratory). Physics and Chemistry of the Earth, 32, 232-240.

Muyzer, G., E.C. De Waal and A.G. Uitterlinden. 1993. Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. Appl. Environ. Microbiol. 59(3), 695-700.

NWMO. 2005. Choosing a way forward. The future management of Canada's used nuclear fuel. Nuclear Waste Management Organization. (Available at www.nwmo.ca)

Onstott, T.C., T.J. Phelps, F.S. Colwell, D. Ringelberg, D.C.White, D.R. Boone, J.P. McKinley, T.O. Stevens, P.E. Long, D.L. Balkwill, W.T. Griffin and T. Kieft. 1998. Observations pertaining to

the origin and ecology of microorganisms recovered from the deep subsurface of Taylorsville Basin, Virginia. Geomicrobiol. 15, 353-385.

Pedersen, K. 2001. Diversity and activity of microorganisms in deep igneous rock aquifers of the Fennoscabdian Shield. In Subsurface Microbiology and Geochemistry. (J.K. Fredrickson and M. Fletcher, Editors), New York, NY, Wiley-Liss Inc., 97-139.

Pfiffner, S.M., J.M.Cantu, A. Smithgall, A.D. Peacock, D.C. White, D.P. Moser, T.C. Onstott and E. van Heerden. 2006. Deep subsurface microbial biomass and community structure in Witwatersrand Basin Mines. Geomicrobiol. J. 23, 431– 442.

Reasoner, D.J. and E.E. Geldreich. 1985. A new medium for the enumeration and subculture of bacteria from potable water. Appl. Environ. Microbiol. 49, 1-7.

Salmon, V., S. Derenne, E. Lallier-verges, C. Largeau and B. Beaudoin. 2000. Protection of organic matter by mineral matrix in a Cenomanian black shale. Organic Geochem. 31, 463-474.

Stephen, J.R., Y.J. Chang, S.J. MacNaughton, S.I. Whitaker, C.L. Hicks, K.T Leung, C.A. Flemming and D.C. White. 1999. Fate of a metal-resistant inoculum in contaminated and pristine coils assessed by denaturing gradient gel electrophoresis. Environmental Toxicology and Chemistry 18(6), 1118-1123.

Stroes-Gascoyne, S. 2005. A review of international experience with microbial activity in bentonite-based sealing materials and argillaceous host rocks. Ontario Power Generation Report 06819-REP-01300-10109-R00. Toronto, Ontario.

Stroes-Gascoyne, S. and C.J. Hamon. 2005. Microbial analysis of fracture surfaces from a sterily drilled granite core at AECL's Underground Research Laboratory. Poster presented at ISSM 2005, Jackson Hole, Wyoming, August 14-19, 2005.

Stroes-Gascoyne, S., B. Schwyn, A. Schippers, S. Poulain, C. Sergeant, C. Le Marrec, M. Simonoff, S. Altmann, T. Nagaoka, L. Mauclaire, C. Vasconcelos, J. McKenzie, S. Daumas, A. Vinsot, C. Baucaire and J.M. Matray. 2005. MA Experiment: Microbial investigations on unperturbed Opalinus Clay samples. Mont Terri Project Technical Note TN2004-44.

Stroes-Gascoyne, S., C.J. Hamon, C. Kohle and D.A. Dixon. 2006. The effects of dry density and porewater salinity on the physical and microbiological characteristics of highly compacted bentonite. Ontario Power Generation Report 06819-REP-01200-10016-R00. Toronto, Ontario.

Stroes-Gascoyne, S., P. Maak, C.J. Hamon and C. Kohle. 2007a. Potential implications of microbes and salinity on the design of repository sealing system components. Nuclear Waste Management Organization Report TR-2007-10. Toronto, Ontario.

Stroes-Gascoyne, S., C.J. Hamon, D.A. Dixon, C.L. Kohle and P. Maak. 2007b. The effects of dry density and porewater salinity on the physical and microbiological characteristics of compacted 100% bentonite. Materials Research Society Symposium Proceedings 985, 505-510.

Stroes-Gascoyne, S., A. Schippers, B. Schwyn, S. Poulain, C. Sergeant, C. Le Marrec, M. Simonoff, S. Altmann, T. Nagaoka, L. Mauclaire, J. McKenzie, S. Daumas, A. Vinsot, C. Beauclaire and J.M. Matray. 2007c. Microbial community analysis of Opalinus Clay drill core samples from the Mont Terri Underground Research Laboratory, Switzerland. Geomicrobiology Journal 24, 1-17.

White, D. C., W.M. Davis, J.S. Nickels, J.D. King and R.J. Bobbie. 1979. Determination of the sedimentary microbial biomass by extractable lipid phosphate. Oecologia 40, 51-62.

White, D.C. and D.B. Ringelberg. 1997. Utility of the signature lipid biomarker analysis in determining the *in situ* viable biomass, community structure, and nutritional/ physiological status of deep subsurface microbiota. In: The microbiology of the terrestrial deep subsurface, (Editors, Amy, P.S. and Haldeman, D.L). CRC Lewis Publishers, Boca Raton USA.

Willey, J.M., L.M. Sherwood and C.J. Woolverton. 2008. Prescott, Harley and Klein's Microbiology, Seventh Edition, McGraw Hill.

Table 1: Composition of Synthetic Porewaters

Element	Shale Synthetic Porewater (SSPW) mol/L	Limestone Synthetic Porewater (LSPW) mol/L
Na	2.40	2.00
K	0.50	0.45
Ca	1.201	0.485
Mg	0.25	0.20
Cl	5.80	3.81
SO_4	0.001	0.005
Recipe	**g/L**	**g/L**
NaCl	140.263	116.886
KCl	37.276	33.548
$CaCl_2.2H_2O$	176.416	70.567
$MgCl_2.6H_2O$	50.825	40.660
$CaSO_4$	0.136	0.681

Table 2: **Culture Results for Cobourg Limestone and Queenston Shale Samples**

Analysis	Limestone (1805)		Shale (1804)	
	CFU/g or MPN/g Regular Growth Medium	**CFU/g or MPN/g LSPW-based Medium**	**CFU/g or MPN/g Regular Growth Medium**	**CFU/g or MPN/g SSPW-based Medium**
Heterotrophic Aerobes				
Crushed Sample	$(6.19 \pm 2.03) \times 10^{1}$	Failed 0*	$(3.31 \pm 3.03) \times 10^{2}$	0*
Powdered Sample	0	0*	n/a	n/a
Heterotrophic Anaerobes				
Crushed Sample	$(3.84 \pm 1.04) \times 10^{1}$	Failed 0*	$(6.67 \pm 11.5) \times 10^{1}$	0*
Powdered Sample	0	0*	n/a	n/a
Sulphate-reducing Bacteria				
Crushed Sample	< 1.5	< 1.5	< 3.03	< 3.03
Powdered Sample	n.m.	n.m.	n/a	n.a.
Nitrate-utilizing Bacteria				
Crushed Sample	5.5	< 1.5	8.36×10^{-1}	$< 2.73 \times 10^{-1}$
Powdered Sample	n.m.	n.m.	n/a	n/a
Nitrate-reducing Bacteria				
Crushed Sample	< 1.5	1.5	$< 2.73 \times 10^{-1}$	$< 2.73 \times 10^{-1}$
Powdered Sample	n.m.	n.m.	n/a	n/a
Iron-reducing Bacteria				
Crushed Sample	3.11×10^{-1}	< 1.5	1.51×10^{1}	< 3.03
Powdered Sample	n.m.	n.m.	n/a	n/a

n.m. = not measured
n/a = not applicable
***NOTE:** Limestone suspension in LSPW and shale suspension in SSPW were plated on regular R2A (LSPW and SSPW-based R2A would not set) for aerobes and anaerobes in these cases

Table 3: **Enrichment Culture Results (Visible Growth) for Limestone and Shale Samples**

Enrichment Medium	Cobourg Limestone Crushed Sample		Cobourg Limestone Powdered Sample		Queenston Shale Crushed Sample	
	Aerobes	**Anaerobes**	**Aerobes**	**Anaerobes**	**Aerobes**	**Anaerobes**
Distilled Deionized Water	-	-	n/a	n/a	-	-
LSPW	-	-	n/a	n/a	n/a	n/a
SSPW	n/a	n/a	n/a	n/a	-	-
R2A Medium (regular)	+	+	+*	n/p	+*	+*
R2A Medium (1/100 regular)	-	-	n/a	n/a	-	-
R2A Medium, LSPW-based	-	-	n/a	n/a	n/a	n/a
R2A Medium, SSPW-based	n/a	n/a	n/a	n/a	-	-

* = Enrichment culture analyzed by DGGE + sequencing
n/a = not applicable
n/p = not performed

Table 4: **Water Content and Water Activity in Cobourg Limestone and Queenston Shale Samples**

Sample	Water Content (%)	Water Activity (a_w)
Limestone Crushed	0	0.182
Shale Crushed	1.01	0.341

Table 5: **Results of PLFA, NLFA and GLFA Analyses**

	Cobourg Limestone	**Queenston Shale**	**Opalinus Clay***	
Viable Biomass (PLFA) cells/g	0	1.0×10^6	4.9×10^4	1.8×10^5
Dead Biomass (NFLA) cells/g	1.0×10^6	5.3×10^5	Failed	Failed
Dead Biomass (GLFA) cells/g	3.4×10^5	3.7×10^7	Failed	Failed
pmoles PLFA/g	0	50	2.4	9
pmoles NLFA/g	51	26.7	Failed	Failed
pmoles GLFA/g	17	1848.2	Failed	Failed
Community Structure (% of total PLFA)				
Firmicutes	0	1.4	0	0
Proteobacteria	0	65.3	0	11.4
Anaerobic Metal Reducers	0	0	0	0
Actinomycetes	0	0	0	0
General	0	33.3	100	88.6
Eukaryotes	0	0	0	0

***** Data from Stroes-Gascoyne and Hamon (2007)

Table 6: **Sequence Results from DDGE Profile for Aerobic R2A 10-day Old Enrichment Culture of Powdered Cobourg Limestone Sample (Figure 1)**

Band	Similar Genus	Similarity Index	Electron Donors	Electron Acceptors	Description
2.1	*Lysobacter spp.*	0.826	Sulfide, Sulfur	Sulfoxide	Members of the genus Lysobacter have the ability to lyse a variety of microorganisms (e.g., bacteria and fungi) through the action of proteases, chitinases, and other lytic enzymes.
2.1	*Xanthomonadaceae*	0.826		O_2	A family of gram-negative proteo-bacteria that is pathogenic to plants.
2.3	*Pseudomonas spp.*	0.974	Small organics	O_2 (NO_3^-)	Pseudomonas can grow very rapidly to take advantage when carbon and oxygen is made available.
2.4	*Pseudomonas spp.*	0.806	Small organics	O_2 (NO_3^-)	Pseudomonas can grow very rapidly to take advantage when carbon and oxygen is made available.

Table 7: Sequence Results from DGGE Profile for Aerobic R2A 6 Week Old Enrichment Culture of Crushed Queenston Shale Sample (Figure 2 1804 AER)

Band	Similar Genus	Similarity Index	Donors	Acceptors	Description
1.1	*Staphylcoccus*	1.000			Pathogenic, produce slime resulting in biofilm formation
1.2	*Staphylococcus*	0.937			Pathogenic, produce slime resulting in biofilm formation
1.3	*Bacillus spp.*	0.974	organics	O_2 (NO_3^-)	Widespread, aerobic heterotroph found in soil,sporeformer
1.4	*Paenibacillus spp.*	1.000	polysaccharides	O_2	Facultative anaerobe, sporeformer
1.5	*Paenibacillus spp.*	1.000	polysaccharides	O_2	Facultative anaerobe, sporeformer

Table 8: Sequence Result from DGGE Profile for Anaerobic R2A 6 Week Old Enrichment Culture of Crushed Queenston Shale Sample (Figure 2, 1804 AW)

Band	Similar genus	Similarity Index	Donors	Acceptors	Description
2.1	*Staphylcoccus*	0.927			Pathogenic, produce slime resulting in biofilm formation
2.2	*Bacillus spp.*	0.948	organics	O_2 (NO_3^-)	Widespread, aerobic heterotroph found in soil,sporeformer

Excised bands not included in this table did not produce phylogenetic matches.

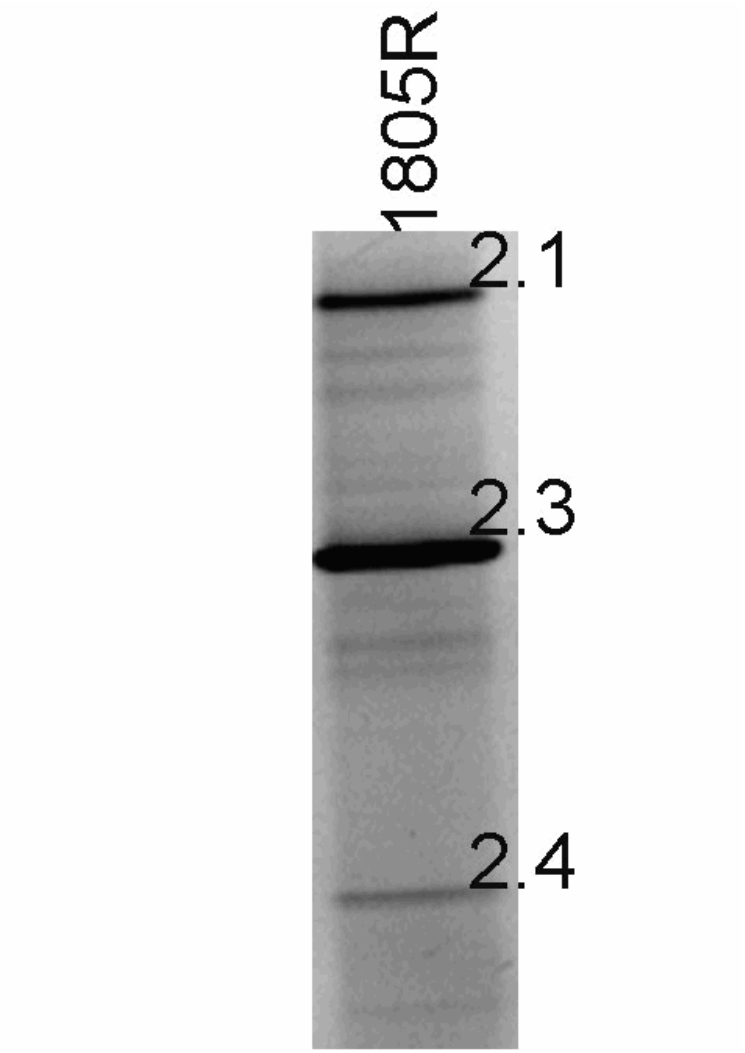

Figure 1: DGGE Gel Band Pattern for 10-day Old Aerobic R2A Enrichment Culture of Powdered Limestone Sample (1805 R)

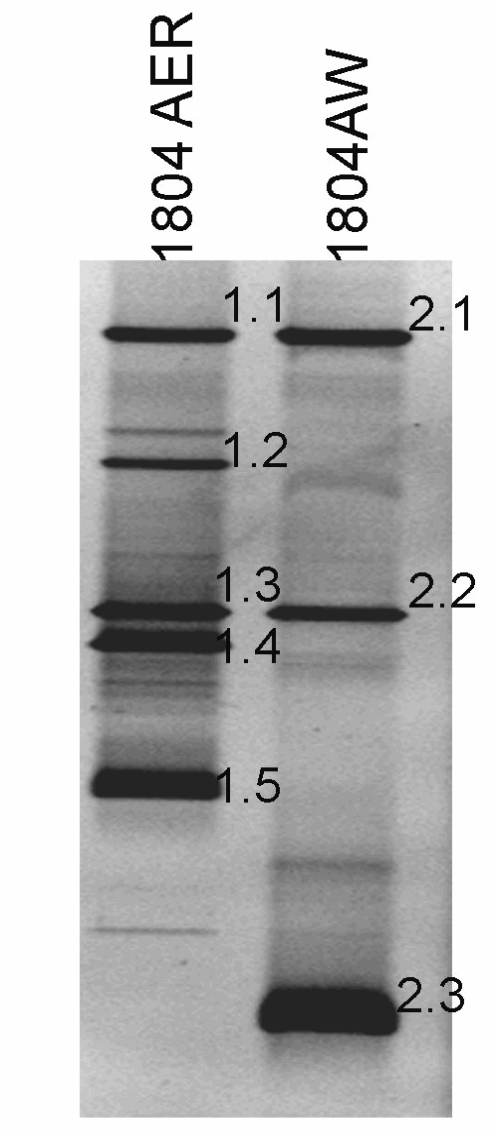

Figure 2: DGGE Gel Band Pattern for 6 Week Old Aerobic (1804 AER) and Anaerobic (1804 AW) R2A Enrichment Cultures of Crushed Shale Core Sample (1804)