The Effects of Elevated Temperatures on the Viability and Culturability of Bacteria Indigenous to Wyoming MX-80 Bentonite

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ABSTRACT

Title: **The Effects of Elevated Temperatures on the Viability and Culturability of Bacteria Indigenous to Wyoming MX-80 Bentonite**
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Abstract

This report describes the results from a two-part study. In the first part, the amount of viable-but-not-culturable (VBNC) cells occurring in saturated compacted Wyoming MX-80 bentonite with a dry density range of 0.8 – 2.0 g/cm^3 (as determined from Phospholipid Fatty Acid (PLFA) measurements), was compared to the amount of culturable cells. It was also investigated if, upon deliberate induction of complete cell death in such bentonite samples (by sterilization at 121^{o}C) the PLFA content decreased accordingly. Results showed large differences between the numbers of VBNC cells and culturable cells in compacted bentonite, especially in samples with high dry density, suggesting that many more viable (but likely inactive) cells than culturable (possibly active) cells were present in these samples. Results also suggested that either the hydrolysis process that degrades PLFA is slow or that biological (enzymatic) activity is needed for PLFA to degrade upon cell death. In case of the latter, PLFA from dead cells may be preserved in environments with low biological activity, such as in highly compacted bentonite with low a_w values and possibly in sedimentary clay-rich environments.

In the second part, the effects of moderately elevated temperature and desiccation (60^{o}C) on the culturability of microorganisms in compacted bentonite were studied, including subsequent recovery of culturability at room temperature. In addition, the effects of higher temperatures (80^{o}C-130^{o}C) on the culturability of microbes in compacted bentonite plugs were investigated. Results showed that the few culturable cells in highly compacted bentonite plugs were not particularly sensitive to a temperature of 60^{o}C and concurrent desiccation. However, the large number of culturable cells in lower dry density bentonite plugs was reduced by up to five orders of magnitude at 60^{o}C. Some culturability remained after exposure to 80^{o}C at all dry densities. At 121^{o}C and 130^{o}C all culturability was below the detection limit for low dry density samples. A very low level of (anaerobic) culturability was observed at high dry density, even after exposure to 130^{o}C. The difference in sensitivity to temperature is thought to be due to the difference in the physiological state of the cells present in the samples (e.g., vegetative cells or spores).The results also showed that the large effects of temperature on culturability in low dry density bentonite were reversible when the heat source was removed and re-saturation was allowed to occur.

The results from the PLFA and temperature studies collectively suggest that microbial cells may remain viable in highly compacted bentonite despite high temperatures, high swelling pressures, desiccation and low water activity. The presence of viable cells implies the potential for increased microbial activity under more favourable conditions in bentonite, such as lower dry density. It is, therefore, important that a high dry density is maintained throughout the bentonite in a repository to keep microbial activity to a minimum. Compliance models can be used to determine the required as-placed dry density of bentonite buffer and gap fillings to achieve specific targets for long-term equilibrium dry densities for various placement room designs.

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1. INTRODUCTION

In the reference design for a deep geological repository, Canada's used nuclear fuel would be encapsulated in corrosion-resistant copper and steel used-fuel containers. These containers would be surrounded by a dense layer of highly compacted 100% bentonite to mechanically support the containers and to retard (by sorption) the transport of any radionuclides if these containers have been breached. One factor that may affect the longevity of the copper and steel containers is the possibility of microbially influenced corrosion (MIC), for instance under biofilms on the metal. Biofilms consist of bacteria embedded in a matrix of exopolymeric substances. In particular, sulphate-reducing bacteria (SRB) can have a detrimental effect on copper materials, because they form sulphides that are corrosive to copper. It has been found that microbial activity is generally depressed in (dense) clay environments (e.g. Fredrickson et al. 1997). Therefore, the compacted bentonite layer can play a role in keeping microbial activity as low as possible near the copper containers, to limit or even prevent significant effects of MIC.

Stroes-Gascoyne et al. (2006, 2010a, 2010b) and Stroes-Gascoyne and Hamon (2008) carried out laboratory experiments with Wyoming MX-80 bentonite plugs to determine to what value of dry density the bentonite layer around the copper containers would have to be compacted to minimize microbial activity in this layer. These bentonite plugs were compacted (at 95% saturation) to a range of dry densities (0.8 – 2.0 g/cm^3), and infiltrated (under pressure) with sterile distilled deionized water or sterile saline solutions of 50 to 200 g NaCl/L (Stroes-Gascoyne et al. 2006, 2010; Stroes-Gascoyne and Hamon 2008) or 50 to 100 g $CaCl_2$/L (Stroes-Gascoyne et al. 2011). During the tests (40 to 90 d), total pressure values were recorded. Upon termination, the bentonite plugs were analyzed for water content, water activity (a_w) and dry density. Swelling pressure values were calculated from total pressure data. Concurrent microbial analyses included culturing for heterotrophic aerobes, anaerobes and sulphate-reducing bacteria (SRB). Average pore size was measured on some samples using mercury intrusion porosimetry (Stroes-Gascoyne et al. 2010).

The physical measurements confirmed that a_w is a function of both dry density and porewater salinity, with the latter becoming the dominant control on a_w at high salinities (> 50 g/L). Swelling pressure values at given porewater salinity were low at dry density values of 0.8 and 1.3 g/cm^3, but increased significantly at dry density > 1.3 g/cm^3. An increase in porewater salinity caused a decrease in swelling pressure for a given dry density. The average pore diameter in bentonite samples with a dry density of 1.6 g/cm^3 appeared to increase slightly with increasing porewater salinity but remained unimodal and in the range of 0.01 to 0.02 µm. At dry densities of 0.8 and 1.3 g/cm^3 and porewater salinities of 0 and 50 g/L, culturability of heterotrophic aerobic bacteria increased by up to four orders of magnitude above back-ground levels in dry, as-supplied bentonite (i.e., (2.1 $\pm$ 0.2) x 10^2 Colony Forming Units (CFU) per g dry bentonite). However, anaerobic heterotrophic bacteria and SRB did not increase significantly above background levels in any of the tests, possibly because the bentonite was stored in air and the experiments were carried out under (initial) aerobic conditions. At higher dry densities (1.6 to 2.0 g/cm^3) and higher porewater salinities (60 to 200 g/L) aerobic and anaerobic culturability remained at, or fell below, the background levels but in all combinations of dry density and porewater salinity tested, some culturability remained. It is likely that these surviving organisms are either (almost) inactive, (nearly) dormant cells, or metabolically inactive spores. With respect to a_w and swelling pressure values, culturability increased exponentially around $a_w \geq 0.96$ but decreased sharply at swelling pressures of $\geq$ 2 MPa. Both a_w and swelling

pressure are influenced by the dry density of the bentonite (Stroes-Gascoyne et al. 2006, 2010a, 2010b; Stroes-Gascoyne and Hamon 2008).

2. CHARACTERIZATION OF MICROORGANISMS OCCURRING IN HIGHLY COMPACTED BENTONITE

The studies by Stroes-Gascoyne et al. (2006, 2010b) were based on culturability. Although in the past culturability was considered equal to viability, it is now well known that not all viable organisms can be cultured. Usually only about 0.01 – 10% of all viable cells in a water or soil sample can be cultured on agar plates or in a liquid medium. Therefore, modern characterization techniques employ extraction, amplification, and sequencing of certain sections of the microbial genome (DNA, RNA) from such samples, followed by identification using an extensive data base. However, it has proven very difficult to extract DNA from clay-rich materials with commercially available kits and methods (e.g., Stroes-Gascoyne et al. 2007; Mauclaire et al. 2007). Therefore, to characterize the microbial populations in clay deposits and in bentonite materials, culture methods have to be relied upon. These include enrichment cultures in which samples with low populations are exposed to dilute nutrients for a long time in order to give dormant organisms sufficient time to reactivate.

Another strategy is to find certain extractable biomarkers that can be used to characterize the microbial community present in clay-rich materials. Biomarkers that appear easily extractable from clay-rich materials are phospholipid fatty acids (PLFA). The determination of the total ester-linked PLFA content of a sample provides a quantitative measure of the viable or potentially viable biomass in that sample (White and Ringelberg 1997). The premise is that a viable organism will have an intact membrane containing PLFA. Upon cell death or cell lysis, cellular enzymes hydrolyse phospholipids, releasing the polar head groups. This hydrolysis can occur within minutes or hours after cell lysis. Therefore, PLFA have a high natural turnover rate and are rapidly degraded in non-viable cells (White and Ringelberg 1997). Also, a careful study of subsurface sediments has shown that the viable biomass, as determined by PLFA extraction and analysis, was equivalent (with a small standard deviation) to estimates based on cellular adenosine tri-phosphate (ATP, the energy units of microbial metabolism) content, cell wall muramic acid, and carefully performed acridine orange direct cell counts (White and Ringelberg 1997). Figure 1 shows the signature lipid biomarkers in microbial cells and Figure 2 shows the conversion of a phospholipid to a diglyceride (from White and Ringelberg 1997).

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 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).

3. TERMINOLOGY AND SURVIVAL STRATEGIES OF MICROORGANISMS IN NATURAL ENVIRONMENTS

Microbes can occur in a number of different physiological states in the environment. Definitions used in this report are:

Viable Cells: Cells that are alive and have intact cell walls. Viable cells include vegetative cells, culturable cells, viable-but-not-culturable (VBNC) cells, dormant cells and spores. Phospholipid fatty acids form part of the intact membrane of a viable cell, and the PLFA hydrolyse quickly upon cell death.

Vegetative Cells: Viable cells with a metabolism that allows cell growth and division (excludes spores and dormant cells). Vegetative cells may or may not be culturable.

Culturable Cells: Those cells that can be cultured on, or in, a growth medium. These are, therefore, viable, culturable cells. Culturable cells have a metabolism (i.e., are vegetative), unless they are spores. Usually only a small portion (0.01 – 10%) of viable cells (in soil or water samples) are culturable on a growth medium. Most viable cells are, therefore, viable but not culturable (VBNC). Viable cells are presumed to be metabolically active unless they are spores. If a culturable cell turns into a VBNC cell it is thought to have a much lower metabolism than its preceding culturable cell.

Viable-But-Not-Culturable (VBNC) Cells: These are viable cells that cannot be cultured, but may maintain (possible very low) metabolic activity. Under deteriorating environmental conditions, a culturable cell may become a VBNC cell analogous to the stress response of differentiating (i.e., spore-forming) bacteria. As a natural progression VBNC cells will then ultimately become fully dormant cells (Mc Dougald et al. 1998).

According to McDougald et al. (1998), cells entering the VBNC state undergo certain definite and predictable changes, which allow these cells to persist in the environment for extended periods of time. These changes include stabilization of the cell wall and membrane, which increases the stability of the cell. Cells entering the VBNC state appear to maintain gross membrane integrity although changes in membrane composition have been reported. VBNC cells may maintain a normal cytoplasmatic membrane, but with a decrease of up to 60% of the major fatty acids compared to culturable cells, and the concurrent appearance of more long-chain fatty acids. Morphology is maintained, including an intact nucleoid and structured cytoplasm, but with a thickening of the cell wall accompanied by loss of permeability by the cytoplasmic membrane. McDougald et al. (1998) propose that this loss of permeability is one of the reasons for loss of culturability.

Dormant Cells: These cells are still viable but likely have no (or a barely distinguishable) metabolism. Dormancy is a surviving mode of some microbes, but different from spores, because it is the microbial cell itself that becomes dormant (i.e., by becoming smaller in volume, dehydrated, with a thickened cell wall and consolidated genomic material). Dormant cells are very resilient. It is possible that dormant cells sustain a very low level of metabolism that allows them to repair damage to their genome, but if this is the case, the metabolic level is not enough for cell growth and division. Dormant cells remain viable (unless they sustain so much damage that it cannot be repaired), but are non-vegetative and not easily culturable unless given ample time in enrichment cultures. Dormancy is probably the end-stage of the VBNC state (before actual cell death), but may still be reversible to a culturable state.

Spores: Spores are a surviving mode of some microbes. A spore is a separate structure, made by vegetative cells upon induction by unfavourable circumstances. Spores are fully dormant cells, with no metabolism (i.e., non-vegetative). Spores are very resilient but can be damaged over time, for instance by naturally occurring radiation, because they cannot repair any damage to their genome. Non-damaged spores remain viable, can be revived to a vegetative state (i.e., cells having a metabolism) and may or may not be culturable (under the right circumstance). Often a special pre-treatment is required to turn spores into vegetative cells.

Dead Cells: Dead cells are cells that have died as a result of sustaining too much damage to any part (including the genome) of their cell components to be repaired. Once a cell is dead, the phospholipid fatty acids (PLFA) in the cell wall hydrolyse and are no longer extractable as PLFA.

Microbes can adapt to a number of negative parameters, including higher temperatures (i.e., thermophiles), radiation (e.g., *Micrococcus radiodurans*), low nutrient conditions (i.e., oligotrophs) and high salt conditions (i.e., halophiles). However, in order for microbes to adapt to, or survive in, unfavourable environments, they must have an active metabolism, to be able to repair any damage done to their genetic material (DNA and RNA) by the negative effects of such factors as temperature, radiation and salinity. Fully dormant organisms or inactive spores do not have that ability, and although such life forms are much more tolerant and resistant to unfavourable conditions (because of their smaller size, thicker cell walls and condensed genetic material), ultimately the damage will be too extensive and the organisms, even in resistant or spore form, will lose viability and die. This may be a very long process, depending on the severity of the conditions, and could possibly have geological time scales in natural environments. For instance, Johnson et al. (2007) state that knowledge of the mechanisms behind long-term cell survival is limited and that it remains unclear how dormancy, a favoured explanation for extended cellular persistence, can cope with spontaneous genomic decay over geological timescales. It is currently not known what level of metabolism is required to maintain functioning repair mechanisms in microbes. However, in their research on long-term survival of bacteria in undisturbed permafrost cores, Johnson et al. (2007) state that they have found strong evidence that such long-term survival is in fact closely tied to low-level cellular metabolic activity and DNA repair that, over time, proves to be superior to full dormancy as a mechanism in sustaining bacterial viability (in frozen, sealed conditions) for up to half a million years. Their data indicate that, despite short-term robustness, fully dormant bacteria are unlikely to be the most persistent cells over time scales of thousands of years in these cold conditions. Instead, bacteria with an active DNA repair mechanism are most likely to persevere.

4. FOCUS OF TEMPERATURE STUDIES

A factor not taken into account in the studies by Stroes-Gascoyne et al. (2006, 2010a, 2010b) thus far is temperature. Although temperatures in bentonite-based materials around containers in a future Canadian repository likely would not reach $\geq 100^{o}$C, and the limit of life is currently thought to be around 125-130^{o}C for microorganisms, it is expected that increased temperatures in the compacted bentonite buffer in a repository would have a further negative effect on any surviving organisms, in addition to the effects of high dry density, low a_w and high porewater salinity.

Chi Fru and Arthar (2008) studied the bacterial communities in compacted Wyoming MX-80 bentonite samples (at a density of 2.0 g/cm^3 or a dry density of 1.6 g/cm^3) from the Long-Term Test (LOT) and the Canister Retrieval Test (CRT) at the Äspö Underground Hard Rock Laboratory in Sweden. Temperatures in the LOT and CRT tests ran as high as 150^{o}C. They carried out direct DNA extractions from the bentonite (one of the few successful DNA extractions from bentonite reported at present to our knowledge), grew heterotrophic aerophilic cultures on agar plates and performed enrichment cultures for SRB and acetogens in suitable growth media. Results suggested that the high temperatures in these tests rather than the high dry density controlled actual microbial survival in bentonite. It appeared that the bacterial population in compacted bentonite after five years of granitic groundwater ingress consisted largely of Gram positive spore-formers while Gram negative bacteria, dominant in the groundwater, were in complete absence in compacted bentonite samples that had experienced ≥ 67^{o}C. In addition, SRB, which are widespread in the Swedish granitic groundwater, were not prominent (although viable) in the compacted bentonite, an important finding for MIC of copper waste containers.

After fusion at room temperature, the number of viable cells (from PLFA measurements) was determined and compared to the number of culturable cells in compacted bentonite in the dry density range of 0.8 – 1.8 g/cm^3. Additionally, experiments were carried out to investigate if induction of complete cell death in bentonite samples (by a standard sterilization method at 121^{o}C) would be reflected in the PLFA content (i.e., also decreased drastically).

Since non-uniformly elevated temperatures will be an integral part of a future repository, the effects of moderately elevated temperature (60^{o}C) on the culturability of microorganisms in compacted bentonite were studied. These studies included the temperature-induced desiccation effects on culturability and the subsequent possible restoration or recovery of culturability at room temperature. Since temperatures in a repository could reach close to 100^{o}C and in some international repository designs even higher (e.g., up to 130^{o}C in Switzerland), experiments to assess the temperature sensitivity of microbes in compacted bentonite plugs in the higher temperature ranges (80^{o}C-130^{o}C) were also carried out. These experiments included determining the effects of the physiological state of the microbes in bentonite (i.e., vegetative, culturable, VBNC, or spores) on the extent of loss of culturability upon a temperature increase in the range of 80^{o}C to 130^{o}C.

5. MATERIALS AND METHODS

All experiments were carried out with compacted bentonite plugs, prepared according to a standard procedure:

- The bentonite used was Wyoming MX-80 bentonite.
- The bentonite was compacted into ethanol-sterilized pressure cells to a target dry density between 0.8 and 2.0 g/cm^3.
- The bentonite plugs were either about 2.0 cm high with a diameter of 3.2 cm (small pressure cells) or about 2.3 cm high with a diameter of 5 cm (large pressure cells).

- Before compaction, the bentonite was mixed with the infiltration solution such that, after compaction, the bentonite would be at about 95% saturation. The infiltration solution was sterilized, distilled deionized water.

- During the experiments, the plugs were infiltrated under pressure to saturation with the infiltration solution.

- Total pressures were recorded during the experiment from which swelling pressures could be calculated.

The infiltration solution used is referred to as infiltration water or porewater in this report, although the latter term is not strictly correct. The bentonite porewater may be somewhat different from the infiltration water, because of the possible presence of various soluble salts in the as-supplied dry bentonite.

After saturation and termination, the plugs were extruded onto sterilized foil, wrapped and taken to the laboratory for immediate analysis:

- The plugs (or divided plugs) were weighed and measured to determine actual dry densities.

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

- Water content was determined by subsequently drying this subsample in an oven at 110^{o}C to constant weight.

- Aerobic and anaerobic heterotrophic bacteria were cultured on R2A medium (Reasoner and Geldreich 1985).

- Sulphate-reducing bacteria (SRB) were cultured on modified Postgate B medium (Atlas 1993).

- For experiments focused on PLFA measurements, the PLFA extraction procedure employed by Microbial Insights (described by White and Ringelberg (1997)) involved extraction of PLFA in organic solvents, followed by analysis using capillary gas chromatography and mass spectrometry (GC/MS).

- Swelling pressures were calculated from total pressures recorded during the experiment with a correction for input pressure.

5.1 EXPERIMENT 1: PHOSPHOLIPID FATTY ACID (PLFA) MEASUREMENTS IN COMPACTED MX-80 BENTONITE INFUSED AT ROOM TEMPERATURE

The purpose of this experiment was to measure viable cell numbers based on PLFA analysis and compare these with culturable cell numbers in compacted saturated bentonite plugs. Bentonite plugs, compacted to target dry densities of 0.8 g/cm^3 (sample 1729), 1.3 g/cm^3 (sample 1730), 1.6 g/cm^3 (sample 1731) and 1.8 g/cm^3 (sample 1732) were prepared in the large pressure cells because of larger material requirements for PLFA analysis. The plugs were infused at room temperature under pressure with sterile distilled deionized water for

approximately 6 weeks (44 days). After termination of the experiment, the plugs were extruded onto sterile foil, sub-sampled for culturing, and subsequently wrapped tightly in sterile foil and plastic and frozen. These frozen plugs, together with a dry, as-supplied bentonite sample (1733), were sent (in a cooler on icepacks) to Microbial Insights (MI), Rockport, Tennessee, for PLFA analysis. The sub-samples were analyzed for water content, water activity ($_w$) and dry density as well as cultured for heterotrophic aerobes, anaerobes and SRB.

5.2 EXPERIMENT 2: PERSISTENCE OF PLFA AFTER CELL DEATH IN COMPACTED SATURATED LOW DRY DENSITY BENTONITE

The objective of this experiment was to investigate if, upon deliberately causing complete cell death in bentonite samples by a standard sterilization method at 121^{o}C in an autoclave, the PLFA content also decreases significantly. Two clay plugs (1834, 1835) were prepared in the large pressure cells because of the larger material requirements for PLFA analysis. A low target dry density of 1.0 g/cm^3 was chosen because at such a low dry density at least a relatively large percentage of the viable microbial cells in the bentonite plugs are likely in a vegetative and culturable state. After termination of the infusion with distilled deionized water, a small sub-sample of one plug (1834) was analysed immediately for water content, a_w, dry density, heterotrophic aerobes, anaerobes and SRB. The remaining portion of this plug (1834) was wrapped in sterile foil and plastic and frozen immediately for PLFA analysis. The second plug (1835) was wrapped in foil, placed in an autoclave pouch and autoclaved at 121^{o}C for one hour. After removal of this plug from the autoclave, it was left to cool for about 24 h at room temperature to give any surviving microbes chance to revive and to give potential PLFA hydrolysis reactions time to complete. The plug (1835) subsequently was analyzed as per the first plug (1834). The bentonite samples were shipped to MI for PLEA analysis by overnight courier in coolers containing ice packs.

5.3 EXPERIMENT 3: PERSISTENCE OF PLFA UPON CELL DEATH IN DRY AS-SUPPLIED BENTONITE

The purpose of this experiment was to determine viability (by PLFA analysis) and culturability in dry as-supplied non-compacted Wyoming bentonite (1836) before and after autoclaving (at 121^{o}C) for 30 minutes and subsequent recovery at room temperature for 24 h. It is likely that these dry as-supplied bentonite samples contained largely spores and/or dormant organisms (at a level of about 220 culturable cells/g; Stroes-Gascoyne et al. 2006) that would be less sensitive to high temperatures than vegetative cells. The pre- and post autoclave bentonite samples (1836 and 1836c) were analyzed as for experiment 1 (except for anaerobes), while part of the autoclaved sample (1836c) was frozen and shipped to MI for PLFA analysis. As-supplied bentonite was analyzed previously for PLFA content (sample 1733, experiment 1, Section 5.1)

5.4 EXPERIMENT 4: PERSISTENCE OF PLFA UPON CELL DEATH IN A LIQUID ENRICHMENT CULTURE OF BENTONITE

The purpose of this experiment was to determine the persistence of PLFA in liquid enrichment cultures of bentonite before and after autoclaving. An enrichment culture of 1 g dry as-supplied Wyoming bentonite in 100 mL liquid R2A medium was prepared, in order to obtain an

abundance of vegetative and culturable cells. The enrichment culture was performed in duplicate and after eight days of incubation at room temperature, one culture (1836a) was analyzed for culturable aerobes (on R2A plates). The second culture (1836b) was autoclaved for 30 minutes (at 121°C), left to cool for 24 h at room temperature and then also analyzed for culturable aerobes (on R2A plates). This autoclaved liquid enrichment culture (1836b) was sent to MI for PLFA analysis. For the non-autoclaved enrichment (1836a), no PLFA analysis was obtained. The PLFA-based biomass in this non-autoclaved sample was assumed to be at least as large as the amount of culturable cells found on the R2A plates.

5.5 EXPERIMENT 5: HEATING OF COMPACTED BENTONITE PLUGS TO 60°C AFTER INFUSION AND EQUILIBRATION AT ROOM TEMPERATURE

The purpose of this experiment was to determine the effect of heating to 60°C on culturable cells in bentonite plugs compacted to a variety of dry densities. Five bentonite plugs (1718, 1719, 1726, 1727 and 1728) were prepared in the small pressure cells to target dry densities of 0.8, 1.3, 1.6, 1.8 and 2.0 g/cm^3 and infused with sterile distilled deionized water at for 77 to 79 days at room temperature. Upon termination of these experiments, the plugs were extruded onto sterile foil and divided in half. One half of each plug (1718, 1719, 1726, 1727 and 1728) was analyzed for water content, a_w, dry density, heterotrophic aerobes, anaerobes and SRB. The other half of each plug (1718-60, 1719-60, 1726-60, 1727-60 and 1728-60) was wrapped tightly in sterile foil and plastic (to reduce water loss as much as possible) and heated for 8 days in an oven at 60°C, prior to analysis for water content, a_w, dry density, heterotrophic aerobes, anaerobes and SRB.

5.6 EXPERIMENT 6: INFUSION OF COMPACTED BENTONITE PLUGS AT 60°C

The purpose of this experiment was to determine microbial culturability in compacted bentonite plugs infused with sterile distilled deionized water at 60°C. Bentonite plugs with target dry densities of 0.8, 1.3, 1.6, 1.8 and 2.0 g/cm^3 (1736, 1734, 1738, 1735 and 1737) were prepared in the small pressure cells and infused with sterile distilled deionized water at 60°C (in a water bath) for 51 or 52 days, and analyzed subsequently for water content, a_w, dry density, heterotrophic aerobes, anaerobes and SRB.

5.7 EXPERIMENT 7: INFUSION OF BENTONITE PLUGS AT 60°C WITH SUBSEQUENT RECOVERY DURING INFUSION AT ROOM TEMPERATURE

The purpose of this experiment was to determine the recovery of culturable cells in compacted bentonite plugs infused at room temperature, after initial infusion at 60°C. A low dry density of 1.0 g/cm^3 was chosen to avoid the negative effects of dry density on culturability. Two bentonite plugs (1750 and 1778) with a target dry density of 1.0 g/cm^3 were prepared in the large pressure cells and infused with distilled deionized water at 60°C (in a water bath) for 40 days, after which plug 1750 was terminated, sampled (i.e., by taking scrapings from the exterior of the plug, and by taking material from the top and bottom sections of the plug) and analyzed for water content, a_w, dry density, heterotrophic aerobes, anaerobes and SRB. The infusion of plug 1778 was continued for 76 days, but now at room temperature, to determine if any negative temperature effects on the culturability in plug 1750 subsequently could be reversed at room temperature. Upon termination, samples from the top, middle and bottom section of this

plug were analyzed for water content, a_w, dry density, heterotrophic aerobes, anaerobes and SRB.

5.8 EXPERIMENT 8: DESICCATION OF COMPACTED BENTONITE PLUGS AT 60°C FOLLOWED BY SUBSEQUENT REHYDRATION THROUGH INFUSION AT ROOM TEMPERATURE

The purpose of this experiment was to determine the effects of heat (60°C) and desiccation (at 60°C to constant weight) on the culturability in bentonite clay plugs with target dry densities of 0.8, 1.0 and 1.2 g/cm^3. Low densities were chosen to determine the effects of temperature and desiccation only, without the added detrimental effect of high dry density on culturability. Subsequently, the possibility of recovery of culturability of microbes after the heat- and desiccation treatment was studied in this experiment. This was accomplished by rehydration of the dried-out bentonite plugs. Such a process would occur slowly in a repository as temperatures which initially could be as high as about 95°C in the compacted bentonite near the containers, driving water away from the containers, would moderate to levels that allow water to return and re-saturate the bentonite near the containers.

A total of 12 plugs were prepared in the small pressure cells at 95% saturation, four each with target dry densities of 0.8, 1.0 and 1.2 g/cm^3. One plug of each density (1768, 1767 and 1766) was analyzed immediately after preparation (< 24h) for water content, a_w, dry density, heterotrophic aerobes, anaerobes and SRB. The second plug of each density (1771, 1770, 1769) was wrapped in plastic, placed in a Teflon container and stored at room temperature for 65 days followed by analysis for water content, a_w, dry density, heterotrophic aerobes, anaerobes and SRB. The remaining two plugs of each dry density were placed in an oven at 60°C and dried to constant weight for 20 days. The top (T) and bottom (B) parts of one dried plug of each density were analyzed subsequently (1774 T, B; 1775 T, B; 1776 T, B) and the second dried plug of each dry density was rehydrated by infusion (under pressure) in small pressure cells with sterile distilled deionized water at room temperature for 40 days. After termination of these rehydrated plugs, samples from top (T) and bottom (B) parts of these plugs were analyzed (1779 T, B; 1780 T, B; 1781 T, B) for water content, a_w, dry density, heterotrophic aerobes, anaerobes and SRB.

5.9 EXPERIMENT 9: HIGH TEMPERATURE SURVIVAL OF CULTURABLE CELLS IN COMPACTED BENTONITE PLUGS

The objective of this experiment was to investigate what effect the physiological state of the microbes in compacted bentonite (i.e., vegetative, dormant or spores) may exert on the extent of loss of culturability upon a temperature increase in the range of 80°C to 130°C; and to establish at which temperature all culturability ceases in compacted bentonite plugs.

For this experiment, a total of eight compacted bentonite plugs were prepared with target dry densities of 0.8 g/cm^3 (1863, 1864, 1865 and 1866) and 1.6 g/cm^3 (1859, 1860, 1861, 1862) in the small pressure cells. The eight plugs were saturated for 27 days, at ambient laboratory temperature with distilled deionized water. After termination of the tests, the bentonite plugs were extruded onto, and wrapped in, sterilized foil and plastic, and taken immediately to the laboratory, where all plugs were measured and weighed. Two plugs (i.e., plugs 1863 and 1859,

one of each dry density) were analyzed immediately for water content, a_w, dry density, heterotrophic aerobes, anaerobes and SRB. The other six plugs were placed (wrapped tightly in sterile foil) in small Teflon jars with a tight screw cap, and placed in an oven at 80°C for two weeks. After two weeks at 80°C, two plugs (i.e., plugs 1864 and 1860, one of each dry density) were analyzed for water content, a_w, dry density, heterotrophic aerobes, anaerobes and SRB. The remaining four plugs were left in the oven, which was turned up to 121°C. After two weeks at 121°C, two plugs (i.e., plugs 1865 and 1861, one of each dry density) were analyzed again for water content, a_w, dry density, heterotrophic aerobes, anaerobes and SRB. The two remaining plugs (1866 and 1862) were then subjected to 130°C in the oven for one further week, after which they were analyzed for water content, a_w, dry density, heterotrophic aerobes, anaerobes and SRB.

6. RESULTS

6.1 EXPERIMENT 1

In this experiment viability was assessed, on the basis of PLFA measurements, in as-supplied dry bentonite and in compacted bentonite plugs infused with distilled deionised water. The PLFA-derived biomass, i.e., equivalent cell numbers/g in plugs 1729 (0.8 g/cm^3), 1730 (1.3 g/cm^3), 1731 (1.6 g/cm^3) and 1732 (1.8 g/cm^3), as well as in sample 1733 (dry as-supplied bentonite) is given in Table 1. The results of the measurements of water content, a_w, and culturability of heterotrophic aerobes, anaerobes and SRB in these plugs are also included in Table 1. Figure 3 shows a comparison between culturable organisms and PLFA-derived viable organisms in the plugs, and suggests that an increase in dry density reduces culturability by up to four orders of magnitude but that viability (based on PLFA equivalents) is affected at most by a factor of 1.5 to 2. This suggests that, although culturability (and by deduction activity) is reduced strongly by high dry density, the actual number of viable cells and, therefore, the potential for increased bacterial activity (e.g., if dry density is reduced) is not affected significantly in the compacted samples.

Details about the microbial community structure are given in Figure 4 and Table 2. Table 3 gives details of the PLFA-based structural groups. Proteobacteria (37 - 41%) and anaerobic metal reducers (1.2 - 1.3%) are similarly represented in all samples. Proteobacteria (Gram-negative bacteria) are typically fast growing, utilize many carbon sources and adapt quickly to a variety of environments. The fact that the percentage of this group is similar in all samples indicates not much shift in the status of gram-negative (more sensitive) organisms in any of these samples. Anaerobic metal reducers include micro-aerophiles and anaerobes such as sulphate- and iron-reducing bacteria. The fact that they are present in low and similar percentages in all samples suggests that the environment in the bentonite plugs was not very suitable (i.e., too aerobic) for metal and sulphate reduction. Despite these similarities, several subtle shifts occurred in the community structure in these clay plugs. Eukaryotes form a considerably larger percentage (8.5%) of the total population in the dry as-supplied bentonite sample (1733) than in the compacted, saturated samples (1.1 – 3.6%), with the lowest percentage occurring in sample 1729 with a dry density of 0.8 g/cm^3. Concurrently, sample 1729 (0.8 g/cm^3) has the highest population of culturable organisms as well as PLFA-derived biomass and shows an observable increase in the percentage of both Firmicutes (Gram-positive bacteria and anaerobic Gram-negative fermenting bacteria) and SRB/Actinomycetes, compared to the other compacted samples and the dry as-supplied bentonite. Such increases

in sample 1729 suggest the development of a more anaerobic (fermentative) environment in the low dry density (0.8 g/cm^3) bentonite plug. This is likely caused by the much larger culturable (and hence probably active) aerobic population in this sample, which would decrease the amount of O_2 present, thereby increasing the suitability of the environment for more anaerobic organisms such as Firmicutes and SRB/Actinomycetes (Table 2). These increases occurred at the expense of the percentage of general fatty acids (Nsats; Table 2 and 3), which occur in all organisms. A reduction in this percentage suggests the development of a more diverse population in sample 1729, compared to the other compacted and as-supplied bentonite samples. The higher dry density samples (1730, 1731 and 1732) show similar percentages for Firmicutes, SRB/Actimnomycetes, general Nsats and Eukaryotes that are comparable to the dry as-supplied bentonite, suggesting that no significant microbial activity occurred in these samples. This is corroborated by the much lower culturability in these samples.

The membrane of a microbe adapts to changing conditions, and these changes are reflected in the PLFA composition. Environmental conditions (such as desiccation or toxicity) may disrupt the membrane and some bacteria respond by making *trans* fatty acids instead of the usual *cis* fat acids, in order to strengthen the cell membrane, making it less permeable. The physiological status of the Proteobacteria (Gram-negative) in the clay samples is indicated by the Slowed Growth and Decreased Permeability ratios in Table 2. These ratios are determined by division of the amount of fatty acid induced by environmental conditions by the amount of its biosynthetic precursor. A marked increase in either of these ratios suggests a change in environment, to less favorable conditions for Proteobacteria. An increase in the Slowed Growth ratio suggests a change in conditions that are not supportive of rapid (healthy) growth, often due to reduced available substrate. An increased ratio for Decreased Permeability suggests that the environment has become more toxic or otherwise unsuitable. The Slowed Growth ratios in Table 2 are similar for all bentonite samples, indicating similar growth conditions for Proteobacteria in all samples. This is corroborated by the fact that the percentage of Proteobacteria in all samples is similar. The Decreased Permeability ratio is markedly larger in the dry as-supplied bentonite, likely reflecting the lack of water in this sample as corroborated by a very low value for a_w (Table 1).

6.2 EXPERIMENTS 2, 3 AND 4

Persistence of PLFA after cell death in compacted low dry density saturated bentonite (Experiment 2), in dry as-supplied bentonite (experiment 3) and in liquid enrichment cultures of bentonite (experiment 4) are shown in Table 4 and Figure 5. It is immediately obvious that autoclaving reduces aerobic culturability to zero in all samples (no colonies on plates). Therefore, depending on the sample, culturability dropped by two to more than eight orders of magnitude, while PLFA levels decreased by less than one order of magnitude (i.e., only about a factor of two to six).

These results suggest that although culturable cells completely disappear (as expected) upon autoclaving, PLFA persists at high levels, even after a “resting” period of 24 h. This suggests either that the resting period of 24 h was insufficient for complete hydrolysis of the polar head groups of the PLFA (Figure 2), or alternatively that active microbial cells or enzyme activity are needed to degrade the PLFA of dead cells. Such enzymes are inactivated upon autoclaving. Therefore, the premise that finding PLFA in a sample implies that this sample contains viable cells with intact cell walls, may perhaps be valid only for samples from a biologically active

environment. If enzymatic activity is needed to hydrolyse the phospholipids (as discussed in the introduction) and this enzyme activity ceases abruptly (as is the case upon autoclave treatment), such hydrolysis does not appear to occur (at least not rapidly), implying that PLFA from dead cells could possibly be preserved in biologically inactive environments, although it is not clear for how long.

6.3 EXPERIMENT 5

In this experiment, bentonite plugs were infused at room temperature with distilled deionised water, followed by heat treatment at 60°C. Results for culturable aerobes are given in Table 5 and Figure 6. The 0.8 g/cm^3 plug (1718) contained almost 10^7 CFU (aerobes)/g bentonite after 77 days but showed a 10^5 drop in CFU aerobes/g after eight days at 60°C (in an oven). Water loss in this plug during heating was 8.6%, leaving the water content still high enough to support a large population. Therefore, the large drop in culturability is likely an indication of the sensitivity to elevated temperature of the revived, culturable and possibly actively growing, vegetative cells in the 0.8 g/cm^3 plug. The aerobic culturability in the 1.3 g/cm^3 plug was about 10^4 CFU/g after 77 days and this level dropped very little (about 25%) after 8 days at 60°C. This result is unexpected and not easy to explain. The much lower aerobic culturability in the 1.6 g/cm^3 and 1.8 g/cm^3 plugs dropped by a factor of 5 to 6 upon heat treatment while the culturable population of aerobes in the 2.0 g/cm^3 sample remained essentially unchanged. This is likely an indication of the insensitivity of spores or virtually dormant cells to treatment at 60°C. These results suggest that the sensitivity to temperature between revived vegetative (possibly actively growing) cells, more or less dormant cells and spores can be very different.

6.4 EXPERIMENT 6

In this experiment compacted bentonite plugs were infused with distilled deionised water at 60°C (in a water bath). Results are given in Table 5 and shown in Figure 7, where they are compared with the results from experiment 5 (Section 6.3), i.e., infusion at room temperature and subsequent heating at 60°C. For a dry density of 0.8 g/cm^3, infusion at 60°C results in an aerobic culturability of almost five orders of magnitude lower than the culturability observed for infusion at room temperature. For the 1.3 g/cm^3 sample, a drop of about three orders of magnitude in aerobic culturability is observed. For the 1.6 g/cm^3 sample, infusion at 60°C results in an aerobic culturability of one order of magnitude lower than in the room-temperature-infused sample of the same dry density and for 1.8 g/cm^3 and 2.0 g/cm^3 samples, no change is observed. These results are similar to the results from experiment 5 (also shown in Figure 7) in which room-temperature-infused plugs were subsequently heat treated at 60°C (in an oven). The revived, vegetative cells in 0.8 g/cm^3 and 1.3 g/cm^3 bentonite plugs appear sensitive to the increase in temperature and are most affected. In the 1.6 g/cm^3 sample, there are possibly some vegetative cells, which are sensitive, hence, the drop of an order of magnitude in aerobic culturability. Materials prepared at 1.8 g/cm^3 and 2.0 g/cm^3 contain so little water that revival from the dry as-supplied bentonite to a vegetative state likely does not occur, either pre- or post-compaction. The organisms in these samples remain in almost dormant- or spore form and do not appear to be sensitive to further compaction and elevated temperature (i.e., 60°C).

The swelling pressures (Table 5) for the samples infused at 60°C are in some instances a little lower than for the samples infused at room temperature (depending on the actually measured dry density in each test). This is likely caused by a slight expansion of the metal pressure cells at 60°C.

6.5 EXPERIMENT 7

In this experiment bentonite plugs with a dry density of 1.0 g/cm^3 were infused with distilled deionized water at 60^{o}C (in a water bath) with subsequent recovery during continued infusion at room temperature. The results are given in Table 6 and shown in Figure 8. Infusion at 60^{o}C for 40 days (sample 1750) has reduced aerobic culturability to about 10 CFU/g in all locations of the plug, i.e., the exterior surface and the top and bottom sections of the plug. This is similar to results for the 60^{o}C infused sample with a dry density of 1.3 g/cm^3 in Figure 7 and Table 5. Anaerobic culturability is essentially below detection limit in the 1750 plug (Table 6). The duplicate plug (1778) in this experiment was allowed to recover at room temperature under continued infusion with sterile distilled deionized water for a further 76 days. Results in Table 6 and Figure 8 show a recovery to a culturability of 10^5 CFU aerobes/g for the bottom part of the plug where infusion took place, and a recovery of culturability to 5 x 10^2 CFU aerobes/g in the top and middle of the plug. It is not clear why the top and middle of this plug had much lower aerobic culturability than the bottom part. Infusion with water occurs at the bottom of the plug, but water content and a_w values in the various parts of plug 1778 were quite uniform as shown in Table 6.

6.6 EXPERIMENT 8

In this experiment the effect of desiccation at 60^{o}C (in an oven) on the microbial culturability in compacted bentonite plugs with target dry densities of 0.8, 1.0 and 1.2 g/cm^3, followed by subsequent rehydration through infusion with distilled deionised water at room temperature, was studied. Results from this experiment are compiled in Table 7 and shown in Figure 9 (heterotrophic aerobes) and show that aerobic culturability in as-compacted, 95% saturated clay plugs (i.e., analyzed within 24 h after compaction) does not increase significantly in any of the three dry densities (0.8, 1.0 and 1.2 g/cm^3) employed, compared to the aerobic culturability in dry as-supplied bentonite. However, Figure 9 and Table 7 also show that aerobic culturability in duplicate plugs that were stored (wrapped in plastic and placed in Teflon containers) at room temperature for 65 days increased by up to five orders of magnitude to values of 10^6 to 10^7 CFU/g (although some water loss occurred, 5 – 10%). This is similar to the result for plug 1718 (dry density 0.8 g/cm^3) infused with distilled deionized water at room temperature for 77 days in Table 5 (Figure 6).

Figure 9 further suggests that heating and concurrent desiccation at 60^{o}C does not alter aerobic culturability significantly compared to the culturability in as-compacted plugs (< 24 h) or dry as-supplied bentonite, suggesting that the culturable aerobic population initially present consists of resistant organisms (i.e., spores or dormant cells).

Rehydration at room temperature of the heated and desiccated plugs (by infusion with sterile distilled deionized water for 40 days) increases the culturable aerobic population to between 10^5 and 10^6 CFU/g for all three densities (Figure 9), about an order of magnitude (or less) lower than in the samples stored at room temperature (65 d), suggesting that the heat and desiccation at 60^{o}C has not affected the dormant cells and/or spores initially present significantly because most of them recovered to culturable status, as in the plugs stored for 65 days at room temperature.

Anaerobic culturability (Table 7) in all samples was within half an order of magnitude of the background anaerobic culturability in dry as-supplied bentonite, except in two samples from which nothing could be grown. This is likely due to the aerobic character of the experiments and surviving anaerobes are likely facultative anaerobes or spore-forming organisms. SRB levels (Table 7) also did not differ significantly from background levels in dry as-supplied bentonite, presumably also because the clay plugs were aerobic and not conducive to a significant increase in anaerobic culturability.

6.7 EXPERIMENT 9

This experiment investigated the effect of the physiological state of the microbes in compacted bentonite (with target dry densities of 0.8 and 1.6 g/cm^3, containing vegetative cells or mostly dormant cells or spores, respectively) on the extent of culturability decrease upon a temperature increase in the range of 80^{o}C to 130^{o}C. Results are given in Figure 10 and Table 8 and suggest that heating for two weeks at 80^{o}C (in an oven) drastically reduced both aerobic and anaerobic culturability in all clay plugs to below the levels in as-supplied dry bentonite (i.e., 1.37 ± 0.30) x 10^2 CFU aerobes/g and 5.56 ± 2.94) x 10^1 CFU anaerobes/g, Table 1). A few sporadic colonies were found on the R2A plates from both the low and high dry density plugs after exposure to 80^{o}C. This could suggest the presence of a few thermophilic organisms in the bentonite or, alternatively, the presence of organisms as less temperature sensitive (dormant or spore) cells.

The results in Table 8 also show that at temperatures above 80^{o}C, no culturability remained in the plugs with the low dry density of 0.8 g/cm^3 (i.er., no colonies were found on the R2A plates), but that in the higher dry density plugs of 1.6 g/cm^3, some culturability remained, even after accumulated exposures (in an oven) of two weeks at 121^{o}C (aerobes and anaerobes) and a further week at 130^{o}C (anaerobes), but again at levels below those in as-supplied dry bentonite. This suggests survival in spore form and possibly some protection against loss of all culturability in the compacted clay matrix in the oven treatment, since autoclaving (at 121^{o}C) eliminated all culturability in the dry non-compacted as-supplied bentonite (Table 4 and Figure 5). It may be possible that microbial cells have a different response to the autoclaving process (which is done under pressure and, therefore, with no or little water loss in the sample) than to the heat exposure in an oven. The a_w data in Table 8 show that, despite the fact that the plugs were tightly wrapped in foil and were contained in small Teflon containers with tight-fitting lids, severe moisture loss occurred in these plugs upon heating to ≥ 80^{o}C in an oven. The loss of water could possibly be a factor in the apparent enhanced endurance of culturable organisms in the high dry density compacted bentonite plugs that were heated in the oven to 130^{o}C.

The differences in remaining culturability between the plugs at 0.8 and 1.6 g/cm^3 confirm again that vegetative cells are more sensitive to the detrimental effects of elevated temperature. It appears that inactive dormant cells or spores are much more resistant because they already possess the necessary defence mechanisms, whereas vegetative cells must initiate the production of these at the start of the increased temperatures, which appears to cause significantly more loss of culturability.

7. DISCUSSION

The PLFA results (Experiment 1, Figure 3) have shown that about 1.4 x 10^6 viable cells are present in dry as-supplied bentonite powder, most of these as VBNC cells. Infusion with water at dry densities of 0.8 to 1.8 g/cm^3 does not appear to affect the level of PLFA-indicated viable cells significantly (it remains at a level of 1.5 to 2.3 x 10^6 cells/g). However, the amount of culturable cells increases from about 1.4 x 10^2 CFU/g in dry as-supplied bentonite (Table 1) to 1.4 x 10^5 CFU/g in compacted, saturated bentonite with a target dry density of 0.8 g/cm^3. Even in plugs with low dry density that were not fully saturated (95%), but kept at room temperature for 65 d (Experiment 8, Figure 9), aerobic culturability increased by up to five orders of magnitude, to 10^5 to 10^7 CFU/g. In contrast, in compacted saturated bentonite plugs with target dry densities of 1.3, 1.6 and 1.8 g/cm^3 (Experiment 1, Figure 3) the culturable cells slowly decreased from 1.2 x 10^2 to 6.5 x 10^1 CFU/g (Figure 1). These results suggest that the increase in culturability in 95-100% saturated, low-dry density bentonite is largely (or perhaps entirely) due to a transformation (revival or reversal) of the VBNC cells occurring in the dry as-supplied bentonite, to the culturable state, rather than to significant actual cell division. The observed shift in the PLFA-based community structure in the low-dry density sample in Experiment 1 also suggests revival of VBNC cells. These results again confirm that there is a continued potential for increased microbial activity but only at those locations where conditions would be more conducive to microbial activity, for instance at interface locations in a repository where the dry density of bentonite may be lower than in the bulk environment, or at locations where some of the high dry density could be lost due to swelling into placement gaps or into less dense backfill, or as a result of water erosion at the location of water-bearing fractures. It is, therefore, important that a high dry density is maintained in the bentonite in a repository to keep microbial activity to a minimum. Compliance models (Chandler 2008) can be used to determine the required as-placed dry density of bentonite buffer and gap fillings to achieve specific targets for long-term equilibrium dry densities for various placement room designs.

The results of the PLFA persistence (upon cell death) experiments suggest that PLFA content analysis is a good biomarker for viable cells in saturated or high water activity, biologically active environments. However, in environments that are biologically inactive (including environments with low a_w), PLFA may possibly be preserved for long periods of time, which implies that PLFA-based microbial biomass may include preserved but dead biomass. Therefore, in such environments, PLFA content may indicate potentially viable microbial biomass only. More study is needed on the potential preservation and rate of degradation of PLFA in environments with low a_w values. Other methods have to be found that can determine with more certainty whether such environments contain truly viable cells in the quantities suggested by PLFA content.

The results from the experiments with moderately-increased temperature (60^{o}C, experiments 3, 4, 5 and 6) suggest collectively that a temperature of 60^{0}C, with or without concurrent desiccation, will not permanently decimate aerobic culturability in compacted bentonite samples of lower dry densities (0.8 – 1.3 g/cm^3). Once heat and desiccation effects are removed and the sample is given time to recover at ample water content, culturability restores to within an order of magnitude of levels encountered at room temperature under similar dry density and a_w conditions. The results further show that as long as cells (viable according to the PLFA results) remain in a VBNC state (likely almost dormant cells or spores) in higher-dry density bentonite plugs, they are not particularly sensitive to a temperature of 60^{o}C and concurrent desiccation. It appears that only those cells revived to a culturable state (vegetative cells) prior to exposure to heat and desiccation at 60^{o}C are sensitive to elevated temperature. The results of these

experiments, therefore, suggest strongly that the initial physical state of the microorganisms in the samples has a large impact on the extent of temperature and desiccation effects.

The results of the experiments with elevated temperature in the range of 80°C - 130°C suggest that in both low and high dry density compacted bentonite plugs, some culturability remained after exposure to 80°C. Cells in the low-dry density saturated compacted bentonite plugs did not remain culturable at temperatures of 121°C and higher. However, some (anaerobic) cells in the high-dry density saturated compacted bentonite plugs did maintain sporadic culturability, even after exposure to 130°C (at a level well below that in as-supplied dry bentonite). The differences in remaining culturability corroborate the fact that inactive (dormant) cells or spores are much less sensitive than vegetative cells to the detrimental effects of elevated temperature

Long-term survival of microorganisms in (almost) dormant or spore state was discussed in the introduction. In order for microbes to adapt to, or survive in, unfavourable environments, they must have an active metabolism, to be able to repair any damage done to their genetic material and other cell components by the environmental conditions over time. Such damage would be inflicted by detrimental environmental conditions (including high or low temperatures, lack of water or nutrients, high salinity and naturally occurring background radiation). Fully dormant organisms or inactive spores do not have such repair ability, and although these forms are much more tolerant of, and resistant to, unfavourable conditions (because of their smaller size, thicker cell walls and condensed genetic material), ultimately the damage will be too extensive and the organisms, even in resistant or spore form, will lose viability and die. This may be a very long process, depending on the severity of the conditions, and could possibly have geological time scales in natural environments. Johnson et al. (2007) claim that they have found strong evidence that long-term survival of microbes in permafrost samples is in fact closely tied to the maintenance of low levels of cellular metabolic activity and DNA repair in cells. They conclude that, over time, such low levels of metabolism appear to be superior to a state of full dormancy as a mechanism in sustaining bacterial viability.

8. CONCLUSIONS

The experiments discussed in this report do not give a definitive answer to the question of whether finding high quantities of PLFA in natural clay-rich samples or in highly compacted bentonite implies that correspondingly high amounts of viable cells are present. However, results do suggest that biological (enzymatic) activity may be needed for PLFA to degrade upon cell death. It is, therefore, a possibility that PLFA from dead cells can be preserved in environments that are not conducive to significant biological activity, such as in highly compacted bentonite with low a_w values and in geological environments with very tight pore spaces, both of which will drastically curtail microbiological activity. Any sedimentary clay-rich samples or bentonites from which very little can be cultured, no DNA can be extracted, but which contain abundant PLFA may, therefore, be considered as possibly biologically largely inactive. Other methods have to be found that can determine with more certainty whether such samples contain viable cells in the quantities suggested by PLFA content.

The effects of moderately elevated temperature and desiccation (60°C) on the culturability of microorganisms in compacted bentonite were studied, including subsequent recovery of culturability at room temperature. In addition, the effects of higher temperatures (80°C-130°C) on the culturability of microbes in compacted bentonite plugs were investigated. Results showed that the few culturable cells in highly compacted bentonite plugs were not particularly

sensitive to a temperature of 60°C and concurrent desiccation. However, the large number of culturable cells in lower dry density bentonite plugs was reduced by up to five orders of magnitude at 60°C. Some culturability remained after exposure to 80°C at all dry densities. At 121°C and 130°C all culturability was below the detection limit for low dry density samples. A very low level of (anaerobic) culturability was observed at high dry density, even after exposure to 130°C. The difference in sensitivity to temperature is thought to be due to the difference in the physiological state of the cells present in the samples (e.g., vegetative cells or spores). The results also showed that the large effects of temperature on culturability in low dry density bentonite were reversible when the heat source was removed and re-saturation was allowed to occur.

The results from the PLFA and temperature studies collectively suggest that microbial cells may remain viable in highly compacted bentonite despite high temperatures, high swelling pressures, desiccation and low water activity. The presence of viable cells implies the potential for increased microbial activity in bentonite under favourable conditions, such as lower dry density. It is, therefore, important that a high dry density is maintained throughout the bentonite in a repository to keep microbial activity to a minimum. Compliance models can be used to determine the required as-placed dry density of bentonite buffer and gap fillings to achieve specific targets for long-term equilibrium dry densities for various placement room designs.

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Table 1: Phospholipid Fatty Acid and Culture Results from Experiment 1

Experiment and Sample Number	Measured Dry Density (g/cm^3)	Duration (d)	Infusion Solution	Water Content (%)	a_w	Swelling Pressure kPa	Aerobes CFU/g	Anaerobes CFU/g	SRB MPN/g	PLFA Cell Equiv./g
0.8-DDWL (1729)	0.66	44	DDW	113.85	0.998	175	$(1.41\pm0.05)x10^5$	$(1.82\pm0.7)x10^2$	< 6.6	2.29×10^6
1.3-DDWL (1730)	1.33	44	DDW	37.98	0.980	1925	$(1.21\pm0.47)x10^2$	$(1.94\pm0.84)x10^1$	< 4.4	1.52×10^6
1.6-DDWL (1731)	1.65	44	DDW	23.53	0.896	Failed	$(7.98\pm1.93)x10^1$	$(3.33\pm0.72)x10^1$	< 3.9	1.48×10^6
1.8-DDWL (1732)	1.83	44	DDW	17.65	0.736	Failed	$(6.48\pm2.64)x10^1$	$(2.59\pm1.70)x10^1$	< 3.4	1.61×10^6
Dry Bentonite (1733)	n/a	n/a	n/a	8.93	0.358	n/a	$(1.37\pm0.30)x10^2$	$(5.56\pm2.94)x10^1$	4.1	1.36×10^6

n/a = not applicable
Failed = Measurement failed due to load cell problems

Table 2: PLFA-based Community Structure in Saturated Compacted and Dry, As-Supplied Bentonite Samples (Experiment)

Sample #	1729	1730	1731	1732	1733
Dry Density g/cm^3	0.8	1.3	1.6	1.8	Dry, As-Supplied
Biomass					
Total Biomass (cells/g)	2.29×10^6	1.52×10^6	1.48×10^6	1.61×10^6	1.36×10^6
Community Structure (% Total PLFA)					
Firmicutes (TerBrSats)	22.76	12.89	15.78	13.69	13.11
Proteobacteria (Monos)	37.20	37.18	40.79	36.70	38.58
Anaerobic Metal Reducers (BrMonos)	1.23	1.29	1.22	1.22	1.18
SRB/Actinomycetes (MidBrSats)	5.54	3.79	3.86	3.74	3.64
General (Nsats)	32.19	42.66	35.35	41.06	35.00
Eukaryotes (Polyenoics)	1.08	2.20	3.00	3.57	8.49
Physiological Status (Proteobacteria Only)					
Slowed Growth	0.56	0.65	0.64	0.42	0.58
Decreased Permeability	0.07	0.00	0.06	0.05	0.20

Table 3: Description of PLFA Structural Groups

PLFA Structural Group	General Classification	Potential Relevance to Bioremediation Studies
Monoenoic (Monos)	Abundant in Proteobacteria (Gram-negative bacteria), typically fast growing, utilize many carbon sources, and adapt quickly to a variety of environments.	Proteobacteria is one of the largest groups of bacteria and represents a wide variety of both aerobes and anaerobes. The majority of Hydrocarbon utilizing bacteria fall within the Proteobacteria.
Terminally Branched Saturated (TerBrSats)	Characteristic of Fimicutes (Low G+C Gram-positive bacteria) and some Gram-negative bacteria (especially anaerobes).	Fimicutes are indicative of presence of anaerobic fermenting bacteria (mainly *Clostridia/ Bacteriodes*-like), which produce the H_2 necessary for reductive dechlorination.
Branched Monoenoic (BrMonos)	Found in the cell membranes of micro-aerophiles and anaerobes, such as sulfate- or iron-reducing bacteria.	In contaminated environments high proportions are often associated with anaerobic sulfate and iron reducing bacteria.
Mid-Chain Branched Saturated (MidBrSats)	Common in sulfate reducing bacteria and also Actinobacteria (High G+C Gram-positive bacteria).	In contaminated environments high proportions are often associated with anaerobic sulfate and iron reducing bacteria.
Normal Saturated (Nsats)	Found in all organisms.	High proportions often indicate less diverse populations.
Polyenoic	Found in eukaryotes such as fungi, protozoa, algae, higher plants, and animals.	Eukaryotic scavengers will often rise up and prey on contaminant utilizing bacteria.

Table 4: Results from Experiments 2, 3 and 4: Persistence of PLFA in Bentonite Samples Before and After Autoclave Treatments

Sample Description	Sample Treatment	Culturable Biomass On R2A (CFU/g or mL)		PLFA-based Biomass (cells/g or mL)
		Aerobes	Anaerobes	PLFA
Saturated Bentonite Dry Density 1.0 g/cm^3 (1834)		$(7.48 \pm 0.49)x10^4$	$(7.21 \pm 2.77)x10^2$	$7.98x10^6$
Saturated Bentonite Dry Density 1.0 g/cm^3 (1835)	Autoclaved	0	0	$1.35x10^6$
Dry (as-supplied) Bentonite (1836)		$(1.20 \pm 0.39)x10^2$	nm	$1.36x10^{6(I)}$
Dry (as-supplied) Bentonite (1836c)	Autoclaved	0	nm	$7.47x10^5$
1 g Bentonite in 100 mL Liquid R2A Medium (1836a)		$(6.95 \pm 0.28)x10^8$	nm	$\geq 6.95x10^{8(II)}$
1 g Bentonite in 100 mL Liquid R2A Medium (1836b)	Autoclaved	0	nm	$8.47x10^8$

(I) From Experiment 1 in which the number of culturable aerobes was $(1.37 \pm 0.30) \times 10^2$ CFU/g

(II) PLFA were not measured for the R2A culture, but because culturable aerobes were $(6.95 \pm 0.28) \times 10^8$ CFU/mL, PLFA-derived biomass should be $\geq (6.95 \pm 0.28) \times 10^8$ cells/mL

nm = not measured

Table 5: Results from Experiments 5 and 6: The Effects of Elevated Temperature (60°C) on Microbial Populations in Compacted Bentonite

Experiment and Sample Number	Description	Duration (d)	Measured Dry Density (g/cm^3)	Water Content (%)	a_w	Swelling Pressure kPa	Aerobes CFU/g	Anaerobes CFU/g	SRB MPN/g
0.8-DDWL (1718)	Infused at Room Temperature ½ plug Analyzed	77	0.69	102.30	0.996	120	$(9.50\pm0.50)x10^6$	$(3.42\pm3.99)x10^2$	< 7.9
1.3-DDWL (1719)		77	1.29	43.99	0.989	1250	$(7.67\pm0.54)10^3$	$(2.38\pm2.97)x10^1$	< 4.7
1.6-DDWL (1726)		79	1.57	29.41	0.945	6300	$(1.48\pm0.41)x10^2$	$(1.43\pm0)x10^1$	< 4.4
1.8-DDWL (1727)		79	1.61	23.51	0.888	14450	$(4.76\pm1.65)x10^1$	$(2.38\pm1.65)x10^1$	10
2.0-DDWL (1728)		156	1.81	18.48	0.773	33000	$(3.75\pm2.17)x10^1$	$(3.33\pm1.44)x10^1$	< 3.76
0.8-DDWL (1718-60)	Other ½ of Plug Wrapped in Foil + Plastic; Exposed to 60°C for 8 Days	8	nm	93.52	0.995	n/a	$(5.33\pm4.62)x10^1$	$(4.00\pm2.00)x10^1$	< 4.57
1.3-DDWL (1719-60)		8	nm	40.25	0.981	n/a	$(5.62\pm1.03)x10^3$	$(6.19\pm4.36)x10^1$	< 4.55
1.6-DDWL (1726-60)		8	nm	20.66	0.770	n/a	$(2.96\pm2.31)x10^1$	$(1.85\pm1.28)x10^1$	4.50×10^0
1.8-DDWL (1727-60)		8	nm	16.95	0.630	n/a	$(7.41\pm6.42)x10^0$	$(3.70\pm6.42)x10^0$	< 4.57
2.0-DDWL (1728-60)		8	nm	13.90	0.547	n/a	$(4.44\pm2.22)x10^1$	$(4.81\pm2.31)x10^1$	< 4.55
0.8-DDWL (1736)	Infused at 60°C	51	0.77	88.64	0.995	335	$(6.68\pm1.67)x10^1$	$(5.00\pm1.67)x10^1$	< 0.5
1.3-DDWL (1734)		51	1.31	45.86	0.988	1189	$(3.60\pm6.23)x10^0$	$(1.08\pm1.08)x10^1$	< 3.4
1.6-DDWL (1738)		52	1.61	32.70	0.965	4464	$(1.16\pm0.50)x10^1$	$(3.03\pm5.25)x10^0$	2.73
1.8-DDWL (1735)		51	1.79	21.95	0.851	16972	$(5.90\pm1.47)x10^1$	$(5.90\pm2.55)x10^1$	<4.29
2.0-DDWL (1737)		52	1.92	19.40	0.776	28973	$(5.70\pm2.74)x10^1$	$(4.17\pm2.22)x10^0$	< 3.75

Table 6: Results from Experiment 7: The Effects of Elevated Temperature (60°C) and Subsequent Recovery at Room Temperature on Microbial Populations in Compacted Bentonite

Experiment and Sample Number	Description	Duration (d)	Measured Dry Density (g/cm^3)	Water Content (%)	a_w	Swelling Pressure kPa	Aerobes CFU/g	Anaerobes CFU/g	SRB MPN/g
1.0-DDWL	Sample Infused at 60°C	40	1.10			450			
1750E	Exterior of Plug		nm	52.94	0.989		$(9.53\pm8.25)\times10^0$	$(9.53\pm16.5)\times10^0$	< 4.4
1750T	Top of Plug		nm	57.54	0.992		$1.14\pm1.31)\times10^1$	$(7.69\pm13.3)\times10^0$	< 2.7
1750B	Bottom of Plug		nm	61.43	0.993		$(1.15\pm1.15)\times10^1$	$(8.58\pm14.9)\times10^0$	<3.6
1.0-DDWL	Sample Infused at 60°C + Recovered at RT	40 at 60°C 76 at RT	1.03			~400 (60°C) 350 RT			
1778T	Top of Plug		nm	57.99	0.990		$(4.36\pm2.55)\times10^2$	$(5.29\pm0.51)\times10^2$	< 4.4
1778M	Middle of Plug		nm	57.38	0.990		$(6.55\pm0.92)\times10^2$	$(4.08\pm1.00)\times10^2$	< 4.1
1778B	Bottom of Plug		nm	56.05	0.989		$(1.98\pm0.17)\times10^5$	$(3.62\pm0.30)\times10^2$	< 4.6

nm = not measured RT = Room Temperature

Table 7: Results from Experiment 8: The Effects of Elevated Temperature (60°C), Desiccation and Subsequent Rehydration on Microbial Populations in Compacted Bentonite

Experiment and Sample Number	Description	Duration (d)	Target (Measured) Dry Density (g/cm^3)	Water Content (%)	a_w	Swelling Pressure kPa	Aerobes CFU/g	Anaerobes CFU/g	SRB MPN/g
0.8-DDWL (1768)	As Compacted	0	0.8 (0.64)	101.03	0.99668	n/a	$(3.29\pm0.55)\times10^{2}$	$(1.64\pm0.27)\times10^{2}$	8.2
0.8-DDWL (1771)	Saved at RT	65	0.8 (0.68)	91.42	0.98971	n/a	$(5.37\pm0.36)\times10^{6}$	$(2.79\pm1.32)\times10^{2}$	10.7
0.8-DDWL (1774)T (1774)B	Heated (60°C), Desiccated	20	0.8 (0.81)	66.81 55.96	0.99365 0.99048	n/a	$(6.52\pm2.72)\times10^{1}$ $(7.86\pm2.62)\times10^{1}$	$(1.01\pm0.57)\times10^{2}$ 0	BDL(<5.5) 9.4
0.8-DDWL (1779)T (1779)B	Rehydrated at RT	40	0.8 (0.70)	90.39 95.07	0.99504 0.99425	220	$(6.63\pm0.28)\times10^{5}$ $(7.18\pm0.29)\times10^{5}$	$(1.87\pm3.24)\times10^{1}$ $(9.97\pm9.14)\times10^{1}$	BDL(<8.4) 10.8
1.0-DDWL (1767)	As Compacted	0	1.0 (1.03)	59.17	0.99438	n/a	$(2.93\pm0.23)\times10^{2}$	$(3.47\pm3.75)\times10^{1}$	BDL(<4.4)
1.0-DDWL (1770)	Saved at RT	65	1.0 (1.03)	53.58	0.99075	n/a	$(2.30\pm0.30)\times10^{7}$	$(3.24\pm1.12)10^{1}$	7.2
1.0-DDWL (1775)T (1775)B	Heated (60°C), Desiccated	20	1.0 (1.08)	29.85 30.05	0.94175 0.93941	n/a	$(1.09\pm0.11)\times10^{3}$ $(3.18\pm0.85)\times10^{2}$	$(4.21\pm4.16)\times10^{1}$ $1.54\pm0.91)\times10^{2}$	BDL(<5.7) BDL(<5.2)
1.0-DDWL (1780)T (1780)B	Rehydrated at RT	40	1.0 (1.14)	58.92 63.73	0.99113 0.99275	260	$(1.80\pm0.24)\times10^{6}$ $(5.41\pm0.61)\times10^{5}$	$(1.18\pm2.05)\times10^{1}$ $(1.97\pm2.25)\times10^{1}$	BDL(<5.6) BDL(<4.6)
1.2-DDWL (1766)	As Compacted	0	1.2 (1.21)	40.91	0.98386	n/a	$(8.81\pm2.50)\times10^{2}$	$(4.00\pm0.69)\times10^{1}$	BDL(<3.8)
1.2-DDWL (1769)	Saved at RT	65	1.2 (1.18)	38.53	0.97955	n/a	$(3.11\pm0.43)\times10^{6}$	$(3.71\pm3.71)\times10^{1}$	13.7
1.2-DDWL (1776)T (1776)B	Heated (60°C), Desiccated	20	1.2 (1.45)	22.82 21.68	0.83523 0.81148	n/a	$(1.06\pm1.06)\times10^{1}$ $(1.46\pm0.48)\times10^{2}$	$(3.88\pm2.66)\times10^{1}$ $(2.06\pm0.11)\times10^{2}$	3.8 6.5
1.2-DDWL (1781)T (1781)B	Rehydrated at RT	40	1.2 (1.24)	40.88 44.12	0.98196 0.98684	860	$(4.41\pm0.98)\times10^{5}$ $(1.18\pm0.16)\times10^{6}$	0 $(7.76\pm6.72)\times10^{0}$	BDL(<4.8) BDL(<4.7)

Table 8: Results from Experiment 9: Effects of Elevated Temperature (80°C – 130°C) on Microbial Culturability in Compacted Saturated Bentonite Plugs

Target Dry Density 0.8 g/cm^3	Treatment[1]	Aerobes CFU/g	Anaerobes CFU/g	SRB MPN/g	Water Activity	Measured Dry Density[2] g/cm^3
Plug 1 (1863)	No treatment (RT)	$(9.68 \pm 1.00) \times 10^3$	$(4.06 \pm 4.00) \times 10^2$	<5.4	0.995	0.87
Plug 2 (1864)	Two weeks at 80°C	$(5.43 \pm 9.40) \times 10^0$	$(2.17 \pm 2.41) \times 10^1$	<5.1	0.994	0.90
Plug 3 (1865)	Two weeks at 80°C Plus two weeks at 121°C	BDL	BDL	<3.8	0.120	0.88
Plug 4 (1866)	Two weeks at 80°C Plus two weeks at 121°C Plus one week at 130°C	BDL	BDL	<3.2	0.057	0.93
Target Dry Density 1.6 g/cm^3						
Plug 1 (1859)	No treatment (RT)	$(9.99 \pm 1.99) \times 10^1$	$(4.34 \pm 2.71) \times 10^1$	<3.9	0.955	1.43
Plug 2 (1860)	Two weeks at 80°C	$(4.16 \pm 7.20) \times 10^0$	BDL	<3.9	0.918	1.49
Plug 3 (1861)	Two weeks at 80°C Plus two weeks at 121°C	$(1.55 \pm 0.67) \times 10^1$	$(7.77 \pm 6.73) \times 10^0$	<3.6	0.109	1.44
Plug 4 (1862)	Two weeks at 80°C Plus two weeks at 121°C Plus one week at 130°C	BDL	$(2.13 \pm 0.81) \times 10^2$	<2.4	0.068	1.45

[1] All plugs were saturated at room temperature (RT) for 27 days

[2] Dry densities for plugs 2, 3 and 4 were calculated assuming they had identical initial dry weights

CFU = Colony-Forming Units; MPN = Most Probable Number; SRB = Sulphate Reducing Bacteria

BDL = Below Detection Limit (i.e., no colonies on any agar plates)

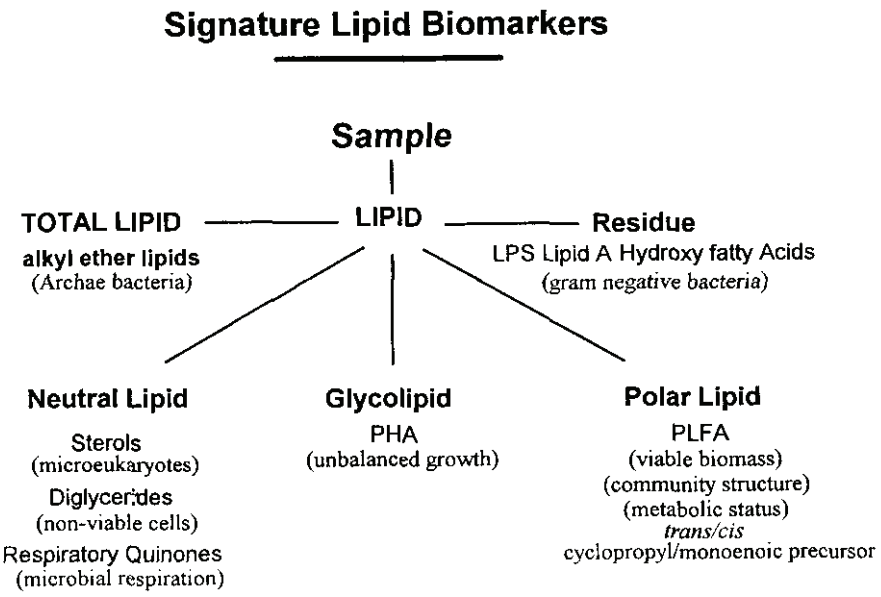

Figure 1: Signature Lipid Biomarkers in Microbial Cells (from White and Ringelberg 1997)

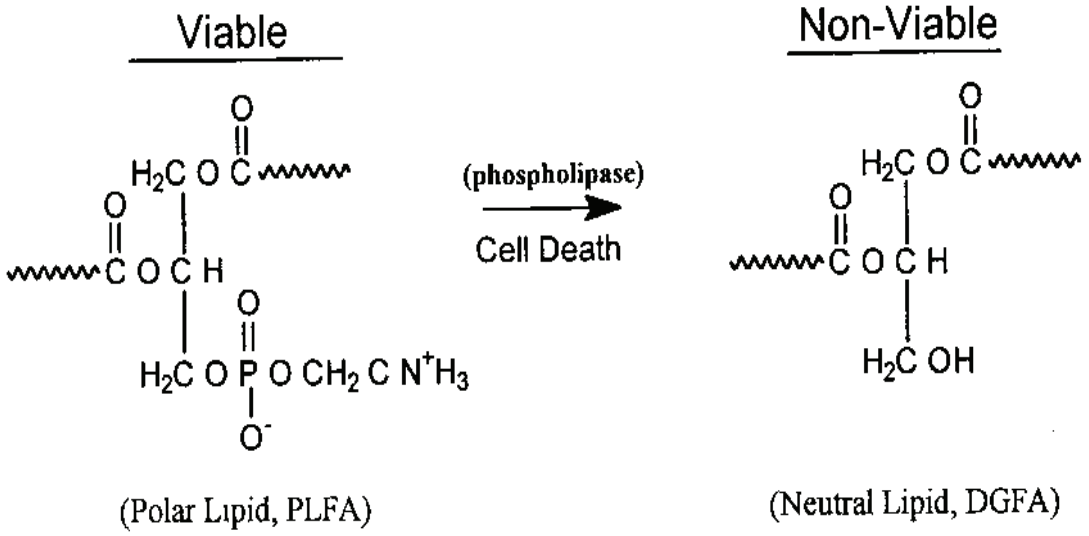

Figure 2: Conversion of a Phospholipid to a Diglyceride (from White and Ringelberg 1997)

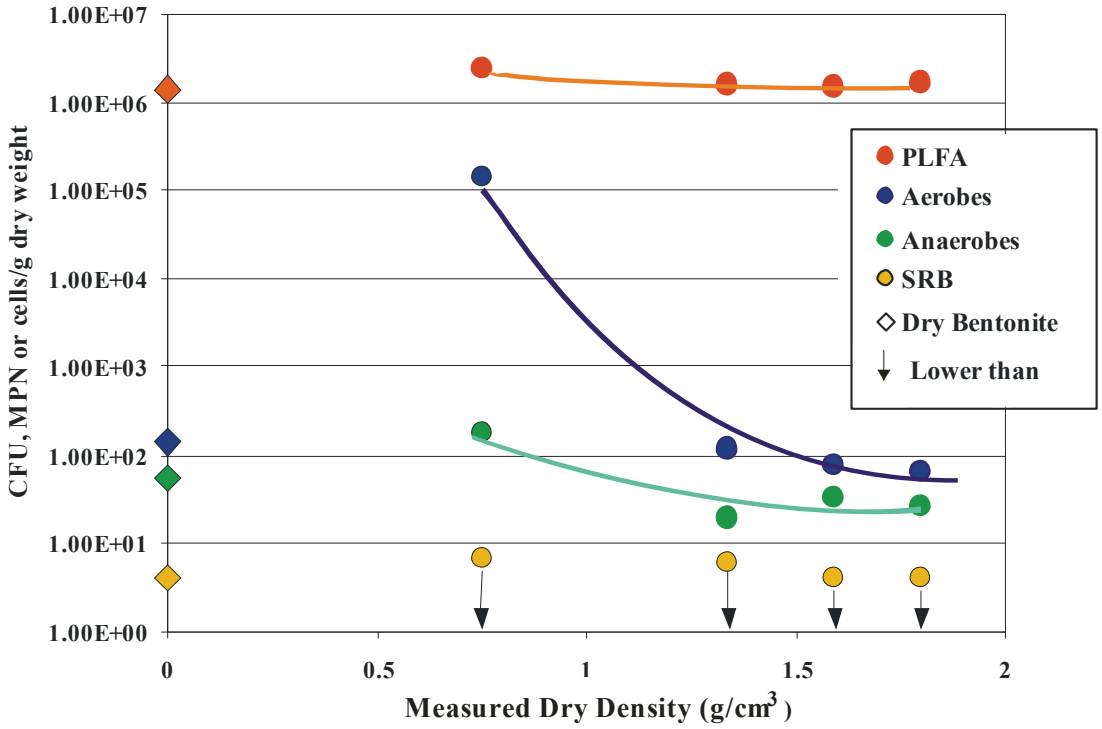

Figure 3: Comparison of Culturable and PLFA-based Biomass in Bentonite Compacted to a Variety of Dry Densities

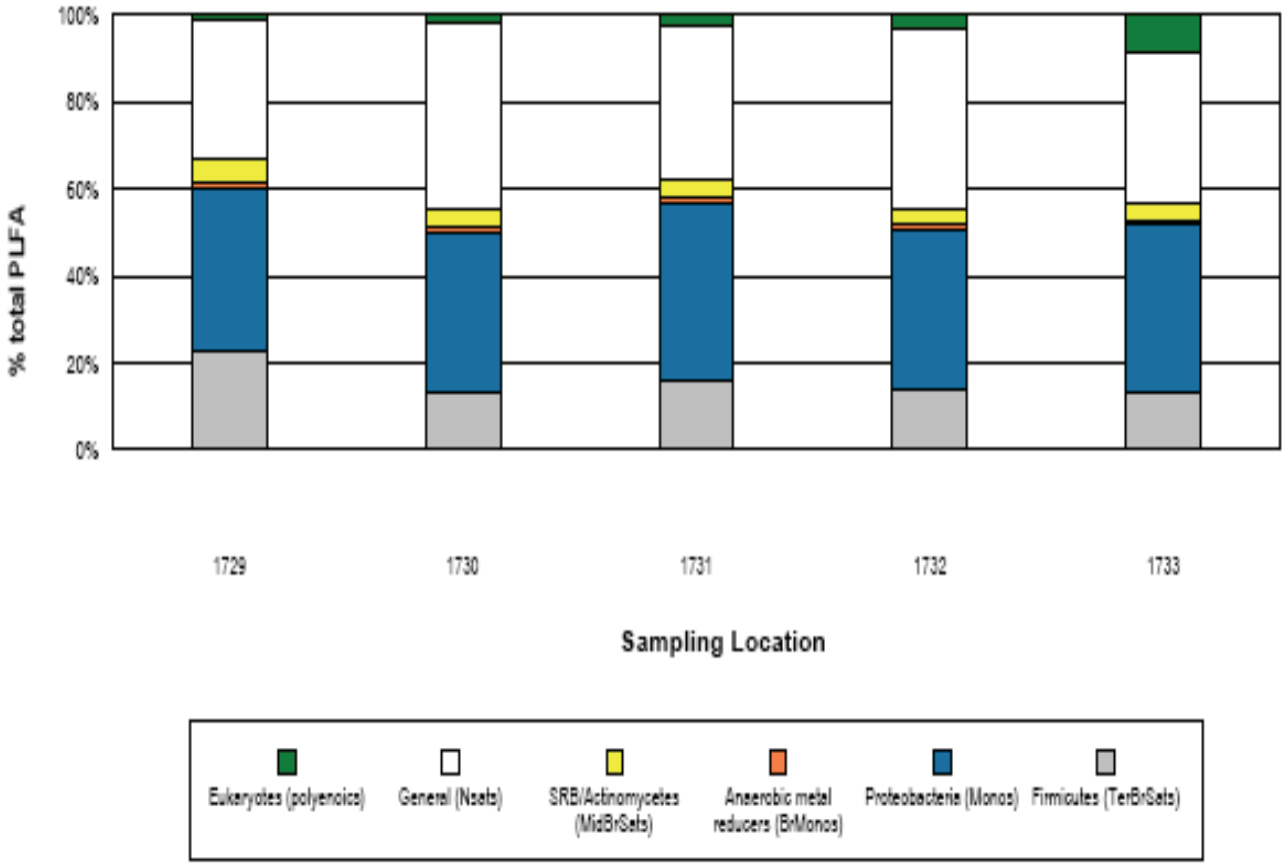

Figure 4: PLFA-based Community Structure in Compacted Saturated Bentonite Plugs of Various Target Dry Densities (1729 = 0.8 g/cm^3; 1730 = 1.3 g/cm^3; 1731 = 1.6 g/cm^3; 1732 = 1.8 g/cm^3; 1733 = as-supplied dry bentonite)

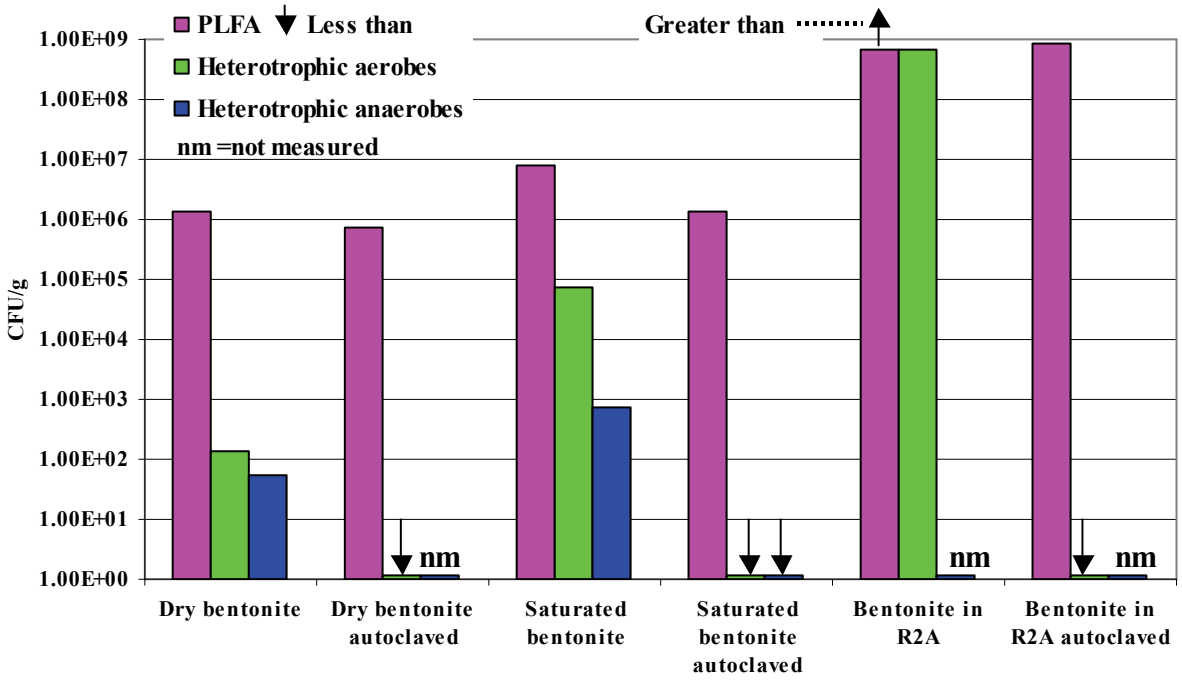

Figure 5: Comparison of the Effects of Autoclave Treatment on Culturable and PLFA-Based Biomass in Bentonite Samples with Different Treatments

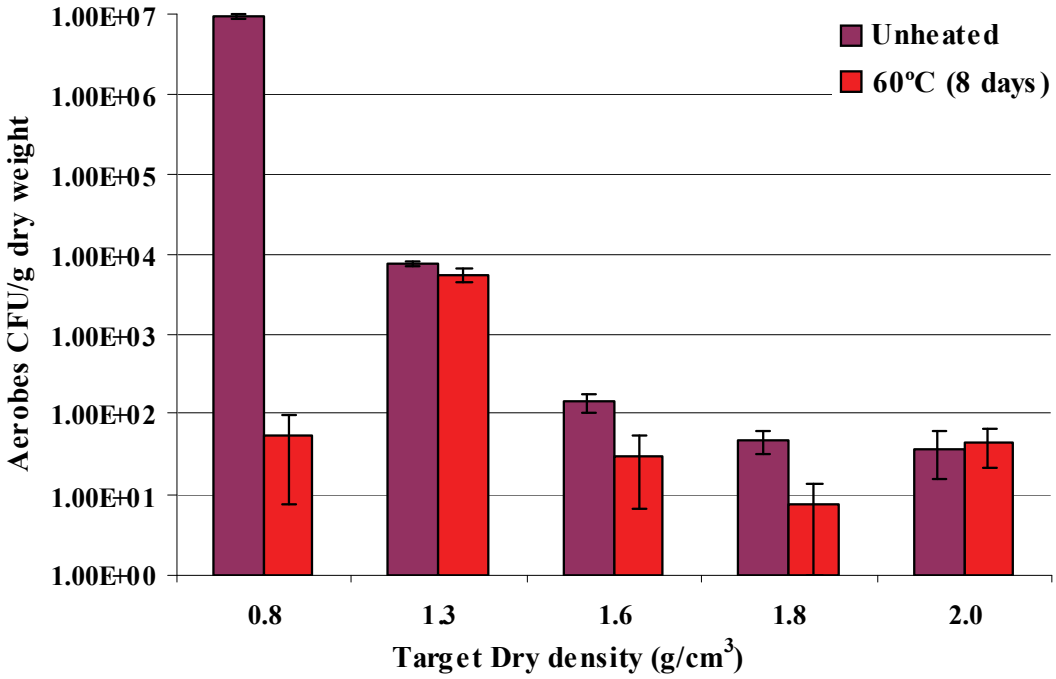

Figure 6: Effect of Heating at 60°C for Eight Days on Aerobic Culturability in Compacted Saturated Bentonite Plugs of Various Dry Densities

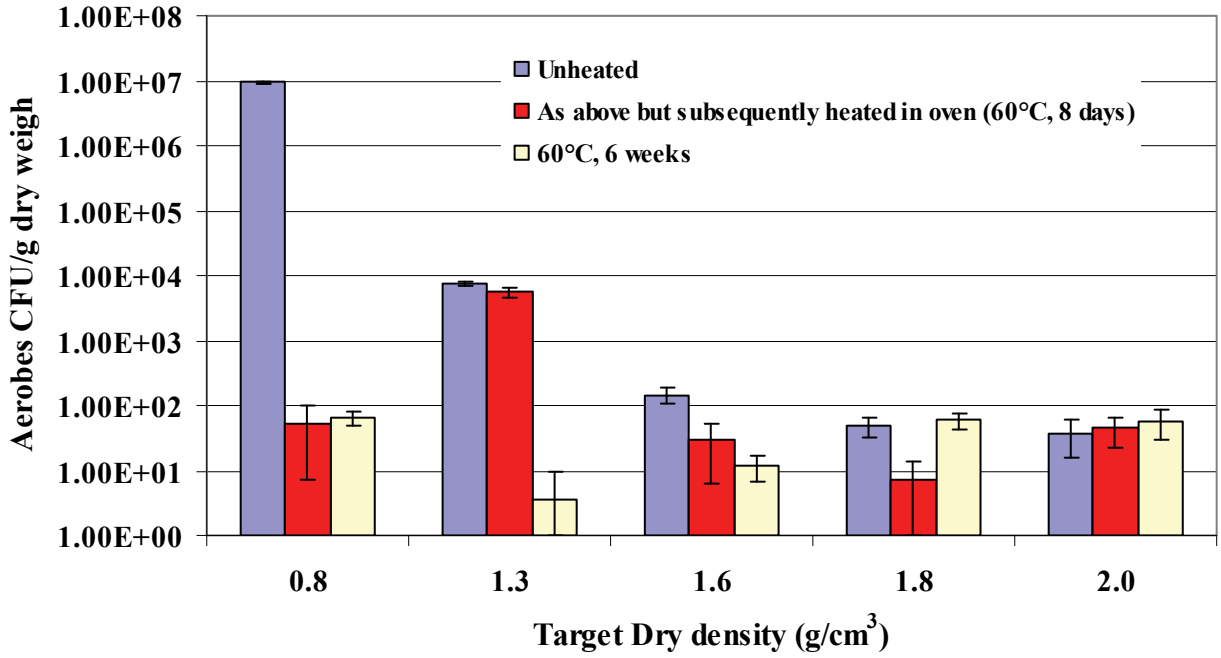

Figure 7: Comparison of Aerobic Culturability in Compacted Saturated Bentonite Plugs of Various Dry Densities after Infusion at Room Temperature and at 60°C

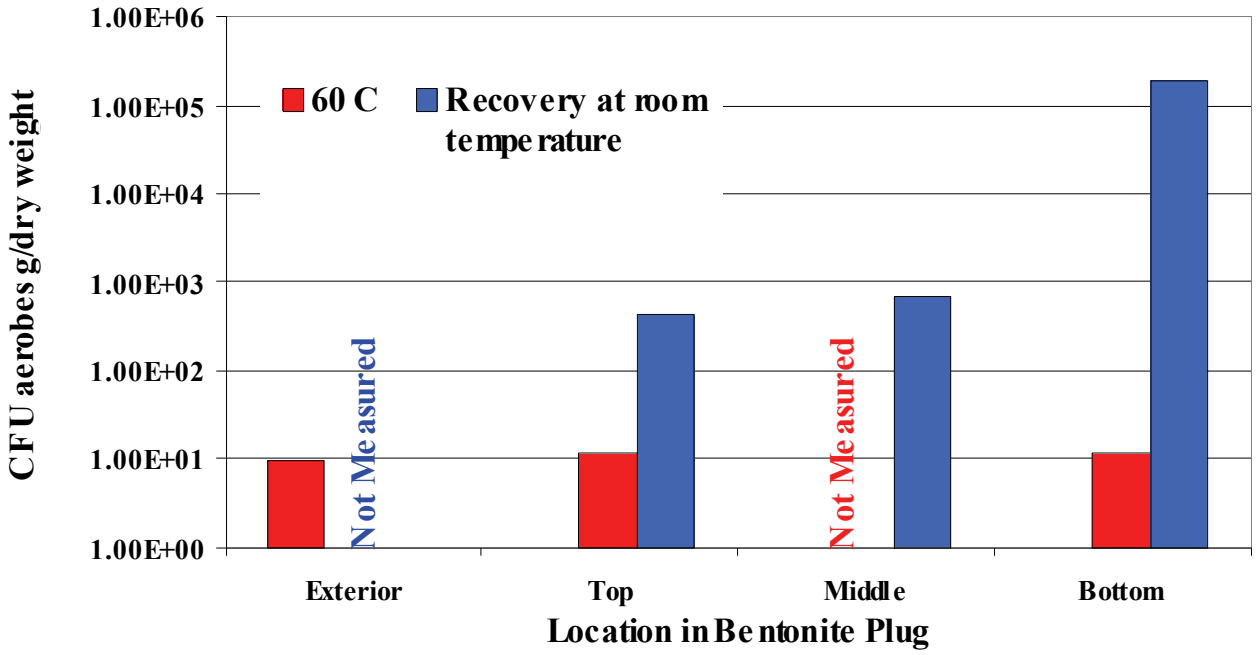

Figure 8: Recovery of Aerobic Culturability at Room Temperature after Infusion at 60°C in Bentonite Plugs with a Target Dry Density of 1.0 g/cm^3

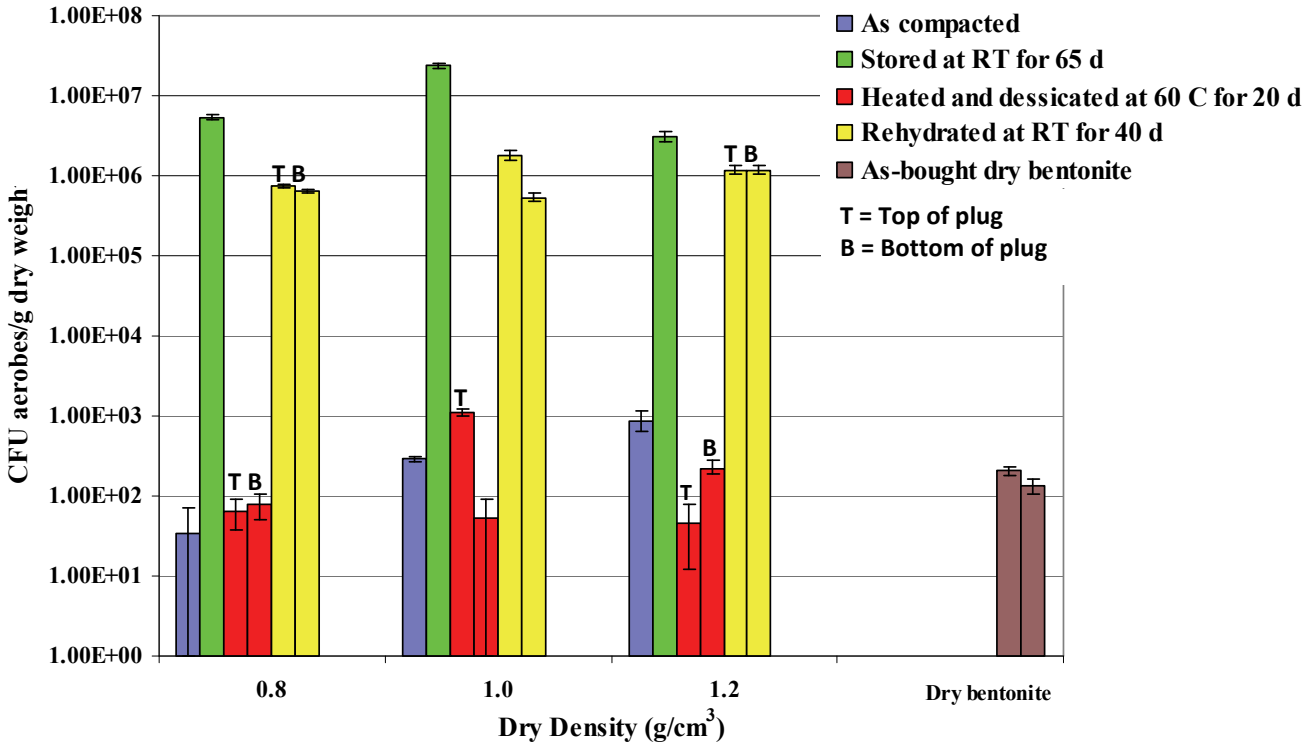

Figure 9: Recovery of Aerobic Culturability after Heat and Desiccation Treatments at 60°C in Bentonite Plugs with Target Dry Densities of 0.8, 1.0 and 1.2 g/cm^3

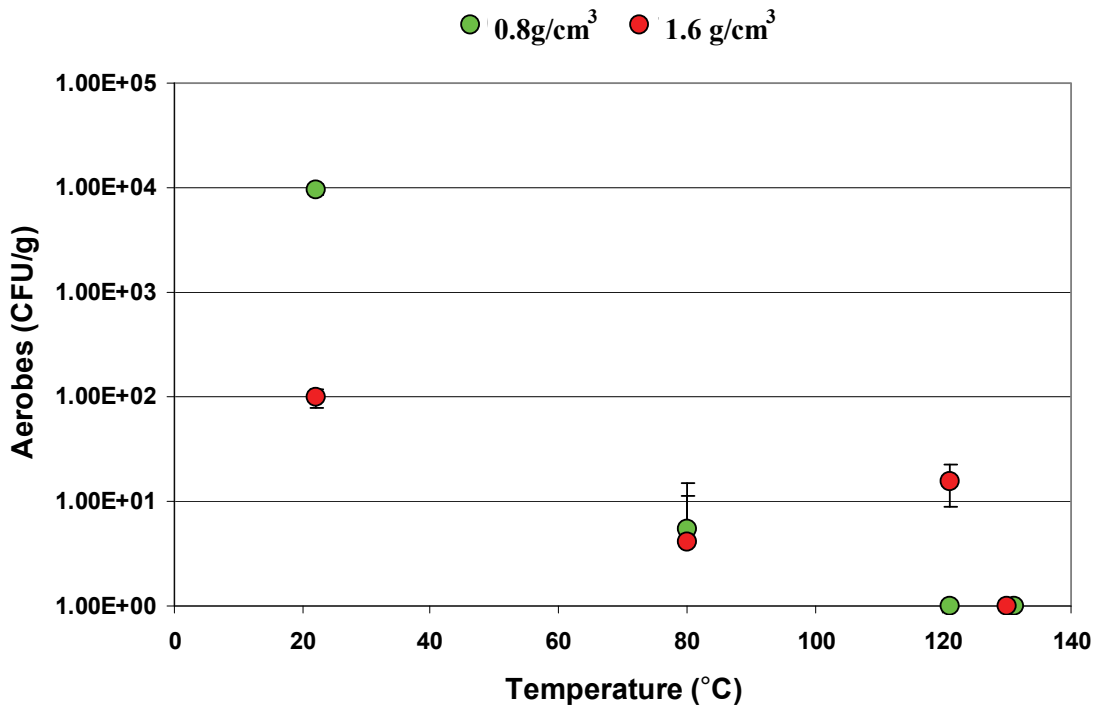

Figure 10: Culturable Aerobes as a Function of Temperature in Low and High Dry Density Compacted Bentonite Plugs