

**MICROBIAL ABUNDANCE, DIVERSITY, AND POTENTIAL ACTIVITY
IN BENTONITE CLAY**

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ABSTRACT

The Canadian deep geologic repository (DGR) concept for long-term safe storage and isolation of used nuclear fuel incorporates a multi-protective engineered barrier system. However, due to the inevitable presence of microorganisms and their metabolic products in a DGR, the integrity of the containers, and hence the repository, might be compromised. Therefore, the emphases of this thesis are to characterize and identify the microbial populations present in bulk and highly-compacted Wyoming MX-80 bentonite, to determine the conditions under which the survival and activity of microorganisms in highly-compacted bentonite clay (one of the engineered barriers) will be minimized or regulated, and to observe the microbial capacity to interact with bentonite particle under nutrient regime (clay-microbe aggregation study). To achieve these, culture-dependent and molecular biology methods (e.g., 16S rRNA sequencing), a range of analytical chemistry assays (e.g., sulfate turbidimetric method), pressure cell studies, microscopic technique (e.g., confocal laser microscopy (CLSM)), particle size analyses and laboratory-scale enrichment (or microcosm) assays were carried out.

Culture-dependent techniques revealed the presence of spore-forming bacterial isolates belonging to phyla *Actinobacteria* and *Firmicutes* in bulk MX-80. Interestingly, when MX-80 bentonite was highly compacted, Gram-positive spore-formers were also identified after being exposed to the collective effect of > 2,000 kPa swelling pressure, 0.96 water activity, oxygen-free environment, and $\geq 1.6 \text{ g/cm}^3$ dry density conditions for ~ 145 days and ~ 8 years. It was determined that microbial culturability was suppressed at or below background level (i.e., $\leq 2 \times 10^2$ Colony Forming Units per g) when the aforementioned parameters were applied and when 50 g/L NaCl solution infiltrated the highly-compacted bentonite (HCB). Sulfate reducing bacteria (SRB) in the HCB, however, were speculated to remain as spores during the incubation period since their microbial counts were similar at different dry densities. The enrichment assays for SRB containing bentonite clay slurry amended with carbon, electron donors and acceptors revealed that lactate was the preferred substrate for sulfidogenesis and that high salinity could impede the same process. Finally, the clay-microbe aggregation study showed that extracellular

polymeric substance (EPS) contribute to the clay-microbe aggregation and that nutrient concentration, carbon substrate type and bentonite concentration affect EPS production.

Overall, these studies are relevant to DGR operations because the results obtained will assist in understanding the potential consequences of microbial interactions with clay minerals.

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DEDICATION

I dedicate this thesis to my beloved husband and parents.

Thank you for all the love and care you have given me throughout the years.

Your endless support and faith have given me the will and confidence to follow my dreams and aspirations.

Thank you for making my world worth living. I will continue to strive for the best and accomplish more to make you prouder.

I love you.

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LIST OF ABBREVIATIONS

AECL	Atomic Energy of Canada Limited
CLSM	Confocal Laser Scanning Microscopy
DGGE	Denaturing Gradient Gel Electrophoresis
DGR	Deep Geologic Repository
EBS	Engineered-Barrier System
EPS	Extracellular Polymeric Substance
HAB	Heterotrophic Aerobic Bacteria
HAnB	Heterotrophic Anaerobic Bacteria
HCB	Highly-Compacted Bentonite
HPLC	High Performance Liquid Chromatography
IRB	Iron Reducing Bacteria
LB	Luria-Bertani liquid medium
MIC	Microbially-Influenced Corrosion
MPN	Most Probable Number
NRB	Nitrate Reducing Bacteria
NWMO	Nuclear Waste Management Organization
PCR	Polymerase Chain Reaction
SEM	Standard Error of the sample Mean
SRB	Sulfate Reducing Bacteria
SRB10	Sulfate Reducing Bacteria – enrichment containing 10g/L NaCl
SRB50	Sulfate Reducing Bacteria – enrichment containing 50g/L NaCl
TC-CC	Treatment C – variation in Carbon and Clay percentage
TC-CNP	Treatment C – variation in C:N:P ratio
TSB	Tryptic Soy Broth
UFC	Used Fuel Container
VBNC	Viable But Not Culturable

1. INTRODUCTION

The Canadian deep geologic repository (DGR) concept for storing used nuclear waste fuel incorporates multiple barriers designed to safely isolate radioactive waste for a period of beyond 1 million years (NWMO, 2005). The repository will be located in a suitable (i.e., either crystalline or sedimentary) host rock formation (NWMO, 2005) at a nominal depth of 500 m. The multi-protective barrier system of the DGR concept includes corrosion-resistant metal containers, highly compacted bentonite clay-based sealing and backfill materials (together considered the engineered barrier system, or EBS), and the surrounding host rock. Highly compacted bentonite clay-based sealing systems are integral to the safe storage and isolation of the highly radioactive used nuclear fuel containers (UFCs) from the surface environment because of their excellent physicochemical, hydro-mechanical and rheological properties (Ravi and Rao, 2013), including low water activity, high swelling pressure and small pore size. While repository design provisions are highly stringent, one factor with potential impacts on repository integrity is the effect of microorganisms, either indigenous or introduced, and their metabolic processes and products.

While it is understood that microorganisms will be present in the EBS when a DGR is commissioned, their control is essential. A variety of undesirable microorganisms in the EBS have the potential to affect the integrity of the system by directly damaging the UFCs through biofilm formation, by generating corrosive metabolic end products which could indirectly damage the container (e.g. sulfide), by producing metabolic gases (e.g., carbon dioxide and methane) that could impact EBS components, and by interacting with, or facilitating the transport of, radionuclides which might escape from a breached container. It is thus important to consider microbial risks that may affect the EBS, and in particular, their potential influences on the performance of the clay barrier and metal UFCs.

This research has focused mainly on the identification and diversity of microorganisms in the bentonite clay material with the use of culture-dependent (enriched microcosm studies)

and culture-independent (16s rRNA high throughput sequencing) methods. The physiology of these organisms was investigated together with the formation and kinetics of metabolites relevant to microbially influenced corrosion (MIC). The effects of swelling pressure, pore-water salinity, clay dry densities and water activity under anaerobic conditions was examined in order to obtain a more complete picture of their effects on the survival and persistence of microorganisms. Lastly, clay-microbe aggregation under nutrient regimes was investigated to determine if how nutrients and clay concentration would affect EPS production and clay-microbe aggregation.

2. LITERATURE REVIEW

2.1 Background

With the rising global demand for energy, the nuclear power industry is expected to grow. Around 13 % (equivalent to over 2500 billion kWh per year) of the world's electricity is generated from uranium, and in Canada, approximately 15.3 % (WNA, 2013) of electricity is generated from nuclear power plants.

In the late 1950s, Atomic Energy of Canada Limited (AECL) developed the “Canada Deuterium Uranium” (CANDU[®]) reactor (Figure 2.1), which uses nuclear fission of uranium as the source of heat, and deuterium oxide, or heavy water, as moderator to control the rate of the fission reactions (Whitlock, 2013; OPG, 1981). By using this technology, Canada could produce as much energy from a single uranium fuel bundle as burning 400 tons of coal (in fossil-fuelled power stations) or 2000 barrels of oil, leading to simpler and lower cost energy generation (Lovering *et al.*, 2016). However, for the past 60 years, 9,400 m³ of used nuclear fuel has been accumulated from the 22 power reactors operating in Canada; an estimated 20,000 m³ of used nuclear fuel will have been accrued by 2050 (LLRWMO, 2012). The Nuclear Waste Management Organization (NWMO) currently devised initiatives to develop a repository to allocate the growing number of used nuclear fuel through the Adaptive Phased Management approach (NWMO, 2005). In the meantime, the used fuel bundles are stored in aboveground facilities licensed for temporary storage.

2.2 Highly radioactive used nuclear fuel

Used nuclear fuel is highly radioactive, particularly within the first few years following its removal from the reactor core. The radiation emitted by used nuclear fuel is caused by the production of a number of fission products after U²³⁵ – a fissile uranium isotope that can readily split after capturing a moving neutron – splits (nuclear fission) (Hahn and Strassmann, 1939). Another source of radiation is the presence of larger transuranic actinides that are formed when uranium, after absorbing a moving neutron, does not split. An example of this would be the

formation of Pu^{240} , a fertile plutonium isotope that is formed when Pu^{239} (fissile) captures a neutron but does not split (WNA, 2012). Pu^{240} can later be recycled into fuel but is unsuitable for making weapons. All radioactive material in used nuclear fuel is unstable and undergoes radioactive decay, sometimes very slowly due to the very long half-life (~4500 million years) of some isotopes. Since the radioactive material undergoes beta decay, in which neutrons are transformed into protons, it may release high-energy non-penetrating beta particles or penetrating gamma radiation, with the latter causing damage at the cellular level due to interactions (primarily free radical formation) with biological material.

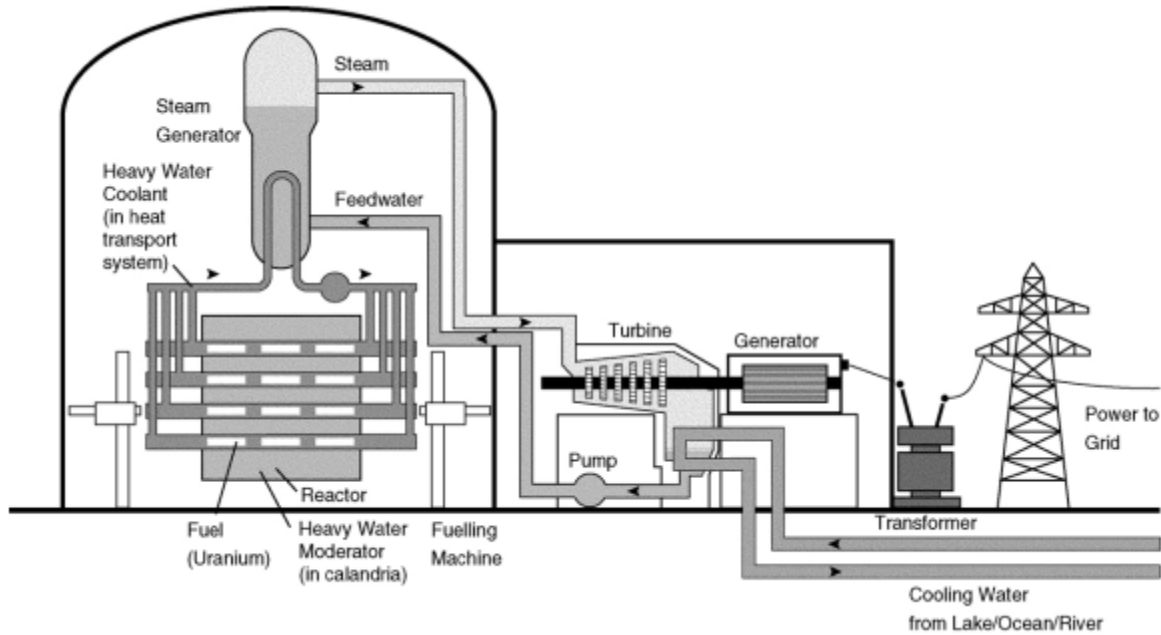


Figure 2.1 Schematic diagram of a Canada Deuterium uranium (CANDU) nuclear power plant. Zirconium alloy tubes containing ceramic uranium dioxide (nuclear fuel pellets) are welded to form fuel bundles, which are assembled in the fuel channels of the reactor vessel. Fission reactions boil the coolant, producing high pressure steam to run the turbines. An electrical generator is linked to the turbines that generate electricity (Garland, 2003).

2.3 The Deep Geologic Repository Solution for used nuclear fuel

Deep geologic repositories (DGR) are used nuclear fuel storage facilities excavated deep within a stable geologic site (Figure 2.2). Countries such as Switzerland, Belgium, France, Finland, Japan, Republic of Korea and Russia all consider the DGR concept to be the safest option for the storage of low- and intermediate-level nuclear waste, as well as for high-level used nuclear fuel (WNA, 2013). In Canada, the DGR approach is also the preferred option for the long-term

storage of highly radioactive UFCs, based on comprehensive examination of the various safety considerations (IAEA, 2006; Kleb, 2013; King, 2006a).

Several countries are considering various host rock options (i.e., hard or soft sedimentary rock, or crystalline rock) to emplace their UFCs (Maak *et al.*, 2010). Some of the sites for potential repository locations have been identified and are currently subject to further investigations, including microbiological studies (e.g., Moll *et al.*, 2015; Thury and Bossart, 1999; Chi Fru and Athar, 2008; Wouters *et al.*, 2013; Urios *et al.*, 2012). Among these chosen sites and formations are the Opalinus clay formation (studied at the Mont Terri Rock Laboratory in Switzerland), the Boom clay in Belgium (studied at the underground research facility at Mol), granite (studied at Äspö Hard Rock Laboratory (HRL) in Sweden), and the Callovo–Oxfordian argillite in France (studied at Bure) (IAEA, 2001b). Once the chosen host rock sites have been excavated, the containers with spent fuel would be encased in either titanium or copper containers (Stroes-Gascoyne and West, 1997) for burial in a DGR at a nominal depth of 500 m. Afterwards, UFCs would be placed in rooms, tunnels or boreholes in the DGR, and then surrounded with highly-compacted bentonite (HCB) clay (Wolfaardt and Korber, 2012). Following placement of UFCs in the excavated rooms, the performance of the repository will be monitored and then it will be backfilled and sealed.

2.3.1 Engineered-barrier system

The engineered-barrier system (EBS) (Figure 2.2) refers to the corrosion-resistant metal containers (e.g., either titanium or copper), bentonite clay buffer materials and sealing and backfill materials. The advantages of the EBS include the protection and isolation of the used nuclear fuel from the environment, as well as the prevention of groundwater access and probable transportation of microbial metabolites to the container surface (Bennett and Gens, 2008; Stroes-Gascoyne and West, 1997; Smart *et al.*, 2011; EC, 2004). The majority of nuclear waste management organizations around the world have adopted an EBS as an integral component of their DGRs (Bennett and Gens, 2008).

2.3.2 Metal containers, backfill and seals

The Canadian DGR concept entails encasing the used fuel bundles in corrosion-resistant UFCs before placing them in the repository (NWMO, 2005). The NWMO's current reference corrosion-barrier material is copper (Maak, 1999), a natural material known for its robust corrosion resistance (NWMO, 2010) under deep geological disposal (reducing) conditions.

Accordingly, an intact container would prevent groundwater from reaching the fuel bundles and would block radionuclides from leaking (NWMO, 2010).

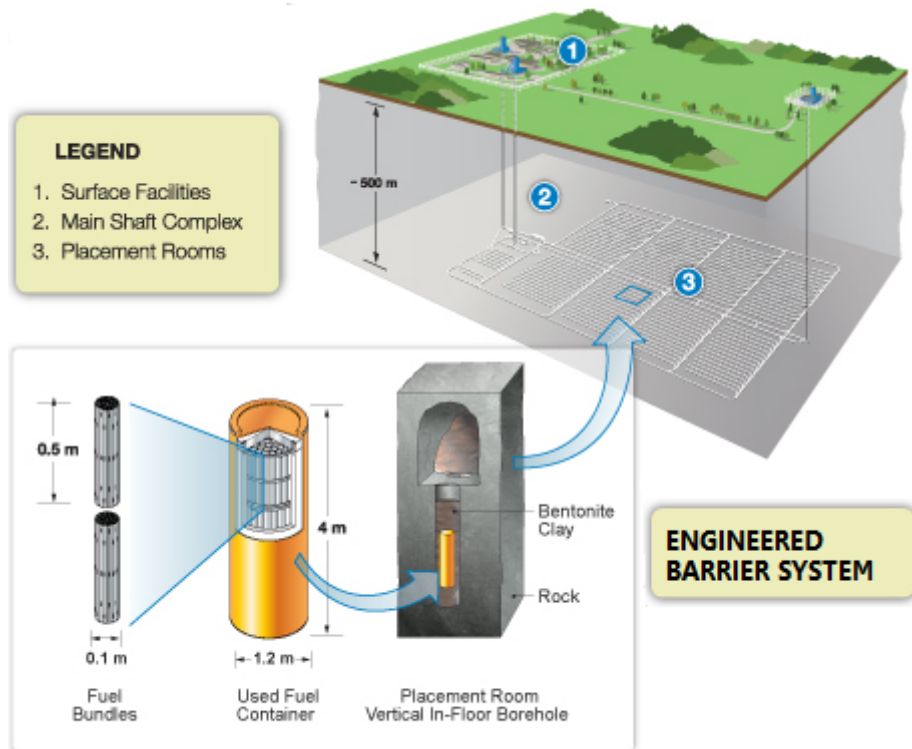


Figure 2.2. Conceptual illustration of a Canadian deep geologic repository design. The image includes the engineered-barrier system for used nuclear fuel bundles (CNSC, 2015).

Clay-based compacted buffer and backfill materials will be used to fill the open spaces once the UFCs have been placed (NWMO, 2010). These materials will also help increase the structural stability of the repository as well as restrict the flow of water within the earlier excavated zones (IAEA, 2001a). While buffer will likely consist of highly compacted bentonite (HCB), backfill may consist of a mix of clay, sand and crushed rock. In addition, the buffer and backfill may be used in combination with high integrity seals, especially when the repository lies in the saturated zone, to limit groundwater flow, and when it is vital to maintain access to certain parts of the repository (IAEA, 2001a). Seals will constrain the expansion of clay barriers surrounding the waste containers and will isolate the emplacement room from access tunnels. It will also permit the clay buffer to take up water, swell, fill gaps and meet its target density and water activity set

points. The combined seal and backfill system within an emplacement rooms will impede water flow and will prevent advective transport of radionuclides.

2.3.3 Bentonite clay as EBS buffer material

The HCB clay in a Canadian DGR will serve as buffer material in the EBS for the subsurface storage of UFCs (Stroes-Gascoyne *et al.*, 2007a, 2010a,b, 2011; Bennett and Gens, 2008). It will surround the UFCs where it will serve as a barrier to advective groundwater flow (Stroes-Gascoyne *et al.*, 2007a). A number of natural and commercial bentonites have been assessed for their suitability for used nuclear fuel storage in Canada (e.g. Karnland *et al.*, 2006; Karnland, 2010; Marshall and Simpson, 2014; Baumgartner *et al.*, 2008).

Wyoming MX-80 bentonite has been selected as the potential sealing material for the EBS because of its excellent physicochemical, hydromechanical and rheological properties (Ravi and Rao, 2013). It consists of montmorillonite (75 wt. %) and other accessory minerals (in wt. %, < 1 kaolinite, < 1 mica, 15.2 quartz, 5-8 feldspar, 0.7 calcite, 0.7 siderite, 0.3 pyrite; Bradbury and Baeyens, 2002). Montmorillonite is a 2:1 hydrous aluminosilicate mineral (Figure 2.3) with an overall negative charge surface. It has a large swelling potential upon interaction with free water that allows it to seal any voids, cracks or fissures in a confined environment such as a DGR. This means that when clay comes in contact with groundwater, the clay swelling process will cause the hydraulic conductivity to become reduced to as low as 10^{-12} to 10^{-14} m/s (Pusch and Weston, 2003) (i.e., mass transport of any solutes would occur solely by diffusion (King *et al.*, 1996)). Since there would be an absence of groundwater flow, the expanded clay buffer would provide excellent isolation properties by retaining radionuclides from escaping any breached containers (Börgesson *et al.*, 1988) and diminishing the possibility of electrochemical corrosion processes on the metal container surface (King, 2007).

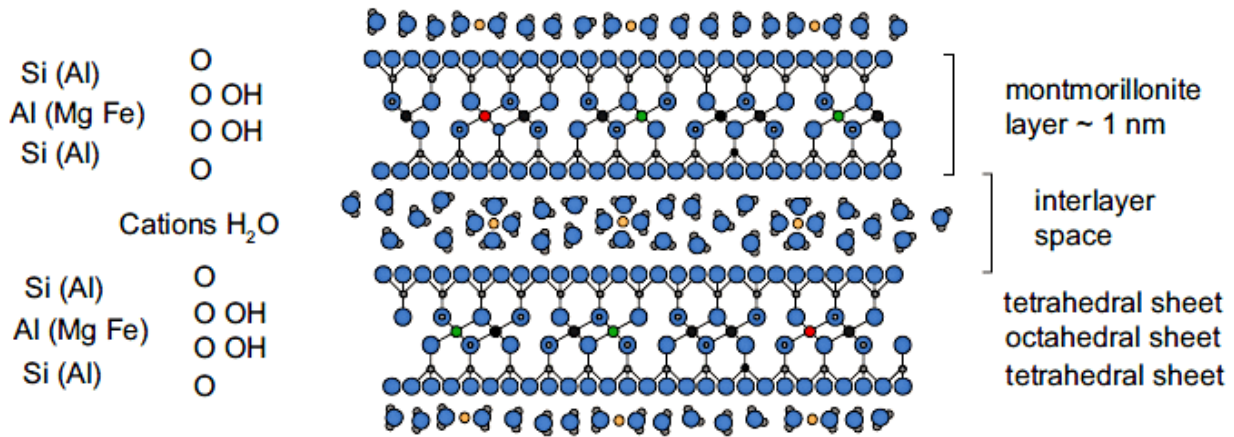


Figure 2.3. Schematic illustration of montmorillonite mineral layers and water molecules. Two tetrahedral silicon sheets sandwich an aluminum octahedral sheet (Karnland *et al.*, 2006). Furthermore, the clay buffer's high swelling pressure ($> 2,000$ kPa) and low water activity ($a_w < 0.96$) at a dry density of ≥ 1.6 g/cm³ have been demonstrated to decrease microbial culturability and viability (Stroes-Gascoyne *et al.* (2006, 2010b) for long periods. Wyoming MX-80 would also act as heat conductor by transferring heat from the used nuclear fuel to the host rock. It would also confine radionuclide migration through sorption, limit microbial activity near the UFC, restrict the rate of mass transport of corrosive species to and from the container, and protect the container against host rock movement or shifting (Stroes-Gascoyne, 2005). Laloui *et al.* (2008) provided an additional list on the functions of clay buffer in a DGR.

2.4 Evolution of the repository environment

It is expected that conditions within a DGR will evolve over time. King (2007) described the evolution of the environmental conditions in a repository in terms of the following: degree of saturation of the repository, temperature, groundwater and pore-water chemistry, redox conditions, microbial activity, mass transport, residual stress and external loads, and gas transport. Detailed reviews on the evolution of DGR and EBS components have also been provided by the NWMO (2012) and King (2006b).

The rate of saturation of the repository depends on the density of the bentonite clay buffer, the local hydraulic conductivity and the container surface temperature. Variations in these factors would affect the length of time it would take to fully saturate the bentonite clay buffer with groundwater, and could range from around a hundred to several thousands of years. Upon placement of the UFCs in the DGR, the temperature at the container surface could rise to 100-120 °C (Maak, 1999), due to the low moisture content of the bentonite material (slow rate of heat

conduction), and partly because of a slightly higher ambient temperature at repository depth. Moisture evaporation would occur and could lead to accumulation of dissolved solutes and precipitation of salts (King, 2006b). After hundreds of years, resaturation would commence due to a UFC temperature drop (i.e., 50-60 °C (Stroes-Gascoyne and West, 1997)) and this would result in an increase in relative moisture content. Thereafter, deliquescence (or substance absorption of moisture) of salt deposits on the surface of the UFCs would occur. Henceforth, the repository would evolve from relatively warm, dry and aerobic to cool, saturated and anaerobic conditions following thousands of years UFC storage in a DGR (King, 2006b). Over time, the EBS pore-water would also equilibrate with the ambient groundwater, through the exchange of ions, pH change, re-dissolution of precipitates and dissolution of minerals, to form a saline solution that would eventually saturate the bentonite clay buffer. Salinity of groundwater is important because it affects the local vapour pressure and thus the water saturation process (PEBS, 2012), water activity (Stroes-Gascoyne *et al.*, 2010b) and bentonite swelling potential (Börgesson *et al.* 1995, 1996).

DGR engineers are aware of the presence of microbial communities in a DGR. As conditions evolve to anaerobic and cool, low water activity, high swelling pressure and high dry density conditions will be established, which are predicted to decrease the number of surviving and viable organisms, or drive cells to enter dormancy, especially in the matrix of the HCB (Stroes-Gascoyne and West, 1997; Stroes-Gascoyne *et al.*, 2010b; Pedersen, 2000; Stroes-Gascoyne *et al.*, 2002). The potential for metabolic activity is only expected to exist in regions that may not be fully sealed with HCB initially. According to Stroes-Gascoyne *et al.* (2010b), these regions include: i) interfaces between HCB and host rock or containers; ii) interfaces between HCB buffer blocks; iii) interfaces between HCB buffer and less dense backfill materials; and iv) imperfectly sealed fractures in HCB buffer.

2.5 Intraterrestrial microbial communities

Microbial communities have been shown to exist several kilometers beneath the Earth's surface. They are found in a variety of subsurface habitats, including continental and marine deep subsurface sediments, deep mines, granitic rock, sedimentary deposits, subsurface aquifers and basalts (Barton and Northup, 2011; Newby *et al.*, 2004; Baker *et al.*, 2003). An attempt was made to calculate the global carbon distribution in terrestrial and intraterrestrial environments (Whitman *et al.*, 1998), and it was suggested that the amount of total carbon in the intraterrestrial

environment is sufficient to support life in the biosphere. The quantities of subsurface microorganisms, however, may differ depending on the location. Approximately 10^3 - 10^6 cells/g sediment (total microbial cell count) have been reported (e.g. Pedersen, 1993; White and Ringelberg, 1998; Wang and Francis, 2005). The physicochemical environment, such as pore size, temperature, pH and water activity as well as availability of organic and inorganic nutrients will affect the activity, diversity and total biomass of subsurface microbes (Wang and Francis, 2005; Stroes-Gascoyne and West, 1997). Subsurface disruption due to human activity or natural causes would also be expected to influence the diversity of the microbial community present in the subsurface (Stroes-Gascoyne and West, 1997). Therefore, a wide array of microbial metabolic processes are anticipated within the environment of a DGR (Ehrlich and Newman, 2009).

The diversity and biomass of prokaryotes in the subsurface are dictated by the type and abundance of carbon and electron donors and acceptors present in the environment. More importantly, only those microorganisms that can adapt, or are adapted, to subsurface conditions would be able to survive (Humphreys *et al.*, 2010). Prokaryotes come in many different shapes and sizes. The smallest prokaryotes may have a diameter of only 0.2 μm , and this characteristic might permit such microorganisms to endure within the matrices of dense subsurface environments (Mauclaire *et al.*, 2007; Stroes-Gascoyne *et al.*, 2007a). Lawrence *et al.* (2000) found a number of Gram-positive and -negative bacteria in the clay zone of an aquitard complex (~86-122 m deep) as well as some sulfur oxidizers and methanogens. Kotelnikova *et al.* (1998) isolated *Methanobacterium subterraneum*, a novel autotrophic species, from Aspö Hard Rock Laboratory (HRL) groundwater in Sweden. Fungi and yeast have also been found in the subsurface, especially in aquifers or groundwater (e.g., Wang and Francis, 2005; Pedersen, 1999). Wolfaardt and Korber (2012) reported on the discovery of flagellated and ciliated protozoa isolated from deep subterranean granitic aquifers in Sweden. The recovery of nematodes from fracture water in South African gold mine was also reported by Borgonie *et al.* (2011). Earlier studies have shown the existence of other subsurface-inhabiting microorganisms (e.g., Ekendahl *et al.*, 1994; Pedersen *et al.*, 1997; Fry *et al.*, 1997).

Oxygen-abundant zones, such as interfaces, transition zones and fractures in a repository may provide refugia to aerobic and other microorganisms. Conversely, as oxygen is depleted, anaerobic microorganisms will come to dominate. A repository introduces harsh conditions to

microorganisms. The low permeability in a DGR introduces space, water and nutrient constraints that would influence the growth and survival of microbes (Stroes-Gascoyne *et al.*, 2002), thus affecting diversity. Humphreys *et al.* (2010) provided a list of extreme conditions and the microorganisms known to tolerate them:

- temperature - Black smoker bacteria are resistant to temperatures as high as 113 °C,
- radiation – *Deinococcus radiodurans* are radioresistant bacteria that can withstand a single dose of 5000 Gy of ionizing radiation without losing viability,
- pressure – *Desulfovibrio desulfuricans* can endure high pressure (~180 MPa),
- pH – nitrifying bacteria are capable of surviving at very high pH while *Thiobacillus ferrooxidans* can tolerate very low pH, and lastly,
- salinity - *Halobacterium halobium* and *Salmonella orianenburg* are resistant to high (50 % salt by weight) and low (70 ppb dissolved salt) salinity, respectively.

It is assumed, therefore, that no environment will remain sterile because microorganisms can survive environmental extremes in a variety of ways (Onstott *et al.*, 2010). It is also appreciated that DGR construction activities and materials will introduce a variety of allochthonous organisms into the EBS, which would then co-exist or compete with indigenous microbes.

2.6 Microbially-facilitated reduction-oxidation processes in the subsurface

Restrictions in an environment's physicochemical factors and energy sources (light or chemical) determine which microbial metabolic processes will be utilized by a particular microorganism (Wang and Francis, 2005; Parnell and McMahon, 2016). In the presence of energy-rich chemicals, heterotrophs (which acquire their energy by oxidizing organic compounds) and autotrophs (which oxidize inorganic compounds for energy) could both dominate. If a light source was available, phototrophs could further proliferate. Generally, energy release involves the transfer of electrons from an electron donor (oxidation of organic or inorganic compounds) to an electron acceptor (reduction of inorganic compound). Some examples of inorganic electron donors include hydrogen, carbon monoxide, ammonia, methane, nitrite, sulfur, sulfide and ferrous iron (Humphreys *et al.*, 2010). The most common electron acceptors are oxygen, nitrate, ferric iron, sulfate and carbon dioxide (Humphreys *et al.*, 2010). Manganate, selenate, uranyl ions, and other oxidized compounds, can also serve as electron acceptors in the subsurface (Sherwood Lollar, 2011).

A DGR setting poses a dilemma to microorganisms, considering its activity-limiting and nutrient-restricted environment. Microbes will accumulate at redox interfaces where energy exploitation is possible. However, autotrophic processes would become slow and less dynamic in the DGR since inorganic electron donors and CO₂ would be limited (Humphreys *et al.*, 2010). Heterotrophs, on the other hand, could feasibly dominate the system because of their ability to utilize a great number of carbon sources. Nonetheless, those microorganisms with alternative metabolic processes (i.e., anaerobic respiration, chemoautotrophy, and fermentation) (Beech *et al.*, 2000; Parkes *et al.*, 2000) that could yield the greatest amount of energy would ultimately dominate (Amend and Teske, 2005; Lovley and Chapelle, 1995). Under DGR conditions, nitrate would be consumed after oxygen, followed by nitrite, manganese (IV), ferric iron, sulfate, and lastly, carbon dioxide (Pedersen, 1999). Nitrate reduction occurs more favourably than sulfate reduction because the Gibbs free energy indicate a more spontaneous reaction than sulfate reduction (see Table 2.1). While Table 2.1 is an oversimplification of the spatial distribution of the electron acceptors in the subsurface (Aller, 1982), the order of consumption of electron acceptors is a useful reference for modelling biogeochemical processes in a DGR (Wang and Francis, 2005).

Table 2.1. Terminal electron-accepting processes observed in the subsurface. Adapted from Kristensen (2000) and Lovley and Chapelle (1995).

Zonation	Type of Respiration	Reaction	Reduction Potential (V)*	Gibbs free energy (kJ/mol)**
Oxic zone	Aerobic respiration	$O_2 \rightarrow CO_2$	+ 0.82	- 479
Oxic zone	Nitrate reduction	$NO_3^- + H^+ \rightarrow N_2$	+ 0.71	- 453
Suboxic zone	Iron reduction	$Fe_2(OH)_3 + 2H^+ \rightarrow Fe^{2+}$	+ 0.77	- 228.3
Fermentation zone	Fumarate respiration	Fumarate \rightarrow succinate	+ 0.03	- 86.0
Sulfate zone	Sulfate reduction	$SO_4^{2-} + 2H^+ \rightarrow HS^-$	- 0.22	- 152.2
Methanogenic zone	Methanogenesis	$H_2 + CO_2 \rightarrow CH_4$	- 0.24	- 131

* values were adapted from Weber *et al.* (2006).

** values were taken from Thauer *et al.* (1977).

2.7 Intraterrestrial adaptation of microbes: growth, activity and survival

Microbial growth in the DGR is dictated by the availability of nutrients and the physicochemical properties of clay. The physical properties of the clay buffer, such as low pore diameter (~0.01 μ m) (Volckaert *et al.*, 2004), high temperature (70-90 °C) (Volckaert *et al.*, 2004), high swelling pressure (> 2,000 kPa) (Stroes-Gascoyne and West, 1997; Stroes-Gascoyne, 2010; McMurry *et al.*, 2003; Pusch and Weston, 2003), low hydraulic conductivity (<1x10⁻¹² m/s) (Smart *et al.*, 2011), clay dry density of at least 1.6 g/cm³ (Stroes-Gascoyne *et al.*, 2007a; Stroes-Gascoyne *et*

al., 2010a, 2010b; Stroes-Gascoyne and Hamon, 2008) and a water activity of <0.96 (Jay *et al.*, 2005; Stroes-Gascoyne *et al.*, 2011; Stroes-Gascoyne and Hamon, 2008), among other parameters, would be expected to collectively suppress microbial proliferation, growth and/or survival.

Microorganisms have evolved in myriad ways to resist harsh environmental conditions. Depending on the condition, some microorganisms may perish, while some could remain dormant (inactive state), or enter a “resting state” (sporulation) until the necessary nutrients and conditions became available. Inactive or dormant microorganisms may further become viable-but-not-culturable (VBNC) (Onstott *et al.*, 2010). Many microorganisms can achieve a low energy-requiring metabolic state in order to survive. Such microorganisms can adapt to, or survive in, extremes such as those found in dense clays. The genera *Bacillus* and *Clostridium* (and possibly including *Negativicutes* (Marchandin *et al.*, 2009)) are capable of undergoing morphological changes to form endospores when deterioration of environmental conditions occurs. *Bacillus*, for instance, commits to sporulation after it fails to deal with starvation in different ways, such as cannibalism (Gonzalez-Pastor *et al.*, 2003) or formation of a genetically-competent state (i.e., developing resistance against antibiotics or compounds toxic to cells) (Veening *et al.*, 2006). De Hoon *et al.* (2010) summarized the morphological stages of the sporulation cycle of *Bacillus subtilis*. Accordingly, an endospore is established when the forespore, which is formed after asymmetric division of the cell, matures. The maturation process is regulated by transcription factors that are triggered in a cell-specific manner (De Hoon *et al.*, 2010). Fully-formed spores are resistant to heat, desiccation, radiation and oxidation; however, once environmental conditions become favourable, spore germination occurs (Setlow, 2003) and vegetative growth resumes. It was reported that previously unknown species of *Alicyclobacillus* (spore-forming) and *Sphingomonas* (non-spore forming) genera (Poulain *et al.*, 2008), and *Desulfosporosinus* spp. (spore-forming) (Ratto and Itavaara, 2012), have been isolated from bentonite clay.

Biofilm formation provides homeostasis when microbes exist in fluctuating and harsh environmental conditions. Biofilm formation has been reported to involve three stages: i) attachment, ii) maturation, and iii) dispersion (Klausen *et al.*, 2003). Costerton *et al.* (1999) explained that the initial binding of bacteria (planktonic cells) on a surface is reversible, but once the attached monolayer of cells multiply and become encased in an extracellular polymeric

substance (EPS), their adherence to the surface becomes more permanent. As soon as the biofilm reaches maturity, it may then disperse either by shear forces, nutrient scarcity or by genetic programming that mobilizes planktonic cells.

2.8 Biogeochemical processes relevant to a DGR

Microbes are present in almost all subsurface habitats at varying population densities; thus, their overall activities have the potential to influence the functionality of the EBS in a DGR. Microbial activities can include the formation of a microenvironment that may lead to biomineralization, radionuclide sorption onto organisms, microbial degradation of metabolites and gas production (McCabe, 1990). The production of microbial end products such as sulfide, hydrogen, carbon dioxide, methane, formate, acetate and lactate via microbial degradation of carbon present in backfill or by anaerobic corrosion of metal containers may also impact a DGR. Therefore, it is important to identify these microorganisms, their metabolic activities, as well as factors for their control.

2.8.1 Reductive dissolution of bentonite

Direct contact between clay minerals and microbes could generate reactions that would affect the physical and chemical features of the clay minerals (Dong, 2012). Among those reactions is the biological reductive dissolution of structural Fe (III). The reduction process involves the presence of Fe (III) (electron acceptor), organic carbon (electron donor) and microorganisms (Meleshyn, 2014; Dong, 2012). Meleshyn (2014) and Perdrial *et al.* (2009) proposed four distinct mechanisms for Fe (III) bioreduction. In one of the mechanisms, the iron-reducing bacteria, *Shewanella putrefaciens*, was able to sequester ferric iron from nontronite (an iron-rich smectite) and reduce it inside the cell even if nontronite contained < 0.19 % organic carbon (Perdrial *et al.*, 2009). However, no bioreduction was observed in MX-80 bentonite. Hofstetter *et al.* (2014) reported on the reducibility of MX-80 bentonite and found that MX-80 requires a higher E_H -range to reduce iron. This is attributed to the low Fe content in MX-80 bentonite, which was found to be only at 2.3 wt.-%.

Reduction and dissolution of Fe (III) in bentonite are intimately related since both are triggered by the microbial demand for Fe (III). Previous studies have shown that upon reduction of Fe (III) in bentonite, Fe (II) was released to the environment (Jaisi *et al.*, 2008; Perdrial *et al.*, 2009). However, the release of Fe (II) and its sorption in clay minerals and microbes may hinder Fe (III) reduction (Dong, 2012). The consequences of these reactions could ultimately affect the

bentonite physically and chemically. These include the irreversible reduction of Fe (III) on the surface, an increase in the interlayer spacing, and a reduction in swelling capacity of the bentonite material (Kostka *et al.*, 1999; Stucki and Kostka, 2006). For these effects to occur, microorganisms would have to be active, and thus at growth-permitting regions (e.g., interfaces) and not within the growth-restricting HCB matrix.

2.8.2 Biofilm formation

Biofilm formation would create a stable microenvironment favourable for microbial growth and would also provide protection against toxic compounds (Templeton *et al.*, 2009). In the EBS clay buffer, biofilm formation would not be expected to occur to a significant extent, unless the integrity of the system was disrupted. However, it is still possible that biofilms will develop within specific locations of the DGR, for instance, at interfaces between the host rock-clay buffer and container-clay buffer, and within fractures and fissures. At these locations, biofilms might form due to increased availability of space, which would permit enhanced cellular interactions probably because of better access to nutrients and water at clay-rock interfaces. It has been noted that the presence of biofilms in the EBS may affect the integrity of the system (Stroes-Gascoyne and West, 1997) in the following ways: i) decreasing the adsorption capacity of the rock in terms of radionuclides, ii) inducing corrosion on the containers (Little *et al.*, 1991), iii) plugging of pores and decreasing the permeability of the backfill materials, and iv) aiding in radionuclide migration in any DGR fractures and at interfaces. Recent studies have confirmed that biofilms could cause clogging or plugging of pore spaces (Wolfaardt *et al.*, 2007; Perdrial *et al.*, 2009), cement-weathering and deterioration caused by biofilm metabolites (Stroes-Gascoyne *et al.*, 2007b).

2.8.3 Microbially-influenced corrosion

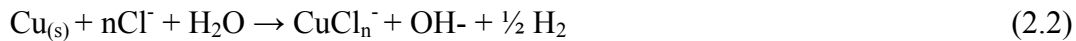
Microbially-influenced corrosion (MIC) is an electrochemical processes that allows microorganisms to transfer electrons from zero-valency metals to electron acceptors (Meleshyn, 2014). Corrosion of the metal canister in a DGR is of concern because it might compromise its containment function. Microbes may directly (through their metabolic action at the container surface) or indirectly (through production and diffusion of corrosive metabolites through the buffer to the container) cause corrosion of metal containers (Scully and Edwards, 2013; Little and Wagner, 1996). It has been suggested that the proliferation of microbes near the container is unlikely as long as the clay buffer remains intact and functional (Stroes-Gascoyne and West,

1997). Microbes may potentially cause corrosion of the three different types of metal containers specified by various international DGR programs – copper (Canada and Finland), copper-iron (Sweden) and iron/steel (Japan, France, Belgium, Germany, Switzerland and United Kingdom) (Wolfaardt and Korber, 2012). Scully and Edwards (2013) indicated that there are three corrosion processes of copper in oxygen-free water.

i) Presence of pure water



ii) Presence of water and high chloride concentrations



iii) Presence of water and sulfides (sulfate-reducing bacteria)



or



Kwong (2011) mentioned that the anaerobic copper corrosion process shown in equation 2.1 is thermodynamically unfavourable; thus, very unlikely to happen. Equations 2.3 and 2.4, on the other hand, are more likely to happen, especially under anaerobic conditions (King *et al.*, 2002). Dissimilatory sulfate-reducing bacteria can use H_2 or organic compounds to reduce nitrate, sulfite, thiosulfate or fumarate to survive (Little and Lee, 2007). Several studies have supported the use of hydrogen or organic matter as electron donors for SRB (Stroes-Gascoyne and King, 2002; Kwong, 2011; Pedersen, 2010). Scully and Edwards (2013) provided several possible reactions pertaining to equation 2.2, and indicated that chloride ions, which could come from saline groundwater, may play a role in the formation and properties of surface films on copper, which could most likely cause negative effects on the surface of the container.

The corrosion of iron or steel occurs when elemental iron is oxidized to ferrous iron (equation 2.5). In the presence of water and oxygen (equation 2.6), ferrous hydroxide (equation 2.7) would be generated. Further exposure of ferrous hydroxide to water and oxygen would generate ferric hydroxide (equation 2.8). Corrosion may continue even if oxygen has been consumed by corrosion and by microbes through consumption of water to produce hydrogen. When the reduction of water (equation 2.9) is coupled with iron oxidation, ferrous hydroxides, ferric hydroxides, or ferric oxides may be formed. The resulting physical damage is the rusting of the

surface of the container, which could eventually lead to a breach in the canister and possible release of radionuclides.



Little and Lee (2007) provided a summary table of microorganisms (e.g., *Desulfovibrio*, *Desulfomonas*, *Acidithiobacillus ferrooxidans* and *Pseudomonas*) that can contribute to MIC. The authors also comprehensively showed how the metabolites produced by bacteria can cause corrosion. One example is where sulfate ion is used as terminal electron acceptor to produce hydrogen sulfide (HS^-) by SRB. In this case, the hydrogen sulfide may cause hydrogen sulfide-induced stress corrosion cracking. Little and Wagner (1996), in addition, provided examples of corrosive chemical species such as sulfide, ammonia and acid, that could be microbially produced and could affect the containers.

2.8.4 Microbial gas production

Gas production in a DGR includes anaerobic metal corrosion, microbial degradation of organic material and radiolysis of water (Capouet *et al.*, 2015). Yet, it is anticipated that microbial gas production in a DGR for high level waste (highly radioactive) would be improbable since microbial activity and carbon sources will be kept at minimum (Sauzeat *et al.*, 2001; Stroes-Gascoyne *et al.*, 2010b). The production of H_2 generated as the product of iron corrosion (equation 2.9) is one of the most energetic substances in the subsurface and is also the major concern in a DGR. It could drive microbial processes, like methanogenesis and acetanogenesis, to produce methane (methanogenesis) and acetic acid (acetogenesis) (equations 2.10 and 2.11) (Pedersen, 1999; Stevens and McKinley, 1995; Stevens, 1997; Pedersen, 2000). H_2 production could also be generated in the corrosion of copper by sulfide as shown in equation 2.3.



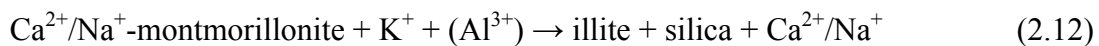
In an environment with limited supply of organics, H_2 could also be used to reduce Fe (III) (Amend and Shock, 2001) and enhance the activity of SRB when dissolved organic matter is limiting (Chautard *et al.*, 2013). Based on the deep hydrogen-driven biosphere hypothesis, such

an environment could theoretically permit autotrophic microorganisms to proliferate (Pedersen, 1999). Fe (III) reduction coupled with oxidation of lactate or acetate would produce CO₂ (Amend and Shock, 2001) and SRB reactions could also generate H₂S corrosive gas and more CO₂ (Libert *et al.*, 2011). The generation of carbon dioxide could enhance the solubility or mobility of radionuclide in the buffer (Hersman, 1997).

Fermentation is an anaerobic process that produces a variety of end products including, for example, hydrogen, carbon dioxide, acetate, ethanol, lactate, and formate. Fermentation is the process where complex organic matter is broken down in to simpler compounds in the absence of terminal electron acceptors. However, it is not likely for the EBS environment to sustain prolonged fermentation because there would be an insufficient amount of organic material coming from the backfill or clay buffer for microbes to utilize. This is due to the higher rates of aerobic or anaerobic reactions, which would utilize organic materials to support their function, leaving an inadequate quantity of organic matter for fermentation to persist over extended time periods (Wang and Francis, 2005).

2.8.5 Biotransformation

Bioconversion of smectite to illite (equation 2.12) (SKB, 2006) may also affect the properties of the bentonite buffer. The conversion of montmorillonite to illite would affect interactions with water and the resultant swelling pressure of the clay. Upon conversion to illite, water is released causing fewer and bigger pores. As a result, the hydraulic conductivity of the clay buffer is affected, potentially affecting the overall functionality of the buffer.



Temperature (300 to 350 °C), pressure (100 MPa) and time (4 to 5 months), in the absence of microbial activity, have been hypothesized to be the major factors affecting the smectite to illite reaction (Mulligan *et al.*, 2009). However, it has been determined that the microorganisms can dissolve smectite by reducing Fe (III) at room temperature, at 101 kPa over a period as short as 14 days (Kim *et al.*, 2004). Hama *et al.* (2001) also indicated that SRB caused a reduction in clay permeability because SRB promoted formation of secondary clay minerals. Nevertheless, Wolfaardt and Korber (2012) suggested that microbial smectite to illite conversion process would not likely be significant for the functioning of the clay buffer because the conditions in the bentonite matrix are highly inhibitory to microbial activity.

2.8.6 *Microbe-radionuclide interactions*

In the event of UFC failure, microorganisms would have direct access to radionuclides, and may also inhibit or accelerate radionuclide transport. This is more likely to occur if the surrounding regions are less dense or contain fractures. Microbial colonization and activity at these regions could therefore affect the solubility, speciation and sorption of radionuclides (Francis, 2006) – e.g., uranium and plutonium. For instance, the presence of *Thiobacillus ferrooxidans* in uranium ore has been reported to oxidize reduced compounds of uranium (UO₂ and uranium sulfate) (Wang and Francis, 2005). Bioaccumulation of uranium was also observed at the cell surface of *Halomonas* species. Extended x-ray absorption fine structure (EXAFS) analysis, which was used to determine the functional groups involved in uranium-bacteria complexation processes, showed that uranium was associated to the cell surface through phosphate complexes and ligands (Francis *et al.*, 2004). Microbial reduction of soluble U (VI) to insoluble U (IV) has been observed in some *Clostridium* and *Shewanella* species (Francis *et al.*, 2004; Ganesh *et al.*, 1997; Min *et al.*, 2005). Complexation of *Pseudomonas fluorescens* with curium (III) has been observed and could, therefore, influence the migration of actinides from nuclear waste repository (Moll *et al.*, 2008).

Sorption of Pu (VI) into the bacterial cell because of microbe-radionuclide interaction has been observed (Panak *et al.*, 2002; Ohnuki *et al.*, 2010). Panak *et al.* (2002) observed the reduction of Pu (VI) to Pu (IV) before forming complexes with the phosphate group at the bacterial surface of *Bacillus sphaericus*. Bioreduction of Pu (VI) to Pu (IV) has also been detected in other species such as *Shewanella putrificiens* and *Geobacter metallireducens* (Neu *et al.*, 2005; Newsome *et al.*, 2014; Perdrial *et al.*, 2009). Vilks (2009) discussed how brine solutions, especially naturally-saline groundwater present in a granitic DGR, would saturate the host rock and bentonite and thereafter affect sorption of radionuclides in the host rock and clay minerals. According to Vilks (2009), efficient adsorption of radionuclide to the bentonite could retard the transportation of radionuclides to the surface. Vilks *et al.* (2011) subsequently found that U (VI) and Eu (III) were highly sorbed (through complexation) in bentonite saturated with Na-Ca-Cl brines, the Canadian Shield groundwater composition. Francis (2006), Wolfaardt and Korber (2012) and Wang and Francis (2005) provided more in-depth examples on the consequences of microbe-radionuclide interactions in the subsurface.

2.9 Impact of DGR conditions on microbes

Upon placement of highly radioactive UFCs, the DGR would quickly develop harsh conditions, making it difficult for microorganisms to evolve or adapt to their new environment and likely resulting in a biologically-inactive clay buffer zone immediately surrounding the containers (McMurry *et al.*, 2003). The used nuclear fuel contains beta-gamma emitting fission products, alpha- and gamma-emitting actinides (Garisto *et al.*, 2009) and neutron-emitters (Wolfaardt and Korber, 2012). These forms of radiation are highly energetic (i.e., ionizing) and thus would inflict deleterious effects on microbes through immediate damage to DNA, RNA and cell membranes if thin-walled UFCs were to be used. In the presence of water, radiolysis would occur, and would generate highly-reactive oxygen species (free radicals), causing even greater damage to DNA and lipid membranes (Wallace, 1998). Stroes-Gascoyne and West (1997) elaborated on the studies conducted to examine the likelihood of creating biologically inactive zones in the EBS. Their studies looked at the radiation sensitivity of the native microbial population in buffer material, including those that were capable of forming spores. It revealed that radiation, temperature, moisture content and water activity had individual detrimental impacts on microbial survival in the EBS. For instance, Stroes-Gascoyne *et al.* (1995) irradiated untreated and microorganism-inoculated buffer (50 % bentonite, 50 % sand) samples with gamma-radiation from a ^{60}Co source (dose rate of 6 kGy/h). The obtained D_{10} values (i.e., total dose required to reduce the amount of viable cells by 90 %) ranged from 0.34 to 1.68 kGy. One radiation-resistant species was found to survive radiation dose of 4 kGy upon exposure. However, once more than 4 kGy of radiation dose was applied, the number of cells in the microcolonies was reduced to single viable cell. The results indicated that the dose required to sterilize the region adjacent to the used nuclear fuel container was dependent on the resistance of the native or introduced microorganisms present. When an intensive microbial sampling program was conducted during the decommissioning of the Buffer/Container Experiment (BCE) at the AECL Underground Rock Laboratory (URL) (Stroes-Gascoyne *et al.*, 1996), the authors found that viable microorganisms disappeared at 50-60 °C when the moisture content was <15 %, in 50 % sand – 50 % bentonite buffer with a maximum density of 2 g/cm³. Stroes-Gascoyne and Hamon (2010) also reported on the effects of elevated temperature on bacterial viability and culturability. Stroes-Gascoyne *et al.* (1996) observed that a water activity (a_w) of ~ 0.96 (most bacteria cannot survive at $a_w < 0.90$ -0.95 (Brown, 1990)) caused a large decrease in the number

of culturable bacteria. The results of this study were in agreement with studies by Motamedi *et al.* (1996) and Pedersen and Karlsson (1995) who observed similar outcomes for SRB survival. Bentonite clay swelling ability is dictated by the surface charge of the clay particles, along with its cation exchange capacity (Stotzky, 1986; Mortland, 1986). In the EBS, groundwater is adsorbed, causing the clay to expand, and the more compacted the clay, the greater the swelling potential and hence subsequent pressure (Craig, 1987). Stroes-Gascoyne *et al.* (2006) determined that a clay dry density of $\geq 1.6 \text{ g/cm}^3$, which generates a resultant pressure of $\geq 2,000 \text{ kPa}$ and $a_w \approx 0.96$, caused the number of viable cells to remain at background levels (i.e., $\leq 2 \times 10^2$ Colony Forming Units per g) in highly compacted bentonite clay when porewater salinity was $> 50 \text{ g NaCl}$. However, this result holds for MX-80 Wyoming bentonite clay, other bentonite clays may provide different parameter values (Stroes-Gascoyne, personal communication). Hedin (2006) similarly supported this premise by suggesting that a swelling pressure of 2,000 kPa or greater would eliminate microbial activity in compacted clay. Pedersen (2000) was able to isolate *Desulfovibrio aespoeensis* and *Desulfomicrobium bacalatum* from the Aspö Hard Rock Laboratory (HRL) (Sweden), but found that these species were unlikely to survive if a total density higher than 1.8 g/cm^3 was achieved in the buffer. Furthermore, a porewater salinity of $> 50 \text{ g NaCl per L}$ or $\geq 50 \text{ g CaCl}_2 \text{ per L}$ (Stroes-Gascoyne and Hamon, 2008, Stroes-Gascoyne *et al.*, 2010a) was also demonstrated to be sufficient to keep the number of culturable microbes at or below background levels.

The effect of small clay particle size is also relevant in terms of microbial activity. For instance, Mauclaire *et al.* (2007) found it very unlikely for bacterial transport to occur because of the restrictive pore size ($< 0.2 \text{ }\mu\text{m}$) in the Opalinus clay formation. Stroes-Gascoyne *et al.* (2010b) suggested that if the pore size is smaller than a starved or ultramicrobial cell size ($0.2\text{-}0.4 \text{ }\mu\text{m}$), it would negatively affect microbial activity. Frederickson *et al.* (1997) found no evidence of microbial activity in shales with a pore throat size of $< 0.2 \text{ }\mu\text{m}$. The authors added that subsurface microorganisms require interconnected pore throats ($> 0.2 \text{ }\mu\text{m}$) to sustain microbial activity. Additionally, a conceptual model proposed by Pedersen (2000) suggested that as saturation of bentonite clay buffer with groundwater increased, the number of microbes would decrease significantly during swelling such that only a few viable cells would survive a fully compacted (full compaction density of 2 g/cm^3 and water content of 26 %) clay buffer. As a result of the Pedersen (2000) study, sulfate-reducing bacteria were found to decrease in number

with depth, and activity declined as full compaction was achieved. Although some spore-forming bacteria would survive, they would eventually die out. Also, since the pore size of the intact clay buffer would be 100 to 1000 times smaller than the microbes, it is very unlikely that new microorganisms would be introduced, due to mechanical filtration effects of the matrix.

The DGR, depending on the rock formation, could have inadequate nutrients, which would affect the density of microbial population in the subsurface. Stroes-Gascoyne and West (1997), for instance, indicated that granitic host rock formations would be expected to be nutrient-poor. Other studies have revealed the presence of organic, inorganic and carbon-containing compounds in bentonite clay deposits, but at low concentration (McMurry *et al.*, 2003; Stroes-Gascoyne, 1989; Sheppard *et al.*, 1997). However, these conditions could change as nutrients would be introduced into a DRG during construction, operation and closure. Sources of allochthonous microbes (mentioned earlier) would also come from the buffer and groundwater. Analyses of emplaced material, including used fuel, metal containers, buffer and backfill materials, were conducted to determine whether these materials could provide nutrients to support microbial growth (Stroes-Gascoyne, 1989). It was found that while nitrogen and phosphorus were present, they would still be the key growth-limiting factors in the EBS. Sources of nitrogen and carbon could also come from the blasting residues from excavation processes, drill water, service water, and spilled oils, lubricants grease and paint (Stroes-Gascoyne and West, 1997; Meleshyn, 2014).

2.10 Approaches and analytical methods for subsurface microbiological analyses

The application of appropriate methods for subsurface microbiological analyses is relevant since, from time to time, traditional microbiology procedures might not be applicable or yield representative results due to the unique physical and chemical properties of the environment. Generally, depending on the objective of the study, the most suitable methodological procedure or analysis must be applied.

2.10.1 Culture-based approaches

Culture-based approaches have been used for several decades to study subsurface microbial diversity, as well as to characterize microorganisms in potential DGR sites (Hallbeck and Pedersen, 2008; Poulain *et al.*, 2008; Vreeland *et al.*, 1998; Chi Fru and Athar, 2008). These methods employ the use of general and selective media to isolate microorganisms from various environmental samples (Bull, 2004) under a broad range of culture conditions (APHA, 1992). However, in doing so, it is also important to provide an environment similar to the

microorganisms' original habitat in order to obtain representative results (Zinder and Salyers, 2001). Estimated numbers of culturable microorganisms can be obtained by directly counting the number of microorganism on solid media or by using liquid media in conjunction with the Most Probable Number (MPN) technique (Oblinger and Koburger, 1975). The use of total number of cells and culturable cell counts to directly and indirectly identify the number of culturable cells are also considered useful. Hallbeck (2009) explained these methods in detail. It is important to note that while culture-based approaches isolate microorganisms that are the most culturable, these are not necessarily the most functionally-relevant species (Rappe and Giovannoni, 2003). Hence, one cannot always correlate the absence of various microorganisms determined using culture-based analyses with their environmental significance (Humphreys *et al.*, 2010). It was estimated that only 0.01 to 0.1 % of the total microbial cell counts observed using fluorescence microscopy form colonies on agar plates (Ferguson *et al.*, 1984). Torsvik *et al.* (1990) reported that only 0.3 % of microorganisms in soil are culturable in comparison to total cell counts, while only 0.25 % in sediments (Jones, 1977). This limitation highlights the need for a more efficient means of acquiring more representative microbial community from a specific environment. Accordingly, the use of culture-independent methods to overcome culture-based biases, and underestimation of biological diversity, have undergone rapid growth.

2.10.2 Culture-independent methods

Culture-independent methods preclude the need to isolate and cultivate microorganisms, and further do not impose biases based on whether an organism is “culturable” or not on a particular growth medium. Molecular approaches to characterize microbial community diversity and to study their phylogeny include DNA/DNA or DNA/RNA hybridization, DNA fingerprinting, and 16s ribosomal RNA (rRNA) analysis (Amann *et al.*, 1990; Saylor and Layton, 1990; Stahl, 1997; Ward *et al.*, 1992). Each method requires the extraction of nucleic acids from the environmental sample followed by amplification using the polymerase chain reaction (PCR) before further analysis. DNA fingerprinting, for instance, involves the extraction and purification of DNA. The DNA is digested using restriction enzymes into fragments of varying length, before subjecting them to gel electrophoresis where the bands obtained from the unknown species are compared to resulting bands of known species (Jeffreys *et al.*, 1985a,b). Direct sequencing (e.g., pyrosequencing), on the other hand, is also a DNA sequencing method wherein an immobilized DNA template will have its complementary strand synthesized enzymatically through sequential

addition of deoxyribonucleotides (A, C, G and T). Once the nucleotide solution complements the unpaired base in the DNA template, a light is produced and detected (Mostafa *et al.*, 1998). Thereafter, the sequences of the template DNA are determined for comparison with DNA databases. These methods generally broaden the percentage of the target microbial community covered because none of the methods requires microbial culturing. Moreover, because the majority of the subsurface microorganisms have not been cultured yet, these methods simply have become standard methods for identifying these microorganisms (Ehrlich and Newman, 2009).

2.10.2.1 DNA extraction and target genes

Obtaining representative DNA extracts from the microbial subsurface community is important, but may be challenged by low nucleic acid yield, adsorption of cells to the sediment particles and co-extraction of organic matter and clay materials (Wolfaardt and Korber, 2012). The nature of the subsurface matrix therefore plays a key role with respect to DNA extraction success, with matrices with high clay content being particularly problematic (Ogram *et al.*, 1987). Direct cell lysis methods in which cells are lysed within the sediment have been found to yield more DNA (Leff *et al.*, 1995; Luna *et al.*, 2006) than separating cells from the sediment matrix before lysis (cell extraction). The most common direct cell lysis protocol used for difficult sediments is the Zhou method (Zhou *et al.*, 1996) used in conjunction with bead-beating (Webster *et al.*, 2003).

As briefly described by Hallbeck (2009), obtaining samples for molecular microbiological investigations involves: i) extraction of DNA from an environmental sample, ii) amplification of target DNA using the polymerase chain reaction (PCR), and iii) PCR amplicon characterization (e.g., cloning, gel electrophoresis or DNA fingerprinting). The 16S rRNA gene is universally present in bacteria and archaea (Coenye and Vandamme, 2003), and can be used for phylogenetic comparisons (Woese, 1987). It is ideal for such use because it contains conserved and hypervariable regions that enable alignment and then comparison, respectively, with different organisms or groups of organisms.

2.10.2.2 DNA fingerprinting methods

PCR products may also be characterized using denaturing gradient gel electrophoresis (DGGE) (Humphreys *et al.*, 2010). In DGGE, the PCR products of the same length but different base pair (bp) combinations are separated on a polyacrylamide gel with an increasing linear denaturing gradient (Muyzer *et al.*, 1993). The denaturants are a mixture of urea and formamide (Muyzer *et*

al., 1993; Muyzer and Smalla, 1998). During denaturation, the double-stranded DNA separates or melts at a specific denaturant concentration and separation will continue until the GC clamp (GC-rich sequence around 40-60 nucleotides long) is reached, so as to prevent complete denaturation and strand separation. The fragment containing the GC clamp will generate a Y-shaped DNA structure and will migrate only to a certain position in the gel. The resulting band-pattern reflects the microbial diversity of the sample (Muyzer *et al.*, 1993).

The PCR-DGGE method has been used to profile microbial communities from microbial mats and biofilms (Muyzer *et al.*, 1993; Muyzer and Smalla, 1998), water (Wu *et al.*, 2006; Lawrence *et al.*, 2008) and soil (Maarit, *et al.*, 2001; Allison *et al.*, 2007). According to Duarte *et al.* (2012), DGGE has been used for: i) analysis of complex communities, ii) monitoring shifts in microbial populations, iii) discovery of sequence heterogeneities, iv) comparison of DNA extraction techniques, v) screening of clone libraries, and vi) determination of PCR and cloning biases (Muyzer and Smalla, 1998). However, there are limitations to the use of PCR-DGGE, including: i) only small fragments (~500 bp or less) can be separated (Muyzer and Small, 1998), ii) inaccuracies in band species representation due to different DNA having similar mobility rates due to identical GC content (Muyzer *et al.*, 2004), iii) introduction of contaminants during DNA isolation and PCR amplification (Muyzer *et al.*, 2004), and iv) possible intra-specific and intra-isolate heterogeneities (Duarte *et al.*, 2012) of rRNA genes which may give rise to multiple banding patterns for a single species (Michaelsen *et al.*, 2006; Nakatsu *et al.*, 2000).

2.10.2.3 Metagenomics

Metagenomics is the study of the metagenome, or all genetic material, present in an environmental sample. Metagenomic-based methods involve direct isolation of DNA from environmental samples, and provide a thorough assessment of the overall microbial biodiversity and improve estimates of comparative microbial abundance (Kennedy *et al.*, 2011). Several metagenomic techniques are currently available for sequence-based and functional analysis of microbial genome (Wolfaardt and Korber, 2012; Sherwood Lollar, 2011; Simon and Daniel, 2011; Handelsman, 2004). For instance, shotgun Sanger sequencing has been used to sequence microbial DNA fragments from environmental samples (Venter, *et al.*, 2004; Tyson *et al.*, 2004). The massively-parallel 454 pyrosequencing, Applied Biosystems SOLiD™ 3, Ion Torrent and Genome Analyzer of Illumina (Wolfaardt and Korber, 2012; Bentley, 2006; Margulies *et al.*,

2005) platforms are among the high-throughput systems that offer a more sensitive means of analyzing microbial environmental samples that traditional DNA fingerprinting cannot cover.

2.10.3 DGR laboratory simulation studies

Culture-based methods can be further analyzed through enrichment cultures and microcosm studies. Microcosm studies impose selected microorganisms to similar environmental conditions as found at their original habitat (Humphreys *et al.*, 2010). This is important since it allows for replicating an environment where, for instance, the interaction of specific microorganism with radionuclides can be examined (Boukhalifa *et al.*, 2007). Enrichment cultures are used to examine particular functional groups of microorganisms in the environment (Sherwood Lollar, 2011) by culturing them in conditions that are specific for their growth. As a result, these particular microorganisms will dominate the culture. Chi Fru and Athar (2008) used a modified anaerobic medium enriched with lactate to grow SRB and acetogens from Äspö groundwater and Wyoming MX-80 bentonite samples. Microcosms/enrichments can be conducted in sealed-batch systems or flow-through systems where nutrients can be continuously added (e.g., Nikolova *et al.*, 2001; West *et al.*, 1998; Kelly *et al.*, 1998). Stroes-Gascoyne *et al.* (2010b) have demonstrated the use of pressure-cell systems to simulate conditions in the EBS of a DGR.

2.11 HYPOTHESES FOR THIS STUDY

1. Survival of indigenous microorganisms in clay implies possession of metabolic pathways, or other strategies, that are beneficial to prolonged adaptation to extreme conditions,
2. Physical forces and availability of energetically-spontaneous carbon sources, and clay mineral and groundwater chemical composition, regulate microbial activity and culturability in the buffer material, and
3. Microbial activity and culturability define significant biogeochemical processes present in the bentonite buffer material and DGR.

2.12 OBJECTIVES FOR THIS STUDY

1. To characterize the microbial communities present in natural and commercial clays with the goal to elucidate their potential to impact the functionality of the engineered barrier system (EBS) by generating corrosive products (e.g., hydrogen sulfide) and by potentially forming aggregates at water-liquid interfaces.
2. To investigate microbial activity (e.g., survival and growth) under simulated DGR conditions. Information obtained from this investigation will aid in the understanding of the potential for MIC and microbial influence on bentonite clay adsorption capacity that is part of the EBS.
3. To develop appropriate model systems to study the potential for microbes to survive and proliferate at DGR interfaces.

3. DETECTION OF INDIGENOUS BACTERIA IN COMMERCIAL BENTONITE

Interface:

This work was done in collaboration with Dr. Alexander Grigoryan at the University of Saskatchewan.

Daphne Jalique (DJ) performed the isolation of bacteria and extraction of DNA from colonies. Moreover, DJ assisted in directly extracting DNA using the Swiss and Modified Swiss protocols as well as contributed to the results, data analyses and interpretation.

Dr. Alexander Grigoryan performed preliminary work on DNA extraction directly from bentonite samples using commercial kits. The application of the Swiss and Modified Swiss DNA extractions methods, described in Appendices 10.2 and 10.3, on bentonite samples were mainly performed by Dr. Grigoryan.

Dr. Darren Korber provided guidance and assisted with experimental design, provided editing of the section, and held the research grant that supported this work.

3.1 Abstract

Anaerobic and aerobic microorganisms may exist in natural and commercial clay materials. The identification and characterization of these microorganisms are important because determining the biodiversity of microorganisms would assist in the validation or improvement of the EBS design. Since clays present a challenge to existing DNA extraction protocols, it is desirable to improve methods for nucleic acid recovery. Two direct cell lysis protocols have been introduced in this study, the Swiss and the Modified Swiss protocols. It was determined that the Modified

Swiss protocol is more effective at extracting DNA directly from commercial and natural clay samples than is the Swiss protocol. However, the presence of humic acids restricts efficient production of amplifiable PCR products. The limitations of the Modified Swiss protocol illustrate the need to seek other means to effectively isolate bacteria from commercial and natural clay samples.

3.2 Introduction

The diversity of microorganisms in an environment regulates the relevant biogeochemical processes affecting the system. However, the accurate identification of microbial communities present in a specific environment poses a challenge since the majority of bacteria cannot be cultured (Amann *et al.*, 1995; Roose-Amsaleg *et al.*, 2001). Culture-based approaches have been traditionally used to characterize microorganisms and to study microbial diversity in materials associated with a DGR (Hallbeck and Pedersen, 2008; Poulain *et al.*, 2008; Vreeland *et al.*, 1998). The application of general and selective media allows isolation of a wide range of microorganisms under varying conditions (APHA, 1992; Zinder and Salyers, 2001). Recent improvements in cultivation procedures and culture media preparation have been achieved to simulate natural environments in order to maximize cultivation of microorganisms. For instance, Zengler *et al.* (2005) have devised a novel microbial cultivation technique by encapsulating single cells within gel microdroplets under low nutrient flux conditions, and allowing them to grow and form into microcolonies. The microencapsulation technique was able to provide more than 10,000 bacterial and fungal isolates per soil/seawater sample. Nevertheless, not all microorganisms are cultivable, and many remain unculturable. The majority of microorganisms that remained unculturable are either not suited to the imposed growth conditions due to extreme adaptability to their environment of origin, or have entered the viable-but-not-culturable (VBNC) state (Onstott *et al.*, 2010; Roose-Amsaleg *et al.*, 2001).

The extreme conditions associated with a DGR would make laboratory culturing of the microbial community more challenging (Ogram *et al.*, 1987). Extracting DNA from subsurface bentonite samples would also be problematic due to low nucleic acid yield, adsorption of cells to the sediment particles and co-extraction of organic matter and clay materials (Wolfaardt and Korber, 2012). Direct cell lysis from clay is the preferred method for extraction of DNA from bentonite clay material (Roose-Amsaleg *et al.*, 2001). The method has been found to yield more DNA because cells are lysed within the sediment (Leff *et al.*, 1995; Luna *et al.*, 2006). The most

common direct cell lysis protocol used for difficult sediments is the Zhou method (Zhou *et al.*, 1996) which is used in conjunction with bead-beating (Webster *et al.*, 2003). Rochelle *et al.* (1992) used direct lysis to extract DNA from ~3-million year-old sediment. The authors extracted DNA from different depths, and were able to recover a total bacterial density of 2.7×10^7 bacteria per g as determined by acridine orange direct counts.

The objective of the current study is to identify and characterize microbial communities in natural and commercial clays in order to determine their potential impact on the overall functionality of the DGR. Two direct lysis protocols were initially tested to directly isolate DNA from natural clay and commercially available bentonite samples (Swiss protocol (Appendix 10.2) and the Modified Swiss protocol (Appendix 10.3)). ; however, neither of the protocols was successful in extracting DNA. As a result, culture-dependent methods were used to isolate the bacteria, followed by identification using standard molecular techniques.

3.3 Materials and Methods

3.3.1 *Commercial and Natural Clay Sources*

Clay samples used in this study were obtained from the American Colloid Company (Wyoming MX-80 Bentonite), Canadian Clay Product (CCP) Inc. (Canaprill[®]) (subsequently referred to as Avonlea clay) and Mont Terri Rock Laboratory (two core samples of the Opalinus clay rock formation). Appendix 10.1 provides the elemental compositions of the commercial clays used in this study. According to the technical data provided by Sigma Aldrich and the American Colloid Company, the elemental compositions of these products are similar. The Canaprill clay samples were obtained from the clay stockpiles at the CCP site in Wilcox, SK, which were originally excavated from the Truax bentonite quarry in Truax, SK. Clay samples were placed in Ziploc[®] bags and stored in a cooler with gel packs. Upon arrival in the laboratory, the samples were immediately stored at -80 °C until used for testing.

The Opalinus clay core samples (Core BFE-A11) were obtained from the 170-million-year-old clay stone formation at Mont Terri's Underground Rock Laboratory. The first core sample was used for culturing of the clay materials as well as for preliminary DNA extraction trials. The second core sample was used for molecular biology studies.

3.3.2 *Isolation of organotrophic bacteria directly from colonies grown on plates*

Clay samples used for this study were sourced from the American Colloid Company (Wyoming MX-80 Bentonite). Appendix 10.1 provides the elemental composition of this commercial clay.

MX-80 clay was prepared for culturing by suspending 1 g of bentonite in 9 mL of phosphate buffered saline (PBS; 0.01 M NaCl buffered to pH 7.6 with 9 mM Na₂HPO₄ and 1 mM NaH₂PO₄). The suspensions were shaken for 30 min after which serial dilutions (10⁰-10⁻³ in PBS) were made. Aerobic and anaerobic organotrophic bacteria were enumerated on R2A agar (Reasoner and Geldreich, 1985) and anaerobic agar (Brewer, 1942; Cat. No. 253610, Difco™) in triplicate and incubated under aerobic and anaerobic conditions. Isolated aerobic and anaerobic organotrophic colonies growing on R2A and anaerobic agar, respectively, were identified by 16S rRNA gene sequence analysis. The streak plate method, followed by four rounds of subculturing, was used to ensure the clonal purity of the isolates prior to molecular identification. The isolates were selected based on colony colour, size and morphological type. A total of 46 bacterial colonies were selected from the original plates for 16S rRNA-targeted PCR and subsequent identification.

Isolated bacterial colonies were subjected to DNA extraction using the Feldan DNA extraction kit following the manufacturer's protocol (BioBasic, Québec, QC). Thereafter, partial 16S rRNA gene amplification was performed using the polymerase chain reaction (PCR) with *Taq*[®] DNA polymerase (New England Bio Labs, Ipswich, MA) and 8F and 531R universal bacterial primers (Hirkala and Germida, 2004). The final concentrations of the reagents used for each 50- μ L PCR reaction were as follows: 200 μ M deoxyribonucleotide triphosphates (dNTPs), 0.2 μ M of each primer and 1.25 units of DNA polymerase. Two μ L of template, 5 μ L of 10X Standard reaction buffer and 39.75 μ L volume of sterile Hyclone water (Thermo Fisher Scientific, Waltham, MA) were added to each reaction mix yielding a total reaction volume of 50 μ L. PCR amplifications were performed using a Techne TC-412 thermal cycler (Techne Inc., Burlington, NJ) using an initial denaturation step of 5 min at 95 °C followed by 35 cycles of 95 °C for 30 s, primer annealing at 52 °C for 30 s, and strand elongation at 72 °C for 30 s. A final elongation step of 72 °C for 5 min was then applied to ensure all amplified DNA was double-stranded. For sequencing, the ~500 bp PCR amplicons were purified using the Feldan Gel Extraction Kit (BioBasic, Québec, QC) according to the manufacturer's instructions. For identification and phylogenetic analysis, 16S rRNA gene sequences, from the 31 out of 46 bacterial isolates that were successfully PCR-amplified, were compared to all strains on the Ribosomal Database Project (RDP II) database using seqmatch (Cole *et al.*, 2005). Sequences were manually-edited using Chromas Explorer Lite, version 3.3.0 (Heracle Biosoft S.R.L., Romania) and GeneDoc

version 2.7 (Nicholas *et al.*, 1997), and thereafter the trimmed/aligned sequences were compared with the RDP II database. Identification of distinct bacterial isolates was based on percent similarity, where 95-100 % identical sequences were associated with the same taxon. A phylogenetic tree (Figure 3.1) of aligned sequences from RDP II database type- strains and the identified isolates was constructed by the neighbour-joining method (Saitou and Nei, 1987) using MEGA6 (Tamura *et al.*, 2013) with 1000 bootstrap replicates.

3.4 Results and Discussion

Previous attempts to extract DNA from natural clay (e.g., Opalinus clay) by some researchers have been unsuccessful (e.g. Stroes-Gascoyne *et al.*, 2007b). However, the use of Selenska-Pobell DNA direct lysis protocol (Selenska-Pobell *et al.*, 2001) (the basis of the Swiss protocol in this section) has been used to successfully extract DNA from the Opalinus clay formation (Moll *et al.*, 2013) as it was able to recover high molecular weight DNA but at 50-g clay increments. The bacterial community from the Opalinus clay reportedly belonged to the phyla *Firmicutes*, *Bacteroidetes*, *Acidobacteria* and *Actinobacteria* (Moll *et al.*, 2013). Unfortunately, in the present study, the application of the Swiss protocol generated low yields of DNA (Table 3.1), as per Nanodrop analysis (NanoDrop Technologies, Wilmington, Delaware, USA), and no PCR-amplified DNA bands were produced (data not shown). This result is similar to that of Stroes-Gascoyne *et al.* (2007b). As a result, the Modified Swiss protocol was developed for the extraction of DNA from microorganisms because it offers an extra cell lysis step by means of adding lysozyme and SDS right after the cells, organic debris and released DNA had been washed and precipitated with PEG/NaCl solution. The addition of lysozyme and SDS to the cells has been shown to increase DNA yield (Marmur, 1961; Schneegurt *et al.*, 2003). The perchlorate treatment then assists in the dissociation of nucleic acids from proteins. The DNA is separated subsequently from proteins using isoamyl alcohol/ chloroform and phenol solutions.

Extraction and analysis of DNA from Avonlea and MX-80 clay samples using the Modified Swiss protocol revealed that greater amounts of DNA were recovered from Avonlea clays than MX-80 (Table 3.1). The reason for reduced DNA yield from MX-80 may be related to the stronger complexation of this clay with polymers such as nucleic acids (Stroes-Gascoyne *et al.*, 2007b; Poulain *et al.*, 2008). This reduction in DNA yield may also be the consequence of

Table 3.1. Spectroscopic analysis of MX-80 and Avonlea clay samples after DNA extraction using Modified Swiss protocol.

Sample	Nucleic Acid Concentration	260/280	260/230
Avonlea b2	98.6 ng/ μ L	1.40	1.36
Avonlea b6	51.6 ng/ μ L	1.43	1.19
MX801	18.0 ng/ μ L	1.50	2.40
MX802	25.1 ng/ μ L	1.47	2.10

montmorillonite protecting microbes from adverse conditions (Haydel *et al.*, 2008). Masurat *et al.* (2010b) proposed that microbial cells surrounded by bentonite could have their water molecules removed, leaving these microorganisms in a desiccated state, which may induce entry into a VBNC condition in the bentonite clay mineral. It is also possible that phenolics or humics contributed to, or overlapped with, the nucleic acid signal obtained during spectroscopic analysis. Rochelle *et al.* (1992) encountered similar difficulty when an attempt to extract DNA directly from a clay-rich sediment samples gave lower yield of DNA. Rochelle *et al.* (1992) suggested that humic acids might be responsible for reducing the intensity of DNA bands in the agarose gel since they are believed to compete with DNA in ethidium bromide binding. Moreover, such a result was anticipated because larger amounts of humic acids were expected in deeper parts of the sediment. Humics may also play a role in protecting DNA against nuclease activity, which makes it difficult to digest DNA. Attempts to amplify fragments of the 16S rRNA gene from DNA extracted using the Modified Swiss method were initially unsuccessful; however, after considering the possible presence of contaminants in DNA extracts, amplifiable-PCR products were obtained by dilution of the template DNA from Avonlea clays and Opalinus clay (data and gel photo not shown). As a result, the Opalinus clay PCR products were subjected to further analysis (unpublished data). Unfortunately, no PCR-amplifiable product was obtained from MX-80 extracts using a similar approach.

Due to the inability to extract PCR-amplifiable DNA, it was determined that identification of cultured organisms using 16S rRNA gene sequencing offered the best opportunity for extending our understanding of clay-based microbial communities (Poulain *et al.*, 2008; Hallbeck and Pedersen, 2008; López-Fernández *et al.*, 2014). Stroes-Gascoyne *et al.* (2007b) and Pedersen *et al.* (2000b) similarly indicated the use of enrichment cultures to culture and cultivate microorganisms that were well adapted to bentonite clay environments. In this study, a mixture of Gram-positive and Gram-negative spore-forming bacteria were isolated from the MX-80 bulk

samples (i.e., straight from the package) (Figure 3.1) (Manisavagan *et al.*, 2013; Liu *et al.*, 2008; Nakamura, 1987). A total of 28 bacterial isolates were obtained from the R2A plates and anaerobic agar colony sub-cultures. Of these 28 bacterial isolates, 22 belonged to the phylum *Actinobacteria*, while the remaining six belonged to the *Firmicutes*. The results are in agreement with those isolates obtained from other subsurface clay samples (Moll *et al.*, 2013; López-Fernández *et al.*, 2014; Wouters *et al.*, 2013; Urios *et al.*, 2012). Stroes-Gascoyne *et al.* (2006) were also able to isolate a number of heterotrophic aerobes (HAB) and anaerobes (HANB) from uncompacted MX-80 bentonite using culture-dependent methods. The microorganisms isolated in this study could have originated from soil or have been externally introduced as a result of anthropogenic activities, and then survived the harsh conditions of bentonite processing by transforming to VBNC state. Given the processing conditions of MX-80 (commonly processed at 700 °C) (Yohe, 1967), this second scenario is unlikely. For example, *Paenibacillus alginolyticus* is a Gram-positive spore-former that can survive desiccated conditions, but growth can be inhibited by the presence of 3 % NaCl (Figure 3.1) (Nakamura, 1987). *Ornithinimicrobium pekingense*, on the other hand, is a HAB that can proliferate in activated sludge and can grow in an environment with 7 % NaCl (Liu *et al.*, 2008). It is, therefore, probable that these and similar microorganisms could later on dominate the harsh conditions of a DGR because of their ability to undergo sporulation in low-energy, low-water activity environments (Stroes-Gascoyne *et al.*, 2010b). Pedersen *et al.* (2000b) reached similar conclusions when they introduced bacteria to a bentonite clay plug and exposed the material to high temperatures. The Pedersen *et al.* (2000b) study found that the number of bacteria (e.g., *Bacillus* spp., *Pseudomonas* spp. and SRB) became reduced over time, and thus the authors concluded that desiccation was the main factor affecting the reduction of microbial numbers and culturability. This notion was contradicted by Hoehler and Jørgensen (2013), who argued that microorganisms adapted to long-term low-energy survival would come to dominate the system in time and not spore-formers. Their reasoning was that spore-formers imply a cessation of metabolism rather than long-term survival and that regermination will be energetically costly. Therefore, over time, the slow rate of death of spore-formers would lead to complete eradication of life in bentonite, especially in material exposed to DGR conditions. It was further hypothesized that only a naturally occurring microbial community could tolerate the low-energy environment of dense clays, while new microorganisms would not be able to adapt to such conditions (Pedersen *et al.*, 2000a).

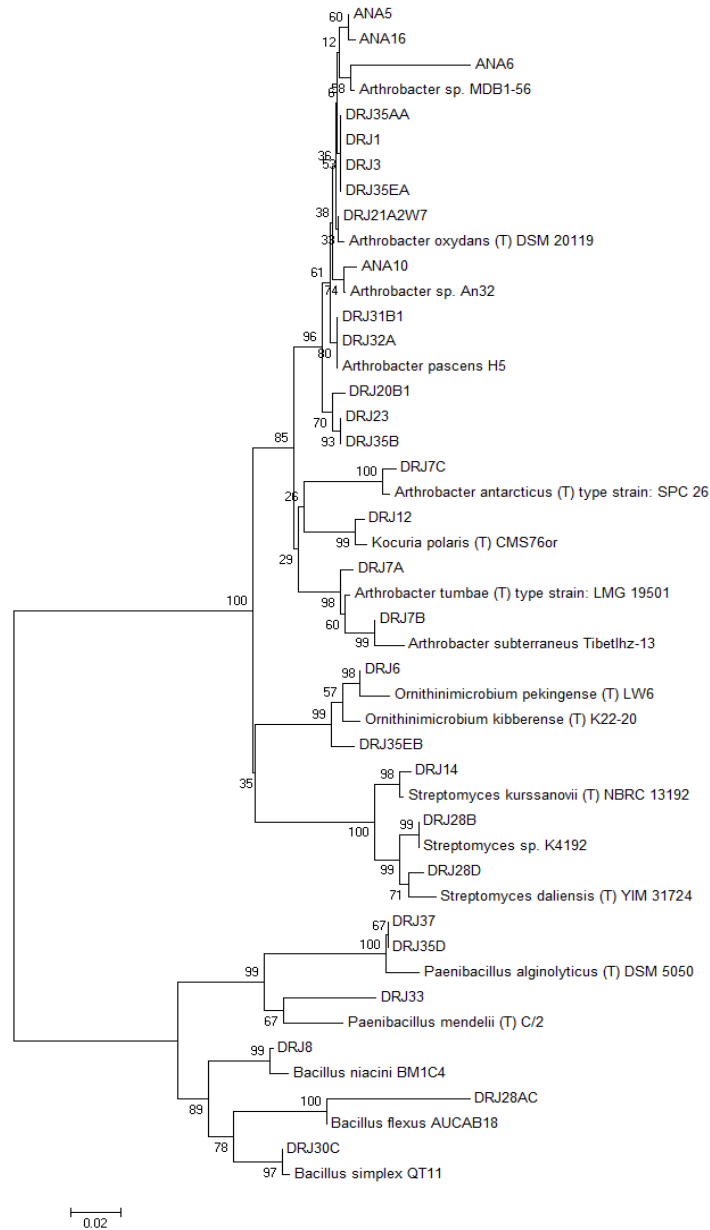


Figure 3.1. Unrooted neighbor-joining tree (using MEGA6 software) of the partial (V1-V3 regions) 16S rRNA gene sequences showing the diversity and distribution of the culturable bacterial isolates recovered from MX-80 bulk clay. These bacterial isolates were selected based on their colony morphologies from either R2A or anaerobic agar plates. The (T) indicates that the bacterial isolate is a type strain. Strain numbers are indicated beside (T). DRJ specifies bacterial isolates obtained from R2A plates, while ANA is designated for those obtained from anaerobic agar plates. The numbers beside ANA and DRJ indicate the number given to the agar plate where the isolate was obtained. The combination of letters and numbers specified is the identification given to each colony present on each agar plate. Sequences were aligned using GeneDoc version 2.7. Bootstrap values greater than 60 are indicated at the nodes. Bar, 0.02 substitutions per nucleotide position.

3.5 Conclusions

DNA extracted directly from Opalinus clay using the Swiss protocol yielded lower concentrations of DNA in comparison with the Modified Swiss protocol. When the Modified Swiss protocol was applied to Avonlea and MX-80 clay samples, Avonlea provided the higher DNA concentration yield followed by MX-80. DNA was recovered from each clay sample using the Modified Swiss protocol; however, the protocol failed to generate PCR-amplifiable products. The presence of humic acids may have contributed to the difficulty in detecting and amplifying DNA. It has been suggested that humic acids would interfere in the binding of ethidium bromide during gel electrophoresis causing faded gel bands and may also play a role in protecting DNA from nuclease activity (Rochelle *et al.*, 1992). Thus, when the DNA extracts from Avonlea, Opalinus clay and MX-80 were diluted, amplifiable PCR products were obtained except for MX-80. It has been hypothesized henceforth that the non-culturable nature of microorganisms naturally-inhabiting MX-80 bentonite and desiccation effects contributed to the difficulty to extract DNA, as well as the ability to obtain a representative microbial community from the same samples. Culture-based technique was applied to resolve the bacteria-isolation problem in this section.

3.6 Connection to Next Section

The inability to extract DNA directly from clay (Section 3) made culture-dependent techniques (Sections 4 and 5) and enrichments (Section 6) necessary to recover indigenous microorganisms from MX-80 bentonite. In the next section, culture-dependent techniques were applied to isolate microorganisms from MX-80 bentonite that were able to withstand simulated DGR conditions. Furthermore, the identification of the parameters that could further decrease bacterial culturability and cell counts in the bentonite buffer component of a DGR's engineered barrier system were investigated.

4. MICROBIAL CULTURABILITY AND DIVERSITY IN AN EIGHT-YEAR OLD HIGHLY-COMPACTED, SATURATED MX-80 WYOMING BENTONITE PLUG

Interface:

This section has been published in *Applied Clay Science*, 126: 245-250, 2016 and was reproduced with the permission. This work was part of a seven-year study initiated by AECL on the effect of the microbially-inhibiting parameters on highly-compacted, water-saturated MX-80 Wyoming bentonite plug.

Dr. Stroes-Gascoyne, Dr. Deni Priyanto, Connie Hamon, Cliff Kohle and William Evenden performed the initial experimental design, setup and preliminary experiments. They also contributed to the results, data analyses and interpretation.

Daphne Jalique performed the extraction of DNA from colonies and molecular analysis of the isolates, and prepared the initial draft of the manuscript.

Dr. Alexander Grigoyan contributed to the design of the molecular studies.

All authors, including **Dr. Stroes-Gascoyne, Dr. Gideon Wolfaardt, Dr. Jennifer McKelvie** and **Dr. Darren Korber**, contributed to the end-of-study analyses and writing of the manuscript.

4.1 Abstract

Highly-compacted bentonite (HCB)-based sealing systems are being developed for potential use in nuclear fuel waste repositories because of their inherent physicochemical and rheological properties. Results from short-duration (40-90 days) studies performed by Stroes-Gascoyne *et al.* (2010b) using highly-compacted (HC), water infused, confined Wyoming MX-80 bentonite revealed that a uniform dry density $\geq 1.6 \text{ g/cm}^3$ (resulting in a swelling pressure above 2,000 KPa, water activity below 0.96 and an average pore size of $< 0.02 \text{ }\mu\text{m}$) showed suppression of microbial culturability. A longer-term (7-year, 264-day) experiment using the same material and conditions was therefore conducted to determine whether these conclusions held over extended periods. Viable cell counts in material from the ~ 8 -yr clay plug approximated those in the original uncompacted bentonite, confirming suppression of microbial growth. Bacteria recovered from the ~ 8 year clay plug were comprised of primarily Gram-positive spore-formers. Together, these results confirmed that HCB suppressed microbial culturability (and presumably activity and growth) over extended time periods, and further revealed that the surviving culturable microorganisms were those adapted to life in low water activity soil environments.

4.2 Introduction

The Canadian DGR concept for storing used nuclear fuel incorporates multiple barriers designed to safely isolate containers and their radioactive contents for up to 1 million years (NWMO, 2005). A key component of Canada's DGR concept includes the use of HCB-based sealing and backfill materials as part of an engineered barrier system (EBS). The Wyoming MX-80 bentonite buffer material consists of montmorillonite (75 wt. %) and other accessory minerals (in wt. %, < 1 kaolinite, < 1 mica, 15.2 quartz, 5-8 feldspar, 0.7 calcite, 0.7 siderite, 0.3 pyrite; Bradbury and Baeyens, 2002), and was chosen due to its excellent physicochemical, hydromechanical and rheological properties (Ravi and Rao, 2013). One of montmorillonite's key properties is a large swelling potential upon interaction with free water, allowing it to seal any voids, cracks or fissures in a confined environment such as a DGR. Given the unavoidable presence of indigenous and introduced microorganisms and their metabolic potentials in a DGR, a primary concern is that microbial activity in the EBS be controlled, since it may cause direct (microbially influenced corrosion) or indirect damage (production of corrosion aggressive products, gases), or interact with, or facilitate the transport of radionuclides, upon breach of any container (Stroes-Gascoyne *et al.*, 2010b).

Studies examining HCB clays have revealed that water activity, swelling pressure, water content, pore size (especially pore throats), and dry density may individually and collectively affect microbial activity and culturability (Chapelle, 1993; Kieft *et al.*, 1997; Hedin, 2006; Stroes-Gascoyne *et al.*, 2006). Short-term studies have shown that for bentonite to suppress the activity of bacteria, as well as the germination of spores, it would have to either: 1) have a water activity of < 0.96 (resulting from a bentonite dry density of at least 1.6 g/cm^3 or a pore-water salinity of $> 60 \text{ g NaCl/L}$), or 2) have a swelling pressure of $\geq 2000 \text{ kPa}$ (Stroes-Gascoyne *et al.*, 2006, 2007a, 2010b). The study described in this section provides insight into the ability of HCB to restrict microbial activity in a DGR over a multi-year timeframe.

4.3 Materials and Methods

4.3.1 Experiment 2063

Commercial bentonite, Wyoming MX-80 (containing $\sim 75 \text{ wt. \%}$ montmorillonite), was compacted at 95 % saturation in an ethanol-sterilized pressure cell to a target clay dry density of 2.0 g/cm^3 (as in Stroes-Gascoyne *et al.*, 2010b). The resulting bentonite plug was about 2-cm high with a diameter of 3.2 cm. The plug was continuously infiltrated with sterile distilled deionized water (DDW) (under pressure) for 2811 days (7.7 years) at ambient temperature ($22 \pm 2 \text{ }^\circ\text{C}$). Total pressure and the pressure of the applied DDW were continuously recorded and were used to calculate swelling pressure.

4.3.2 Plug extrusion, sampling and physical properties

After ~ 7.7 years, the plug (Figure 4.1) was extruded into a sterile cup, wrapped in sterile aluminum foil and plastic, and immediately taken to the microbiological laboratory at CNL-Whiteshell Laboratories for sampling and microbial analyses. Two bentonite subsamples were taken aseptically from the plug using a sterile knife and scalpel: a) from the outside of the plug (top, sides and bottom, representative of interfaces or fractures in the EBS) (sample 2063A), and b) from the inner consolidated region of the bottom half of the plug (representative of the highly-compacted bentonite) (sample 2063C). Water activity (a_w) was measured on crushed clay subsamples using a DecagonTM WP4 Dewpoint PotentialMeter (Decagon Devices, Pullman, WA). Water content (% w/w) was determined by drying the (above) water activity clay material at $110 \text{ }^\circ\text{C}$ until a constant final weight was achieved.

4.3.3 *Microbial analyses*

Microbial analyses were performed on serially-diluted clay suspensions using PBS. For heterotrophic aerobic bacteria (HAB), dilutions were plated on R2A agar plates (Appendix 10.6; Reasoner and Geldreich, 1985) in triplicate and incubated at 30 °C for 5-7 days prior to counting of colonies. Similarly, heterotrophic anaerobic bacteria (HAnB) were cultured on R2A pour plates in an anaerobic chamber (85 % N₂: 10 % CO₂: 5 % H₂) maintained at 30 °C for 28 days before enumeration. Nitrate-reducing bacteria (to nitrite; NRB-NO₂ and to nitrogen gas; NRB-N₂) were enumerated by the three-tube, most-probable number (MPN) method in degassed R2A



Figure 4.1 HCB clay recovered after termination of the ~7.7 yr experiment. The clay plug was subsampled as follows: sample 2063A (outer portion representative of interfacial regions) and sample 2063C (intact portion representative of consolidated high-density regions).

broth amended with 0.1 % sodium nitrate. The MPN tubes were scored after four weeks for gas production (observed in Durham tubes; for NRB-N₂) and for nitrite production (observed using the nitrite detection test: two drops of 0.8 % sulfanilic acid and 0.5 % N,N-Dimethyl-alpha-naphthylamine in 5 N acetic acid and 2-3 mg zinc dust to the sample (clear – positive, pink – negative; for NRB-NO₂). Sulfate-reducing bacteria (SRB) were enumerated by the MPN method in modified Postgate's B medium (Appendix 10.7; Postgate, 1984) at 30 °C for about four weeks. Tubes were then scored for sulfide production based on formation of black precipitate.

4.3.4 *Identification of aerobic bacterial isolates carbon source utilization patterns*

The BiologTM GEN III system (Hayward, CA, USA) was used to evaluate substrate utilization patterns of aerobic bacteria isolated from sample 2063C aerobic (HAB) triplicate plates C1 and C2 of dilution 10⁰. The colonies, which differed in color, shape, consistency and size, were picked using sterile wood toothpicks and streaked onto fresh R2A plates to obtain isolated

colonies. Newly-formed colonies were then streaked onto Biolog Universal Growth plates, and were later resuspended in Inoculating Fluid (IF) to a turbidity of 90 to 98 %, following protocols A and B as per manufacturer's instructions. Turbidity was measured using a Biolog turbidimeter (BiologTM, Hayward, CA). A 100 µL aliquot of the resultant cell suspensions was added to each of the 96 wells of the BiologTM GEN III plate. A computerized plate reader (Biolog MicrostationTM ID system; Hayward, CA) was used to measure the colour development in the plate wells after 4 to 48 hours of incubation. Purple wells were indicative of a positive reaction, and together provided a carbon source utilization pattern for each isolate that could then be compared with the BiologTM database (GEN III database version 6.01).

4.3.5 Identification of bacterial isolates using 16S ribosomal RNA (rRNA) gene sequence analysis

Isolated HAB colonies growing on R2A medium inoculated with clay plug samples were identified by 16S rRNA gene sequence analysis. The streak plate method, followed by four rounds of subculturing, was used to ensure the clonal purity of these isolates prior to molecular identification, which were selected based on colony colour, size and morphological type. A total of 46 bacterial isolates were selected from the original plates for 16S rRNA-targeted PCR and subsequent identification using 8F and 531R universal bacterial primers (Hirkala and Germida, 2004). Purified amplicons were then sequenced, data aligned and edited using standard methods, and compared with the RDP II sequence database using Seqmatch (Cole *et al.*, 2005). A phylogenetic tree comparing RDP II type-strains and identified isolates was constructed by the neighbour-joining method (Saitou and Nei, 1987) using MEGA6 (Tamura *et al.*, 2013) with 1000 bootstrap replicates.

4.4.1 Results and Discussion

4.4.1 Survival and proliferation of microbes in highly-compacted bentonite clays

The water-saturated HCB plug used in this study represented the cool, saturated conditions that would exist in a DGR several hundreds to thousands of years after the initial hot, dry phase. During this later, cooler stage, the water-saturated bentonite would become fully-expanded, thereby increasing the swelling pressure (Stroes-Gascoyne *et al.*, 2002), and mass transport within the clay matrix would be diffusion-dominated (King *et al.*, 1996). Overall, *in situ* repository conditions at this stage are thought to be highly unfavourable for the activity and growth of microorganisms (e.g., Pedersen *et al.*, 2000b).

Table 4.1 provides a comparison of key measured parameters (water activity, water content, dry density, swelling pressure) in sample 2063C with those conducted previously (Stroes-Gascoyne *et al.*, 2010b). Water activity (a_w) and water content data indicate the relative humidity of the system and the amount of free and sorbed water that may be available for bacterial use, respectively. Dry density refers to the degree of compactness of clay in a given volume, whereas swelling pressure is the amount of pressure exerted by saturated expanding clay minerals within a confined volume. It has previously been shown that water activity and swelling pressure in HC MX-80 bentonite used in this study are inversely-related (Stroes-Gascoyne *et al.*, 2002, 2010b), and contribute to microbial activity and growth suppression. Pedersen *et al.* (1995) similarly observed a large reduction in SRB viable counts in HCB after 60 days when water activity and dry density were < 0.96 and 2.0 g/cm^3 , respectively.

Table 4.1. Comparative summary of selected physical properties (water activity, a_w ; water content, %; “target” and “measured” dry clay densities, g/cm^3 ; and swelling pressure, kPa) of sample 2063C and similar data on highly-compacted bentonites, infused with DDW, adapted from Stroes-Gascoyne *et al.* (2010b).

Experiment and Sample No.*	Duration (days)	a_w	Measured Water Content (%)	Target** Dry Density (g/cm^3)	Measured Dry Density (g/cm^3)	Swelling Pressure (kPa)
2.0 – DDW (1663)	54	0.789	19.38	2.0	1.77	31,400
2.0 – DDW (1728)	156	0.773	18.48	2.0	1.80	33,000
2063C	2811	0.874	22.37	2.0	1.70	23,300

* Values reproduced (with permission) from Stroes-Gascoyne *et al.* (2010b) with the exception of sample 2063C, which is from the present long-term experiment, and included for direct comparison.

** Target dry density is the desired dry density determined at the start of the experiment for the clay plug to reach. The measured dry density was derived from the water content in the plug (assuming full saturation) and measured after termination of experiment. DDW = distilled deionized water

Work on the stress-strain relationship in MX-80 was conducted previously to determine the buffer material behavior and physical properties in pressure cells (also known as oedometers) under triaxial loading conditions (Börgesson *et al.*, 1995, 1996, 2010; Dixon, 2000; Baumgartner *et al.*, 2008; Priyanto *et al.*, 2008; Dueck, 2010). When a constrained, consolidated block of bentonite is saturated with water, hydraulic conductivity decreases as swelling pressure increases. High salinity groundwater may cause diminished swelling pressures on saturation, resulting in a reduced ability to resist shear stress (i.e., the bentonite becomes weaker) (Börgesson *et al.*, 1995, 1996) along with a more loosely-packed clay matrix. Reductions in swelling potential under high salinity conditions (Baumgartner *et al.*, 2008; Priyanto *et al.*, 2008)

could, however, be offset by use of higher dry density bentonite (Wang *et al.*, 2012). Karnland *et al.* (2006) and Börgesson *et al.* (2010) observed swelling pressure values ranging from 16,700-20,000 kPa when clay dry densities were $\geq 1.6 \text{ g/cm}^3$, observations consistent with our present swelling pressure (i.e., $\sim 23,300 \text{ KPa}$). The high swelling pressures obtained from highly compacted MX-80 is attributed to the montmorillonite content in bentonite as well as its charge-compensating ions (Karnland *et al.*, 2006). The ~ 8 -year HCB clay plug was infused continually with sterile DDW, resulting in measurements: a_w of 0.874, water content of 22.37 %, calculated dry density of 1.70 g/cm^3 and swelling pressure of 23,300 KPa (Table 4.1), in close agreement with shorter-term experiments also carried out with DDW infusion (Table 4.1). In the likely case of saline groundwater being present in a DGR, any decrease in swelling pressure caused by elevated salt content would not affect the EBS in terms of suppressing microbial growth because salinities greater than 60 g/L NaCl have been shown to inhibit microbial activity and spore germination in clays (e.g., Stroes-Gascoyne and Hamon, 2008), presumably due to salts effect on water activity (i.e., $a_w < 0.96$).

Two subsamples from the 8-year clay plug were analyzed using culture-based techniques and compared with those from reference bentonite powder that was analyzed in 2008 (Table 4.2). Sample 2063A, obtained from the outer plug surfaces (interfacial regions), contained higher numbers of HAB [$(1.08 \pm 0.12) \times 10^4 \text{ CFU/g}$] and NRB-NO₂ ($1.83 \times 10^5 \text{ MPN/g}$). These results were not surprising since higher numbers of culturable bacteria have previously been found at clay-metal interfaces in pressure cells compared with bulk clay samples (e.g., Stroes-Gascoyne *et al.*, 2002, 2007a). Stroes-Gascoyne *et al.* (2010b) suggested that a lower clay dry density at interfaces may provide a more favourable environment (e.g., increased access to water and nutrients). Interfacial regions, therefore, have been identified as regions where enhanced microbial activity may occur in a DGR (Wolfaardt and Korber, 2012), and include: i) boundaries between bentonite-based buffer blocks or transitions between buffer and other sealing materials such as backfill, ii) fractures, and iii) locations where incomplete sealing has occurred, e.g., between bentonite and host rock and between bentonite and containers.

The number of HAB [$(1.28 \pm 0.65) \times 10^2 \text{ CFU/g}$] and NRB-NO₂ ($6.43 \times 10^2 \text{ MPN/g}$) in the highly-compacted sample (2063C) were lower in comparison to the interface sample. Our results thus agree with the findings of Stroes-Gascoyne *et al.* (2010b). Furthermore, the numbers of HAnB, NRB-N₂, and SRB were all low and comparable between samples 2063A and C (Table

4.2). This suggests that conditions within highly compacted regions of the plug were homogeneous and generally inhibitory to microbial activity, and likely lacking in available space, water, nutrients and/or suitable electron acceptors. It is also possible that conditions within the bentonite plug were not sufficiently reducing for these physiological groups to proliferate and that clay plug interfaces contained more O₂, hence the larger numbers of HAB at these locations.

In terms of the critical parameters (i.e., a_w , clay bulk dry density, swelling pressure) known to control microbial proliferation within clays, the conditions in the ~8-yr experiment included an actual calculated dry density of 1.70 g/cm³, which resulted in a measured a_w of 0.87, along with a measured swelling pressure of 23,000 kPa. Figure 4.2A superimposes the ~ 8-year culturable HAB result for sample 2063C (pink dot) on the previous short-term HAB data of Stroes-Gascoyne *et al.* (2010b). It can be seen that any 100% bentonite clay dry bulk density yielding a measured a_w of < 0.96 restricted culturable HAB numbers to < 2.0 x 10² CFU/g (Stroes-Gascoyne *et al.*, 2010b), which approximates the background culturable HAB levels in the reference MX-80 bentonite. Figure 4.2B reveals a similar trend with respect to measured clay dry density (sample 2063C); any 100% bentonite clay plug having a dry bulk density of at least 1.6 g/cm³ held culturable HAB numbers to background levels. Swelling pressures > 2,000 kPa similarly inhibited proliferation of culturable HAB (data not shown).

Overall, our results agree with the earlier findings by Stroes-Gascoyne *et al.* (2010b), giving confidence that the growth of microbes would be suppressed in a properly designed and installed EBS in a future DGR. Although the values reported in this study are for high-density Wyoming MX-80 bentonite, these results can be extrapolated to other bentonites (on the basis of montmorillonite content) (Stroes-Gascoyne *et al.*, 2010b).

4.4.2 Diversity of microbes in highly-compacted bentonite clays

In this part of the study, HAB strains were targeted for identification since the pressure cell used to confine the 8-year clay plug, as well as the previous short-duration plugs, were incubated aerobically. Although we also enumerated HAnB, SRB, NRB-NO₂, and NRB-N₂ we did not isolate or identify these. The aerobic bacterial isolates identified using BiologTM belonged to either the phyla *Actinobacteria* or *Firmicutes*. Both of these phyla include microorganisms commonly found in soil- and human-associated environmental samples and are suggestive of the presence of soil bacteria with high tolerance to environmental stress, spore-formers and probably

some human-origin (contaminant) bacteria (Janssen, 2006; Kuramae *et al.*, 2012). Bacterial 16S rRNA sequence analysis was also used to confirm the BiologTM results, as well as identify those isolates that could not be identified using BiologTM. In our study, 31 of the 46 HAB isolates were successfully identified using 16S rRNA gene sequencing, of which only 13 were unique and all belonged to the phylum *Firmicutes* (two to the order *Bacillales*, five to the genus *Bacillus*, one to the family *Paenibacillaceae* and five to the genus *Paenibacillus*) (Figure 4.3). These Gram-positive microorganisms are common to soil and include spore-formers, thus tend to be resistant to harsh conditions (Goto *et al.*, 2004; Bredemann and Werner, 1933; Elo *et al.*, 2001; Heyrman *et al.*, 2004). Almost all species identified were strictly aerobic, with the exceptions of the facultative anaerobes *P. borealis*, *B. licheniformis* and *B. cereus*. They had remained viable, and hence culturable, after ~8 years of incubation in a highly-compacted, diffusion-dominated bentonite matrix. Organisms such as these would only be able to proliferate to above-background levels if a DGR in some way became compromised, relieving the microbially-suppressive effect of the HCB, or if an influx of nutrients and water from the surrounding environment became available in interfacial regions.

The bacterial isolates in our study are similar to those observed in compacted MX-80 Wyoming bentonite used in a study at the Äspö underground HRL in Sweden (Chi Fru and Athar, 2008). In that study, the authors compared gene sequences from highly compacted Wyoming bentonite with those from *in situ* groundwater in contact with the bentonite (for five years). They detected predominantly Gram-positive *Firmicutes* and *Actinobacteria* in the HCB, whereas the groundwater contained predominantly Gram-negative species. The bacteria isolated in our study (and by others) from HCB have the potential to influence the geochemistry of bentonite. For instance, *Bacillus* species are known to reduce Fe (III) to Fe (II) in bentonite (Stucki and Getty, 1986). *Pseudomonas stutzeri*, present in Chi Fru and Athar (2008) bentonite samples, could produce metabolites able to complex with radionuclides, possibly aiding in their mobilization (Pedersen, 2005; Newsome *et al.*, 2014). *Paenibacillus* species were observed to reduce U (VI) to U (IV) by Lütke *et al.* (2013).

Many of the Gram-positive organisms recovered in our study were capable of forming spores. Stroes-Gascoyne *et al.* (2010b) previously considered the importance of spore-forming microorganisms in EBS clays since spores have the ability to survive under extreme environmental conditions. However, even though many microorganisms can achieve a low

energy-requiring metabolic state (i.e., through sporulation or dormancy), the question of how long such cells can remain viable in dense clays remains a topic for conjecture. Nevertheless, the adaptation of Gram-positive microbes to wetting/drying cycles in soils (where elevated salinity and decreased water activity would occur during desiccation) could facilitate their survival over extended periods in high density, low water activity clays. Chemical and structural properties of clays could also be linked to microbial survival in bentonite. Masurat *et al.* (2010b) and Marshall (1975) proposed that water molecules are removed from microbial cells surrounded by bentonite, desiccating the microorganisms and causing them to enter a viable but non-culturable state.

The prevalence of Gram-positive bacteria observed in the present study is presumably due to the microbially inhibitory parameters dominating the HCB plug environment. In the study by Chi Fru and Athar (2008), the groundwater-saturated HCB was found to predominantly contain Gram-positive, endospore-forming bacteria despite the groundwater itself being dominated by Gram-negative mesophilic microorganisms. These results could signify that the ability to sporulate increases survival under DGR conditions. If the controlling conditions provided by HCB are not maintained (e.g., high swelling pressure and low water activity), other factors such as salinity (e.g., Stone *et al.*, 2016b) and low carbon availability would continue to constrain microbial processes. However, disturbance of this natural environment by drilling has invariably resulted in the presence of thriving microbial populations in the borehole water (e.g., Stroes-Gascoyne *et al.*, 2011); thus, it is unclear whether these bacteria were introduced by disturbance despite stringent precautions, or were originally present in the clay formation. Further work to elucidate these questions is ongoing at MT.

Table 4.2. Physico-chemical and microbiological characteristics of compacted bentonite plug 2063. The results were compared to uncompacted dry bentonite lot 1847.

Sample	1847*	2063a	2063c
Description	Dry bentonite	Outside plug	Inside plug
Water content (% dry wt)	7.55	24.45	22.37
Water activity	0.393	0.913	0.874
HAB (CFU/g)	$(6.24 \pm 2.34) \times 10^2$	$(1.08 \pm 0.12) \times 10^4$	$(1.28 \pm 0.65) \times 10^2$
HAnB (CFU/g)	$(1.74 \pm 0.60) \times 10^1$	$(3.29 \pm 1.43) \times 10^1$	$(1.74 \pm 0.60) \times 10^1$
NRB-NO ₂ (MPN/g)	9.62×10^2	1.83×10^5	6.43×10^2
NRB-N (MPN/g)	<3.14	<7.50	<4.29
SRB (MPN/g)	<3.14	<7.41	<4.26
Dry density** (g/cm ³)	N/A	1.85	1.85
Dry density*** (g/cm ³)	N/A	N/C	1.70
Swelling Pressure (kPa)	N/A	N/A	23,300

* Previous Analysis (2008)

** Averaged for whole plug (using plug thickness of 21 mm)

Continuation...

***Calculated from water content of sample 2063C

Note: CFU = Colony Forming Units; MPN = Most Probable Number; N/A = Not applicable; N/C = Not calculated; kPa = Kilo Pascals

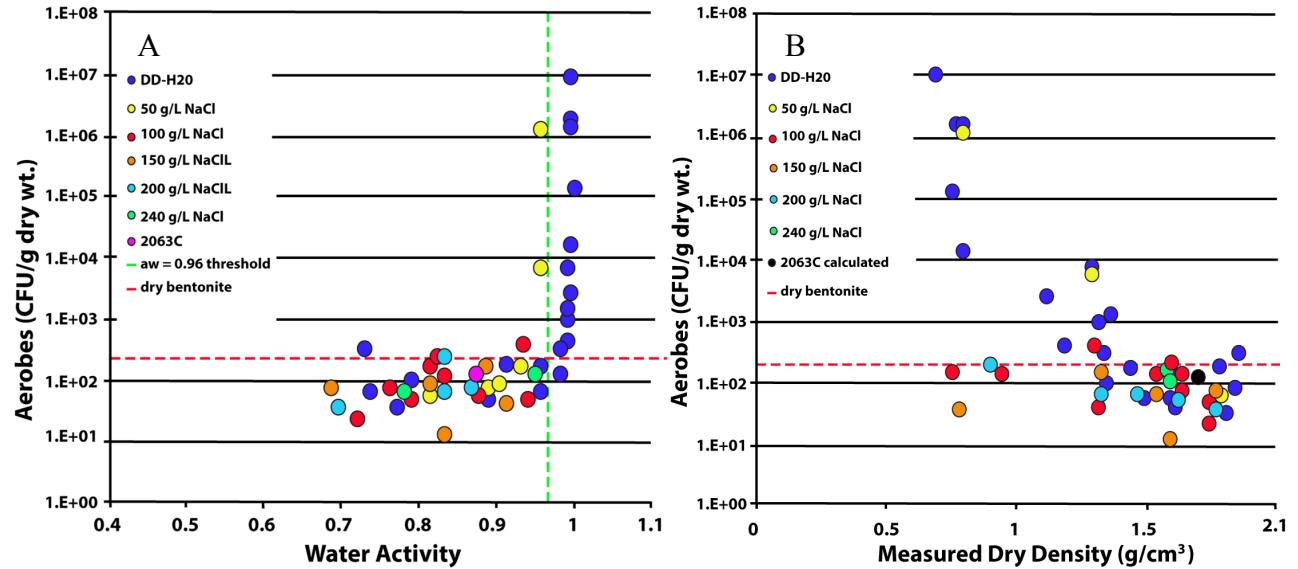


Figure 4.2A, B. HAB (CFU/g) as a function of water activity and measured dry density. (A) Presence of culturable aerobic heterotrophs (CFU/g clay dry weight) as a function of water activity in Wyoming MX-80 bentonite with DDW + saline solution from previously published data (reproduced in part from Stroes-Gascoyne *et al.* (2010b), with permission), onto which has been superimposed the number of culturable aerobic heterotrophic bacteria from sample 2063C recovered from the ~8-year plug (pink circle). (B) Number of culturable aerobic heterotrophs (CFU/g clay dry weight) as a function of dry density in highly compacted Wyoming MX-80 bentonite with DDW + saline solution from previously published data (reproduced in part from Stroes-Gascoyne *et al.* (2010b), with permission), onto which has been superimposed the number of culturable aerobic heterotrophic bacteria from sample 2063C recovered from the ~8-year plug (black circle).

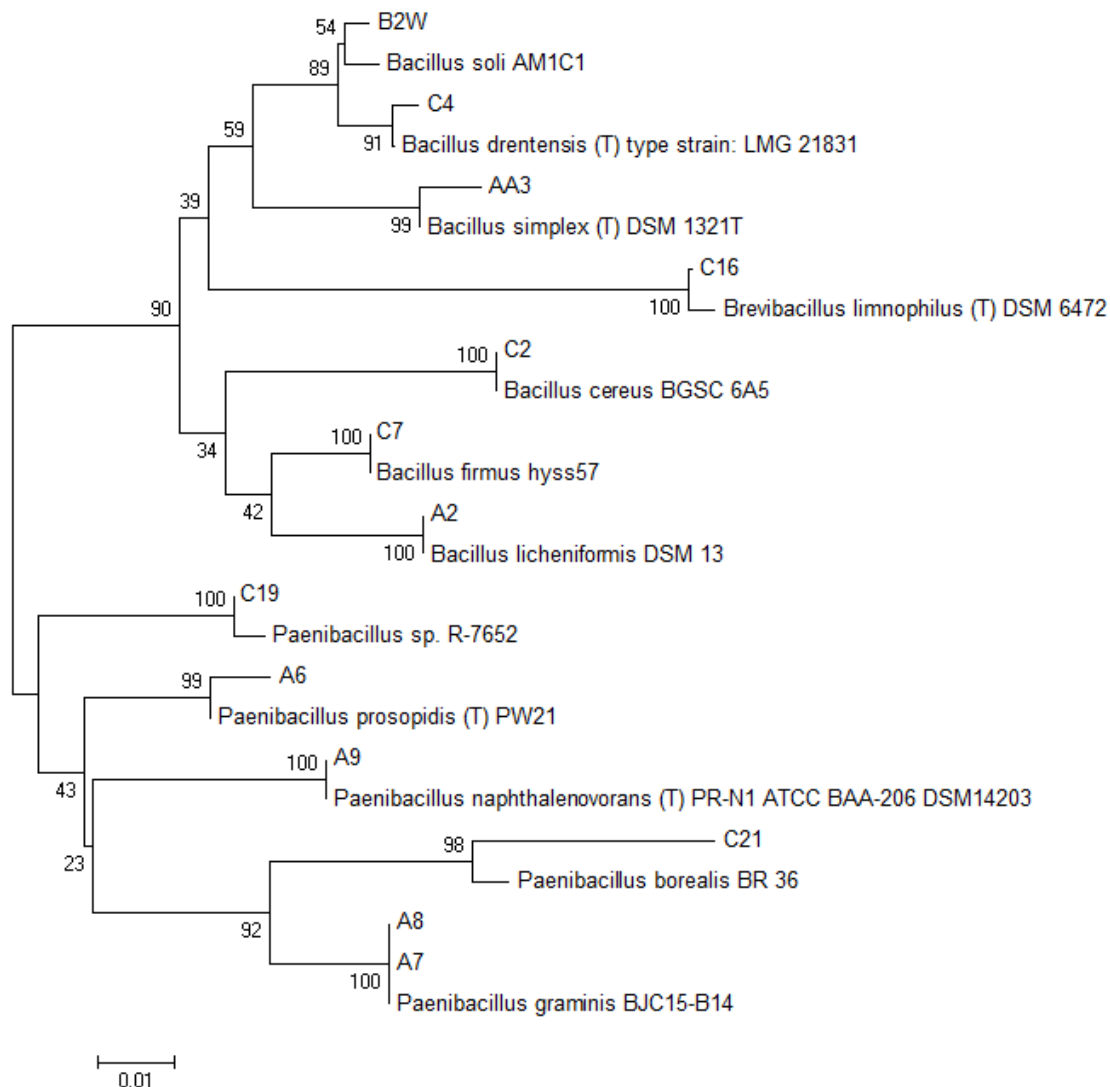


Figure 4.3. Unrooted neighbor-joining tree (constructed using MEGA6 software) of the partial (V1-V3 regions) 16S rRNA gene sequences showing the diversity and distribution of the culturable bacteria from 7.7-year old bentonite clay plug. Identification of isolates was based on percent similarity, where 99-100% identical sequences were associated with the same taxon. The (T) indicates that the bacterial isolate is a type strain. Strain numbers are indicated beside (T) or after the binomial name. The combination of letters and numbers specified is the identification given to each colony present on each agar plate. Sequences were aligned using GeneDoc version 2.7. Bootstrap values greater than 60 are indicated at the nodes. Bar, 0.01 substitutions per nucleotide position.

4.5 Conclusions

The results of this long-term study validate the earlier, short-term findings of Stroes-Gascoyne *et al.* (2010b) wherein highly compacted Wyoming MX-80 bentonite having a dry density of ≥ 1.6 g/cm³, a swelling pressure of $> 2,000$ KPa, and an a_w of < 0.96 suppressed microbial culturability to at or below background levels, and appeared to select for Gram-positive bacteria, including spore-formers, which presumably only became metabolically active when the inhibitory conditions imposed by the HCB were removed (i.e., during culturing). Provided that these inhibitory conditions are maintained in the EBS of a future DGR, the viability of indigenous organisms would be expected to decline over DGR-relevant time-scales, ultimately reaching a point where viable cells may no longer be recovered.

4.6 Connection to Next Section

The previous section discussed the effect of the critical parameters (i.e., water activity, swelling pressure and dry density) on the culturability and population of microorganisms in water-saturated MX-80 bentonite after approximately 8 years under aerobic condition. In the next section, microbial survival and culturability in anaerobic, high-density MX-80 plugs saturated with 50 g/L NaCl, represents a direct comparison to the Stroes-Gascoyne *et al.* (2010b) study, wherein MX-80 bentonite plugs were exposed to simulated DGR conditions under aerobic conditions for 40-90 days. The next section also discusses the effects of critical parameters, including the influence of saline solution on microbial abundance and culturability, but this time, the bentonite plugs were maintained under strictly anaerobic conditions. The results obtained in the next study reveal how anaerobic conditions affect the diversity of microorganisms that are able to survive simulated DGR conditions. The bentonite plugs from the next study, however, were only incubated under such conditions for six months.

5. MICROBIAL SURVIVAL AND CULTURABILITY IN ANAEROBIC, HIGH DENSITY MX-80 PLUGS SATURATED WITH 50 G/L NaCl

Interface:

Daphne Jalique and **Dr. Alexander Grigoryan** equally performed all the preparation, tests, analyses and result interpretation for this section.

Cliff Kohle (AECL) assisted in the setup of the pressure cells, demonstrated the use of data logger, and provided the information needed to calculate dry density and swelling pressure.

Maria Martinez and **Kara Friesen** provided technical assistance to this work.

Dr. Darren Korber provided guidance and assisted with experimental design, provided editing of the section, and held the research grant that supported this work.

5.1 Abstract

A long-term confined highly compacted bentonite (HCB) plug infused with water confirmed that a uniform dry density $\geq 1.6 \text{ g/cm}^3$, swelling pressure above 2,000 KPa, and water activity below 0.96 suppressed microbial culturability. Short-term (40-90 days) studies performed by Stroes-Gascoyne *et al.* (2010b) under aerobic conditions previously showed suppression of microbial culturability since their microbial counts remained at or below background levels (2.1×10^2 CFU/g). Gram-positive spore formers were recovered and were believed to have adapted to low energy life in bentonite. A similar experiment (~6-month duration) was conducted under

anaerobic conditions (inside a custom-made anaerobic chamber) with 50 g/L NaCl as the infiltration solution (simulating DGR saline groundwater). In the three dry densities tested (1.0, 1.3 and 1.6 g/cm³), the 1.6 g/cm³ HCB MX-80 plug resulted in the lowest viable cell counts for HAB, HAnB and SRB. Salinity, together with the microbially inhibiting effect of HCB, effectively suppressed microorganisms in the clay plugs during these short time-frame experiments.

5.2 Introduction

Reduction in microbial culturability background levels has been demonstrated previously in a water-saturated MX-80 bentonite plug after ~8-year incubation period (as discussed in Section 4 of this thesis). Previously, Stroes-Gascoyne *et al.* (2010b) performed similar 40 to 90-day HCB plug experiments wherein the collective effect of low water activity, high dry density and high swelling pressure caused indigenous and/or introduced microorganisms to form spores or to become acclimated to low-energy environments. In those experiments, Gram-positive spore-forming heterotrophic aerobic bacteria (HAB) were isolated and identified from 40 to 90-day bentonite plugs, but do not reflect the potential (and probable) presence of anaerobic microbial biomass in the HCB plug (Stroes-Gascoyne *et al.*, 2010b). Therefore, SRB and HAnB were also cultured using enrichment media and enumerated using plate count and MPN. While these anaerobic organisms were not further analyzed, the fact that they grew on enrichment media indicated that they survived the harsh conditions prevalent in the HCB, such as high swelling pressure (> 2,000 kPa) and low water activity ($a_w < 0.96$), and would thus proliferate in a DGR if conditions became favorable.

The presence of indigenous and introduced microorganisms in a HCB MX-80 plug could potentially impact and contribute to the relevant biogeochemical processes in a DGR. Among these potential impacts are their ability to cause microbially-influenced corrosion (MIC), sorption of radionuclides, gas production and radionuclide mobilization. For instance, the existence of *Paenibacillus* could impact radionuclide mobility/speciation (Moll *et al.*, 2013). Masurat *et al.* (2010a) discovered an indigenous SRB, *Desulfovibrio africanus*, in Wyoming MX-80 using enrichment cultures, which contributed to sulfide production in bentonite. Therefore, it is important to suppress all microbial activity in the EBS of a DGR.

A University of Saskatchewan pressure cell trial study was performed during November 2013 (unpublished data) to serve as a benchmark for this section, as well as to confirm the initial work

performed by Stroes-Gascoyne *et al.* (2010b) on an MX-80 plug under aerobic conditions, as mentioned in Section 4 of this thesis. The outcomes, however, did not correlate well with the Stroes-Gascoyne *et al.* (2010b) results mainly because of the two-log difference between the HAB count and vast difference between the swelling pressures. It was felt that the findings from the trial study are not representative of the microbial counts and physical properties expected for high density MX-80 in pressure cells under aerobic conditions. Despite this, the outcomes of the trial study provided insights regarding the possible biogeochemical processes that may be active within or at the interface of the HCB if more or less nutrients, space and water were available. For instance, the detection and abundance of HAnB and NRB alongside HAB in nitrate-amended media (i.e., media to nourish denitrifying bacteria) specifies nitrate as alternative electron acceptor in the absence of oxygen (Pedersen, 2008) - nitrate could possibly originate from the explosives used in DGR construction or subsurface groundwater (Stroes-Gascoyne and Gascoyne, 1998; Beller *et al.*, 2004; Posiva, 2009). The study also provided some baseline knowledge about the microbiology (i.e., diversity, population) of the MX-80 bentonite when exposed to microbially restricting conditions, such as high swelling pressure, low water activity and high dry density, as well as the operational and logistic considerations.

The purpose of the present study was to assess the effects of swelling pressure, water activity, salinity and dry density on the culturability and proliferation of microorganisms contained in HCB MX-80 plugs under anaerobic conditions. The effect of salinity on microbial culturability was previously evaluated by Stroes-Gascoyne *et al.* (2010b) (40-90 days) but not under strict anaerobic conditions, a factor which could result in the growth of a range of potentially-problematic microorganisms (e.g., SRB, methanogens). Accordingly, triplicate plugs (i.e., three plugs per treatment) were prepared with target dry densities of 1.0, 1.3 and 1.6 g/cm³. All HCB plugs were infiltrated with 50 g/L NaCl solution and incubated in an anaerobic chamber for about six months commencing in May 2014.

5.3 Materials and Methods

5.3.1 Pressure cell setup – incubated from May 2014 to November 2014

Wyoming MX-80 bentonite and sterile infiltration solution (i.e., distilled deionized water containing 50 g/L NaCl) were pre-reduced to achieve anaerobic state. In order to obtain pre-reduced clay, MX-80 clay was held inside the anaerobic chamber for a 48-hour period (10 % H₂:10 % CO₂:80 % N₂ atmosphere). HCB plug preparation followed a similar procedure as in

section 4.3.1 (Experiment 2063), but with dry densities of 1.0 (known as set 1), 1.3 (set 2) and 1.6 g/cm³ (set 3) (in triplicate). The clay plugs (sets 1 to 3) were continuously infiltrated (under pressure) with saline infiltration solution for six months. Moreover, the pressure cells were housed in a sealed chamber constructed of transparent polymethylmethacrylate into which ultra-high purity, O₂-free nitrogen gas was continuously delivered at a rate of 0.002 L per minute (Appendix 10.5), under control of a digital mass flow meter (Model 8160, Matheson, NJ). An overflow valve/water-trap assembly ensured that oxygen exposure was minimized. Swelling pressure was monitored using a Campbell Scientific CR1000 data logger in conjunction with an AM16/32 multiplexer (Campbell Scientific, Edmonton, AB) and compression load cells (model 53, Honeywell Sensotec Sensors, Hoskin Scientific, Burnaby, BC) with different load ratings (1000-10000 lbs).

5.3.2 *Dry density, water activity and water content*

After the experiment, the clay plugs were removed, weighed and measured to determine “calculated” dry densities (Stroes-Gascoyne and Hamon, 2008; Priyanto, 2013). The calculated dry density is computed based on the water content of the clay plug. The formula for the calculated dry density (ρ_{calc}) is:

$$\rho_{\text{calc}} = \frac{Gs \cdot \rho_w}{1 + \left(\frac{p \cdot Gs}{S}\right)} \quad (5.1)$$

where Gs is the specific gravity of bentonite (which is 2.745 for MX-80), ρ_w is the density of water (with salt correction) (1035 kg/m³; calculated with 50 g/L NaCl at 22 °C), S is the degree of saturation (in this case, 95 %) and p is the moisture content of the clay plug (see equation 5.3). The “measured” dry density (ρ_{meas}) is the usual measurement used to describe the dry density of the clay plug at the end of the test. It is derived from the measured dimensions (i.e., total volume) of the plug, the weight of the plug inside the cell and the moisture content (see equation 5.2).

$$\rho_{\text{meas}} = \frac{M \cdot 1000}{\pi r^2 h} \cdot \frac{1}{1 + p} \quad (5.2)$$

where M is the mass of the clay added to the pressure cell (in g), r^2 is the radius of the clay plug (in this case, 16 mm), h is the height of the plug (in mm) and p is the moisture content of the clay plug (see equation 5.3). The water activities for portions of the clay plugs were measured using a AquaLab 4TE water activity meter (Decagon Devices, Inc. Pullman, WA) in accordance with

manufacturer's instructions. Water content was expressed in terms of total evaporable moisture and determined by drying a weighed clay sub-sample at 110 °C (ASTM Standard D2216-10, 2011) to constant weight. Thereafter, total evaporable moisture (p) was calculated based on equation 5.3:

$$p = \frac{(W - D)100}{D} \quad (5.3)$$

where is p the portion of total evaporable moisture content of sample, W is the mass of the wet sample, and D is mass of dried sample.

The effective montmorillonite dry density (EMDD) is a normalizing parameter that allows the comparison of clay materials with different montmorillonite contents (equation 5.4) (Dixon *et al.*, 2002). The EMDD will enable comparison of the results of the present study and that of Stroes-Gascoyne *et al.* (2010b) based on variable clay content, montmorillonite content and dry density. The EMDD values will also assist in predicting the swelling pressure (P_{swell} , kPa) in the plug sets infiltrated with 50 g/L NaCl solution (Dixon, 2000) (see equation 5.5). The EMDD and P_{swell} are calculated as follows:

$$EMDD = \frac{f_m f_c \rho_d}{1 - \frac{(1-f_c)\rho_d}{G_s \rho_w} - \frac{(1-f_m)f_c \rho_d}{G_n \rho_w}} \quad (5.4)$$

Where f_m is the mass fraction of montmorillonite in clay fraction, f_c is the mass fraction of clay in dry solids, ρ_d is the measured dry density of clay, ρ_w is the density of water, G_s is the specific gravity of solids component, and G_n is the specific gravity of non-montmorillonite component.

$$P_{swell} = 0.0015e^{5.2044(EMDD)} \quad (5.5)$$

5.3.3 Microbial analyses - enrichment, cultivation techniques and growth conditions

Enumeration of cultivable microorganisms from clay plugs was performed using the Most Probable Number (MPN) procedure (Koch, 1994). Briefly, mixtures of 2 g of solid clay taken from the plug (Appendix 10.4) in 8 ml of PBS (pre-equilibrated overnight under 10 % H₂:10 % CO₂: 80 % N₂ atmosphere in an anaerobic chamber) were serially-diluted in test tubes. Preparation and dispensing of either samples or media under strictly anoxic conditions were applied for cultivation of strictly anaerobic bacteria (Widdel and Bak, 1992).

For enrichment media preparation, anaerobic organotrophic prokaryotes (HANB) with fermentative metabolism were enriched in a R2A medium (Appendix 10.6). R2A medium amended with 0.1 % NaNO₃ was used to culture denitrifying bacterial growth (NRB). Both

media for fermenting and denitrifying bacteria were prepared under an oxygen-free argon atmosphere. SRB were enriched under a 20 % CO₂: 80 % N₂ gas mixture in Postgate's modified medium B (Appendix 10.7). Acetoclastic methanogens were incubated under 20 % CO₂: 80 % N₂ in Zeikus' medium (Appendix 10.8). Lastly, lithotrophic methanogens were enriched under similar atmosphere in modified Zeikus's medium without sodium acetate. All media were prepared in Hungate-type tubes fitted with butyl rubber septa (Chemglass Life Sciences, Vineland, NJ). The tubes were aseptically inoculated with 1 ml of 10 % clay suspensions in PBS using syringes, and incubated at room temperature (25 ± 2 °C) for 30 days. Microbial activity was also assayed by measuring H₂ (a key by-product of fermenters), monitoring dissolved sulfide (SRB activity indicator), and measuring increases in N₂ and CH₄ (growth factors for NRB and methanogens, respectively). Growth of aerobic bacteria in aerated R2A medium was determined by measuring increases in optical density of R2A relative to an uninoculated control. Viable HAnB and HAB were enumerated using R2A agar (BD; Franklin Lake, NJ) or Tryptone Soy Agar - TSA (BD; Franklin Lake, NJ) plate count procedure following Standard Methods (APHA 9215 C, 2005). Newly formed bacterial colonies were counted after either 7 days (aerobic growth) or 7 and 30 days (anaerobic growth; in the anaerobic chamber under 10 % H₂:10 % CO₂:80 % N₂ atmosphere) incubation at room temperature (25 ± 2 °C). Finally, for comparing the effects of dry densities on the HAB, HAnB and SRB counts, the one-way analysis of variance (or one-way ANOVA) was used (Minitab 17).

5.4 Results and Discussion

The combined effects of swelling pressure, water activity, and dry density on suppressing microbial proliferation in HCB MX-80 have been previously discussed (Stroes-Gascoyne *et al.*, 2010b; Pedersen *et al.*, 2000a). Section 4 of this thesis and Stroes-Gascoyne *et al.* (2010b) performed similar pressure cell studies but over ~ 8-year and 40-90-day incubation periods, respectively. The present study would serve to complement the 40-90-day pressure cell study. It would also attempt to determine if the findings from this section correlate with Stroes-Gascoyne *et al.* (2010b) results. This time, however, the target dry densities used were 1.0, 1.3 and 1.6 g/cm³ (in triplicate), the infiltration solution was 50 g/L NaCl solution, and the condition was anaerobic for ~210 days (6 months).

Table 5.1 shows the average moisture content, water activity, measured and calculated dry density values, EMDD and EMDD-based predicted swelling pressure for plug sets 1, 2 and 3.

The moisture content and water activity values obtained at the interface do not reflect the actual values because the interface region is between the surface of the plug and the inner wall of the pressure cell. These values will therefore not be interpreted in this present study. The measured dry densities appeared to be more accurate than the calculated dry densities (Table 5.1) because it was based on the moisture content, the weight of the plug and its total volume, while the calculated dry density is only based on moisture content. The measured dry density values obtained for the present study will henceforth be used to compare results with Stroes-Gascoyne *et al.* (2010b), and to calculate the EMDD and the predicted swelling pressure of each plug set. The measured dry density values acquired from Stroes-Gascoyne *et al.* (2010b) (Appendix 10.9A – for 50 g/L NaCl) indicate that when dry density values are $< 1.5 \text{ g/cm}^3$, the water activity would be ≥ 0.96 . In the present study, the water activity values for sets 1 and 2 were both 0.95. Hence, it was only appropriate that the measured dry densities were 1.3 g/cm^3 (see Table 5.1). The measured dry density and water activity values acquired for plug set 3 are in agreement with the values provided by Stroes-Gascoyne *et al.* (2010b) since the measured dry density is 1.6 g/cm^3 with water activity is 0.92.

Table 5.1. Average moisture content and water activity (a_w) values obtained from HCB MX-80 plugs with dry densities of 1.0, 1.3 and 1.6 g/cm^3 . The HCB plugs were infiltrated with 50 g/L NaCl for six months in an anaerobic chamber.

Clay plug ID	Target Dry Density	Calculated Dry Density after 210 days	Measured Dry Density after 210 days	EMDD	P_{swell} (kPa) (Dixon <i>et al.</i> , 2000)	Physical Properties	
						Moisture Content, %	a_w
Set 1 – interface	n/a	n/a	n/a	n/a	n/a	63.4 ± 5.71	0.95 ± 0.005
Set 1 – inside	1.0 g/cm^3	1.1 g/cm^3	1.3 g/cm^3	1.1 g/cm^3	441	54.7 ± 4.02	0.95 ± 0.004
Set 2 – interface	n/a	n/a	n/a	n/a	n/a	47.4 ± 8.28	0.95 ± 0.002
Set 2 – inside	1.3 g/cm^3	1.3 g/cm^3	1.3 g/cm^3	1.1 g/cm^3	427	49.6 ± 15.5	0.95 ± 0.004
Set 3 – interface	n/a	n/a	n/a	n/a	n/a	26.3 ± 0.49	0.94 ± 0.006
Set 3 – inside	1.6 g/cm^3	1.6 g/cm^3	1.6 g/cm^3	1.4 g/cm^3	1,940	26.6 ± 1.25	0.92 ± 0.010

Looking at the impact of salinity on the swelling pressure is important since the Canadian repository would likely contain highly saline groundwater. The presence of high salinity groundwater could prompt irregularities on the functionality of the bentonite in the DGR. Previous studies have shown that high salinity solutions decreased the swelling capacity of bentonite (Karnland, 2010; Foster, 1954; Marshall and Simpson, 2014; Mata and Ledesma,

2005). It has been suggested that high salinity could weaken and eventually deform the clay minerals, which could lead to increased void spaces within the bulk bentonite (Börgesson *et al.* 1995, 1996; Baumgartner *et al.*, 2008). However, Dixon (2000) suggested that MX-80 bentonite clay structure is unlikely to be influenced by the changes in swelling pressure due to high salinity water as long as the EMDD is $\geq 1.0 \text{ g/cm}^3$. The swelling pressures in the present study were monitored and were obtained for all the plugs; however, inconsistent results were acquired for sets 1 and 2. As a result, the actual swelling pressures measured were not reported in this study (except for set 3) (Figure 5.1), instead, the estimated swelling pressure (P_{swell}) was calculated, which is based on the EMDD. The P_{swell} estimated in Table 5.1 for sets 1 and 2 were 441 kPa and 427 kPa, respectively. These values were in agreement with Dixon (2000) and Studds *et al.* (1998) in that a minimum of 100 kPa is required to maintain or achieve a dry density $\geq 0.9 \text{ g/cm}^3$ in saline water (containing 10 to 75 g/L NaCl). In the present study, the P_{swell} values estimated were able to maintain a dry density value of 1.1 g/cm^3 in both sets 1 and 2 for 145 days. For plug set 3, the actual swelling pressure was measured but remained unstable over the course of approximately 145 days (Figure 5.1). It can be observed that the swelling pressure is still increasing and, therefore, possible to have reached 2,000 kPa if kept for longer periods of time. The P_{swell} value for set 3 is 1,940 kPa. It is probable that this value would have been the final swelling pressure for set 3 if the swelling pressure stabilized. Despite the fact that the swelling pressure was not stable, the microbial counts remained at background levels ($\leq 10^2 \text{ CFU/g}$) (discussed below), suggesting that the combined effects of salinity and bulk density were sufficient to prevent proliferation of microorganisms above background levels.

In terms of culturability of microbes in HCB MX-80 under three different dry densities, it can be observed that HAB and HAnB microbial counts decreased with increasing dry density (Figure 5.2). In Figure 5.2, the number of microorganisms in plug set 3 (1.6 g/cm^3 measured dry density) are lower compared to plug sets 1 and 2 (1.1 g/cm^3 measured dry density). In set 3, the number of HAB was 10^1 CFU/g at the interface and inside the plug. The HAnB counts for set 3 at the interface and inside the plug were $< 10^0 \text{ CFU/g}$. However, the SRB counts were similar in all plugs. The HAB and HAnB counts for sets 1 and 2 were quite similar altogether as seen Figure 5.2, but higher than those in set 3. The analysis of variance showed that the effect of dry density on the culturable microbial count was highly significant for HAB ($F(2,15) = 67.3, p < 0.001$) and HAnB ($F(2,15) = 32.1, p < 0.001$), but not for SRB ($F(2,15) = 2.5, p = 0.1165$). The statistical

results suggest that SRB is not highly affected by the changes in swelling pressure in the clay plugs. This could mean that the SRB were present as spores and, therefore, not affected by the environmental conditions present in the pressure cells.

Examining Figure 5.2, it is apparent that set 3 with 1.6 g/cm^3 measured dry density influenced the microbial counts the most. In order to compare the HAB microbial counts (within the plug) in the present results with the results of Stroes-Gascoyne *et al.* (2010b), the EMDD was calculated for HCB MX-80 using the measured dry density. Based on Appendix 10.9B EMDD versus CFU/g graph (salinity 50 g/L), the culturable count for HAB is $\sim 10^4$ CFU/g when at 1.1 g/cm^3 EMDD, and $< 10^2$ CFU/g at 1.6 g/cm^3 EMDD. In the present study (Figure 5.2 and Table 5.1), the results were $\sim 10^4$ CFU/g for 1.1 g/cm^3 EMDD and 10^1 CFU/g at 1.6 g/cm^3 EMDD. These observations suggest that the results agree with each other since these values fit well with the Stroes-Gascoyne *et al.* (2010b) data as the microbial counts remained at background level (10^2 CFU/g).

Salinity could also influence the culturability of microorganisms in the HCB by decreasing water activity, or the water available for microbial utilization. Stroes-Gascoyne *et al.* (2010b) have studied this phenomenon and showed that HCB infiltrated with $\geq 60 \text{ g/L}$ NaCl solution, which resulted in water activity of < 0.96 , greatly reduced HAB culturability. When salinity is $< 50 \text{ g/L}$ NaCl, Stroes-Gascoyne *et al.* (2010b) empirically determined that as long as the dry density was $\geq 1.6 \text{ g/cm}^3$, HAB culturability would be suppressed. Hence, the presence of higher salt concentrations are more effective in inhibiting microbial culturability in the HCB. Yet, even though these conditions were met by plug set 3 in the present study (Table 5.1), SRB are still present (see Figure 5.2). As mentioned earlier, it is possible that SRB are present as spores and therefore not affected by the pressure cell conditions. It was only when the SRBs were cultured that they were able to germinate; hence, similar in number in clay plug sets 1 to 3. In terms of HAB and HAnB in set 3, the conditions in the pressure cell seemed to reduced them to below background levels. Nevertheless, the HAB's eventual survival and existence in high density, low water activity and high salinity clays could be attributed to their spore-forming capability since they are adapted to wetting/drying cycles in soils.

Overall, water activity of < 0.96 , dry density of $\geq 1.6 \text{ g/cm}^3$ and swelling pressure $> 2,000 \text{ kPa}$ would suppress microbial culturability and ensure microbial counts to be below the background

level ($< 10^2$ CFU/g). Moreover, high salinity water (≥ 50 g/L NaCl) with at least ≥ 1.6 g/cm³ dry density could further suppress microbial culturability.

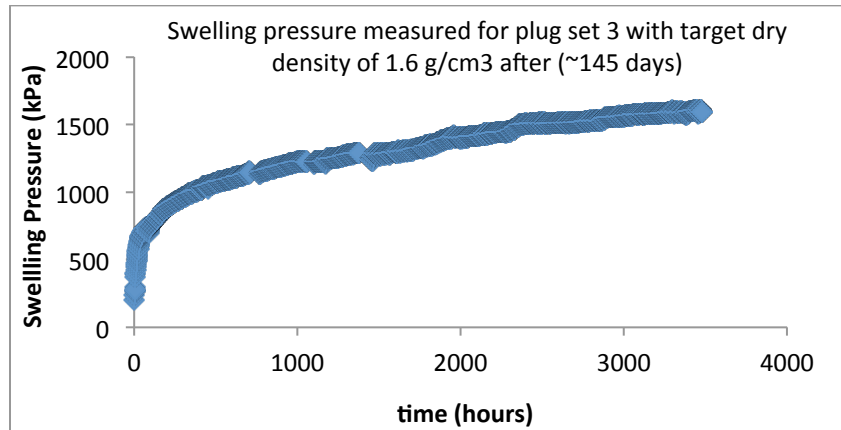


Figure 5.1. Diagram of the actual averaged total pressure (kPa) in set 3 triplicate cells that containing HCB plugs at target dry density of 1.6 g/cm³ and infiltrated with solution of 50 g/L NaCl over 3360 h (~145 days). The swelling pressure was not stable even after 3360 hours, but did not reach the desired swelling pressure of 2,000 kPa by the time experiment was terminated. The swelling pressure is presumed to be still increasing and would have reached desired swelling pressure if kept for longer periods of time. Sets 1 and 2 swelling pressures were not reported due to inconsistent data.

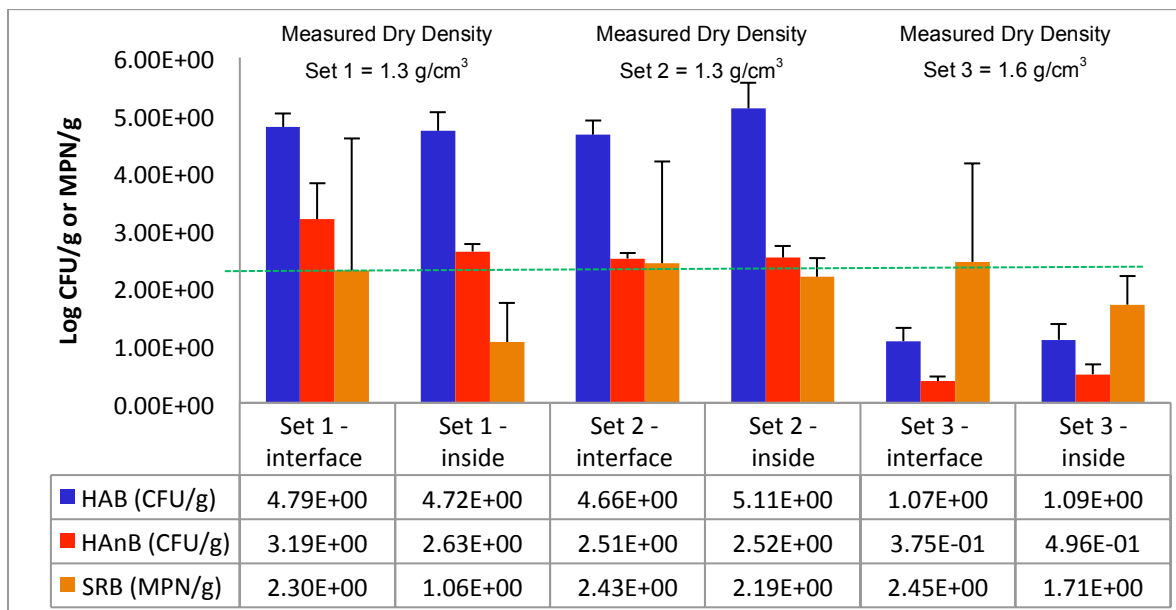


Figure 5.2. Graphical representation of the average microbial count of culturable HAB, HAnB and SRB from HCB MX-80 plug (in triplicate) infiltrated with 50 g/L NaCl solution. Set 1 represents the triplicate plugs containing a target density of 1.0 g/cm³, Set 2 and 3 contain target dry densities of 1.3 and 1.6 g/cm³, respectively. The error bars indicate the standard error of the sample mean (SEM) and examine the statistical significance between the means. Dotted green line represent the background level which is equal to 2.30 log CFU/mL.

5.5 Conclusions

Overall, a dry density of $\geq 1.6 \text{ g/cm}^3$, swelling pressure of $> 2,000 \text{ kPa}$, water activity below 0.96 and salinity of 50 g/L NaCl infiltration solution suppressed microorganisms in the HCB MX-80 plug. The microorganisms recovered were considered physiologically adaptable microorganisms. HAB and HAnB were found to proliferate in environments conducive for growth, but were reduced in number as the conditions became extremely harsh. An example of microbial proliferation under more favourable conditions would be the observed higher numbers of culturable aerobes and anaerobes in lower dry densities (i.e. 1.0 and 1.3 g/cm^3) of clay plugs. Microbial counts reduced even further when dry densities were $\geq 1.6 \text{ g/cm}^3$. The ability of these microorganisms to adapt under simulated DGR conditions could be attributed to their capacity to form spores or transform into a VBNC state. Such ability could probably explain how, after periods of being exposed to harsh conditions, these microorganisms can still emerge from dormancy once a favourably conditioned milieu is introduced such as in culturing.

5.6 Connection to Next Section

Microbial suppression in HCB MX-80 plugs were demonstrated in Sections 4 and 5. These studies provided evidence that, when the combined effects of low water activity (≤ 0.96), high swelling pressure ($> 2,000 \text{ kPa}$), high dry density ($\geq 1.6 \text{ g/cm}^3$) and salinity ($\geq 50 \text{ g/L NaCl}$) the microbial population in the HCB would be unable to increase to numbers above background levels. With respect to a DGR, these findings indicate that microbial activity would be suppressed as long as the critical parameters with respect to dry density are met and maintained over time. However, the question arises what would happen when these critical parameters are compromised, or when lower density regions, cracks, voids or fissures are present? The next section focused on sulfide generation as a result of sulfate reduction by dissimilatory SRB in non-consolidated MX-80 bentonite. Moreover, the effect of 50 g/L NaCl on sulfide generation was also examined.

6. DETERMINATION OF THE RATE OF SULFIDE GENERATION FOR SRB IN NON-CONSOLIDATED WYOMING MX-80 BENTONITE

Interface:

Daphne Jalique and **Dr. Alexander Grigoryan** equally contributed to the design of the experiment. Both performed all the preparation, tests, analyses and result interpretation for this section.

Kara Friesen, Maureen Woytowich and Hayley Kumitch provided technical assistance to this work.

Dr. Darren Korber provided guidance and assisted with experimental design, provided editing of the section, and held the research grant that supported this work.

6.1 Abstract

Highly compacted 100 % bentonite clay serves as a barrier material in a DGR. Suppression of microbial activity by bentonite clay is possible if target values of swelling pressure, bulk density and water activity are met. However, even if targeted bulk clay parameters are achieved the possibility still exists that microbes may be more active in interface regions with lower clay densities and swelling pressures. The experiments presented in this study model conditions at interfacial locations to examine activity and metabolite formation by indigenous, anaerobic clay microbes. Accordingly, anaerobic enrichments with two different salt concentrations [10 g/L (SRB10) and 50 g/L NaCl (SRB50)], and 20 mM sulfate were constructed. Each enrichment contained 10 % (w/v) dry commercial bentonite clay (i.e., MX-80, Canaprill or National

Standard) with either lactate or acetate as electron donors. The enrichments were tested under pH 7.5 or 8.5, and were incubated at 37 and 15 °C for ~120 days. Anaerobic microbial activity was determined through sulfide formation. In SRB10, 12.4 mM sulfide was produced in MX-80 clay at 15 °C (lactate) after 44 days, while 2.8 and 4.3 mM sulfide was observed in the same clay at 37 °C (lactate). The medium with acetate produced < 1 mM sulfide after 65 days at 37 °C, while 4.8 mM sulfide was found in the medium at 15 °C. Canaprill clay (lactate) incubated at 37 °C exhibited the highest sulfide production (~22 mM), whereas the rest of the Canaprill and National Standard clay enrichments showed sulfide production of < 4 mM. These results indicated that the rates of sulfide production and amount of sulfide in all commercial clays was dependent on temperature, organic source and clay type, and that microbial sulfidogenesis preferred lactate as the major electron donor. Culture-independent 16S rDNA PCR-DGGE-based community analysis revealed that *Desulfobulbus*, *Desulfuromonas*, *Pelobacter* and *Pseudomonas* were present in all enrichments. For SRB50, sulfide production was lower (< 2.9 mM sulfide was produced after 120 days in all commercial clays at 15 °C with lactate) compared to SRB10 enrichments. The results suggest that elevated salt concentrations would be inhibitory to sulfidogenesis.

6.2 Introduction

Conditions within the EBS of a DGR are intentionally engineered to present a major hurdle to microbial growth and activity. As shown in Sections 4 and 5 of this thesis, after a HCB plug's exposure to the combined effects of low water activity, high swelling pressure and high dry density, microbial populations remained at background levels (in agreement with the findings of Stroes-Gascoyne *et al.*, 2010b and Pedersen *et al.*, 2000a). In those studies, microorganisms were not completely eradicated, but rather likely entered into a VBNC state, or formed or remained as spores to withstand deleterious DGR conditions. Therefore, it is possible that these microorganisms could once again become active if the system became more favourable for growth. For instance, if fissures/cracks form or if the HCB becomes less dense, available void spaces, hydraulic conductivity and water activity could all increase, allowing increased activity of microorganisms. Moreover, such circumstances could introduce allochthonous microorganisms into a DGR from groundwater (Haveman *et al.*, 1999). The outcomes could possibly affect a DGR in several ways as many conceivable biogeochemical processes may transpire because of increasing microbial activity (Fredrickson and Balkwill, 2006). One possible

occurrence that may emerge would be the over-pressurization of the repository as a result of gas generation coupled with heterotrophic organic degradation processes. The organic degradation processes could also promote mobility of gases, radionuclides and groundwater. Sulfate reduction activity is another process that could indirectly affect the DGR, through the production of corrosive metabolites.

SRB are obligate anaerobic microorganisms that utilize a number of carbon substrates (e.g., lactate or acetate as electron donors) and use sulfate as electron acceptor. SRB produce sulfide, which is corrosive to copper. Sulfide is an active chemical that can blacken sediments in aqueous environments due to ferrous sulfide formation (Pedersen *et al.*, 1995). Moreover, it can be detected by its pungent smell. In a DGR, the presence of SRB could facilitate the corrosion of the metal containers (Enning and Garrelfs, 2014; Lovley *et al.*, 1991; Pedersen *et al.*, 2000b). However, since a DGR environment would be diffusion-dominated, the likelihood that sulfide would be transported to the metal containers are very low. The diffusion of sulfate in bentonite, on the other hand, is faster than sulfide (Masurat *et al.* 2010b). Therefore, if SRB biofilms were formed near or on the canisters, higher rates of sulfate transport may result in substantial canister corrosion. The presence of naturally occurring dissimilatory SRB in Wyoming MX-80 bentonite has been reported. Masurat *et al.* (2010a) were able to isolate *Desulfovibrio africanus* from MX-80 bentonite and suggested that this bacterium survived because it entered a dormant, desiccated state, and once appropriate anaerobic media was provided, the bacteria were revived. They also showed that *D. africanus* remained viable after 20 h of heat treatment (100 °C), indicating that it could tolerate the heat produced by radioactive UFCs. SRB have also been detected in groundwater under anaerobic conditions (Haveman and Pedersen, 2002). Therefore, monitoring SRB activity in bentonite in terms of sulfide production would assist in determining the extent of their capability to compromise a DGR.

The purpose of this study is to investigate SRB activity in anaerobic enrichments containing low-density clay, which will be termed “slurry”, through sulfide generation. Three types of clay were selected (i.e., Wyoming MX-80, Canaprill and National[®] Standard bentonite) which served as the inocula. The enrichments varied in terms of electron donors, pH, salt concentration, and incubation temperature. Sulfate reduction and sulfide production were monitored using the BaCl₂ turbidimetric and methylene blue methods, respectively. In addition, some of the enrichments

were subjected to molecular and biochemical analyses (i.e., DNA extraction, and DGGE and High Performance Liquid Chromatography (HPLC) analyses).

6.3 Materials and Methods

6.3.1 Clay sources and inoculum

Commercial clay products used in this study were obtained from American Colloid Company (MX-80, Wyoming clay), Canadian Product Inc. (Canaprill[®], Avonlea clay), and Bentonite Performance Minerals LLC (National[®] Standard, Wyoming clay) (see Appendix 10.1 for chemical composition).

6.3.2 Media and growth conditions

6.3.2.1 SRB10 Enrichment – Regular Zhilina medium containing 10 g/L NaCl

For the enrichment of SRB in clay, a basal medium for alkaliphilic bacteria was prepared (Zhilina *et al.*, 1997; Appendices 10.10 and 10.11). The medium contained 10 g/L NaCl, and accordingly was labelled SRB10. The Zhilina medium was autoclaved and cooled in an anaerobic dispensing system under a 90 % (vol/vol) N₂, 10 % (vol/vol) CO₂ atmosphere. Prior to dispensing, the pH value was adjusted either to 7.2 or 8.5 using NaOH. For all enrichment bottles (150 mL serum bottles), 90 mL of medium was aseptically dispensed under an anoxic gas phase into the bottles and 10 g of dry, degassed MX-80 bentonite was aseptically added. Thereafter, the bottles were sealed with butyl rubber stoppers and crimped with aluminum rings (Figure 6.1A).

The following conditions were introduced (in duplicate): 1 M sodium acetate and 20 mM sulfate at pH = 7.2, 1 M sodium acetate and 20 mM sulfate at pH = 8.5, 1 M sodium lactate and 20 mM sulfate at pH = 7.2, 1 M sodium lactate and 20 mM sulfate at pH = 8.5, control at pH = 7.2 (with 20 mM sulfate without electron donor; one bottle only) and control at pH = 8.5 (with 20 mM sulfate but without electron donor; one bottle only). In total, 20 enrichments were incubated under stationary conditions at 15 °C and 37 °C (ten bottles per temperature). Sampling was performed every two days for samples incubated at 37 °C, and every three days for samples at 15 °C for 70 days on all clay enrichments. For sampling, a 22 G x 1-1/4” needle and 3 mL syringe (pre-flushed with O₂-free nitrogen gas prior to sampling) were used to obtain 1 mL of sample from the clay-Zhilina medium suspensions. These samples were analyzed for sulfate and sulfide concentration (section 6.3.3). Changes in the consistency, colour of clay slurry and the amount of consumed/formed sulfate/sulfide were documented. Lastly, 1 mL of sample from the

aforementioned enrichment was taken and kept frozen at -80 °C for subsequent assay for organic acids by HPLC (section 6.3.4).

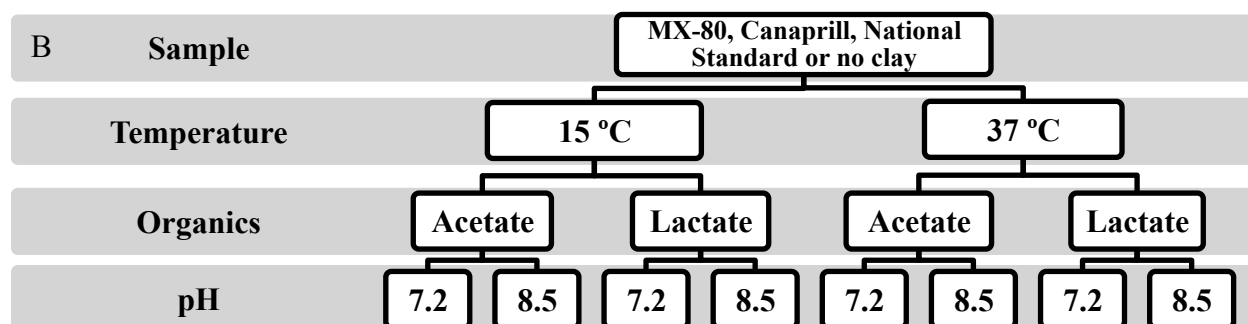


Figure 6.1. SRB10 Enrichment study. A) Enrichment bottles containing 10% MX-80 bentonite and organics stored at 37 °C in duplicate at the completion of the incubation period. B) Schematic diagram showing the various enrichment treatments. A total of 18 bottles per bentonite sample (MX-80, Canaprill, and National Standard) were made including control (no clay).

6.3.2.2 SRB50 Enrichment – Modified Zhilina medium containing 50 g/L NaCl

For this study, the same clay materials and Zhilina media were used, however only pH 7.5 was used (a decision based on the results presented in section 6.4.2, since SRB50 was done after SRB10) and the final concentration of NaCl was 50 g/L, instead of 10 g/L (SRB10 in section 6.3.2.1).

6.3.3 Analytical procedures - sulfate and sulfide determination

For sulfide determination, aqueous sulfide concentrations were determined colourimetrically via the methylene blue method employing N,N-dimethyl-p-phenylenediamine sulfate ($[(CH_3)_2NC_6H_4NH_2]_2 \cdot H_2SO_4$), as modified from Trüper and Schlegel (1964) and Cline (1969). The basic sulfide assay was performed as follows: a 39.5 μ l aliquot of sample was added to 632 μ l 2 % zinc acetate (in 0.1 % w/v glacial acetic acid) to trap sulfide. Thereafter, 789 μ l of pPDA

reagent (0.2% w/v of N, N-dimethyl-p-phenylenediamine sulfate (Eastman Kodak Co. No. 1333, New York, USA) in 20 % w/v H₂SO₄) was added. The mixture was vortexed briefly and then set aside for 1-2 min at room temperature. The sample was then centrifuged for 2 min at 13000 rpm at room temperature. The supernatant was gently removed and transferred to a new microcentrifuge tube (solid-free sample). A 39.5 µl aliquot of 10 % FeNH₄(SO₄)₂·10H₂O (in 2 % w/v H₂SO₄) was then added. The mixture was incubated for 20 min and the absorbance was measured at OD₆₇₀.

Sulfate was assayed using a turbidimetric method, employing BaCl₂. Turbidimetric assays were conducted in one of two ways: 1) by mixing a 50 µl sample with 950 µl of conditioning agent I (a 180-fold dilution in H₂O of 50 mL glycerol, 30 mL concentrated HCl, 75 g NaCl, 100 ml 95 % ethanol and 255 mL deionized water). Excess ground BaCl₂ (~4 mg) was then added and the mixture was vortexed about 30 s to ensure the BaCl₂ was completely dissolved (Nemati *et al.*, 2001). After setting aside for 30 min, the optical density (OD₄₂₀) was measured using a Genesys 10S UV-Vis Spectrophotometer (Thermo Scientific, Massachusetts, USA). Sample concentrations were determined by comparison with a standard curve, or 2) by adding 150 µl of centrifuged turbidity-free sample to 350 µl of deionized (MQ) water to which 500 µl of citric acid-glycerol solution (5 %, w/v, citric acid·H₂O in 60 %, v/v, glycerol) and 125 µl barium reagent (1 % w/v, BaCl₂·2H₂O in 10 %, w/v, citric acid) were added. After the solution was mixed and left to incubate for 30 min, the turbidity was measured at OD₄₃₆ (Cypionka and Pfennig, 1986).

Moreover, sulfate concentrations in MX-80 and Canapril samples were determined using Agilent 4100 Microwave Plasma-Atomic Emission Spectrometer (MP-AES) machine at the Department of Soil Science analytical lab, University of Saskatchewan. For determination of sulfate by means of MP-AES, 8 g of clay (pre-dried at 130 °C for 4 h) was mixed with 25 mL deionized water at either pH = 2 or 7 (adjusted by means of H₃PO₄ [conc.] and NaOH [10 N]). Two different pH values were used in order to verify if Darbi *et al.* (2003) observation of enhanced sulfate leaching at pH = 7 is correct. The mixture was equilibrated overnight at room temperature on a rotary shaker under gas mixture of 5 % H₂, 10 % CO₂ and 80 % nitrogen. Thereafter, clay slurries were centrifuged at 20000 rpm at 4 °C (Sorvall Ultra-Speed Centrifuge, Ivan Sorvall, Inc., Norwalk, CT) for 15 min. About 5 mL of supernatant were then aliquoted and

filtered through a 0.45 µm pre-sterilized syringe filter and analyzed in accordance with the standard operating system in the analytical laboratory.

6.3.4 Analysis of organic acids from clay samples using High Performance Liquid Chromatography (HPLC)

The concentrations of acetic and lactic acids in the samples collected in section 6.3.2.1 were determined using a Varian ProStar High Performance Liquid Chromatography (HPLC) system at the Laboratory of Soil Toxicology at the Department of Soil Science, University of Saskatchewan. The equipment consisted of a Varian Prostar 410 autosampler, Varian Prostar 212 pump system, and the Varian Prostar 325 UV-Vis detector. Each sample was measured at 210 nm after elution through a Hi-Plex H column (6 by 316 mm; Agilent) with 0.01 M H₂SO₄ in deionized distilled water (ddH₂O) as the mobile phase.

6.3.5 Evaluation of sulfide accumulation rates

The rate of sulfate reduction in the enrichments was evaluated using equations 6.1 and 6.2,

$$(S^{2-})_{1/2} = \frac{100}{T_{50}} \quad (6.1)$$

Where $(S^{2-})_{1/2}$ represents the time required (in days) to generate 50 % of the maximum concentration of detected sulfide and T_{50} is the predicted future value of sulfide concentration calculated using the linear regression of the existing values. These initial relative values of sulfide producing activity were then recalculated to consider both time and amount of sulfide produced. In this case, equation 6.2 was used.

$$RA = (S^{2-})_{1/2} \frac{1}{C_{50}} \quad (6.2)$$

where C_{50} represents 50 % of the maximum concentration of detected sulfide from sample. The *RA* index (relative activity index) represents the measurement of sulfide concentration per time (mM per day) and is based on the equation 6.1.

6.3.6 DNA extraction and Denaturing Gradient Gel Electrophoresis (DGGE) analysis

SRB10 enrichments (samples from 18 bottles) were centrifuged in 3-ml aliquots for 20 min at 4 °C and at 4000 × g. The combined pellets from replications of each type enrichment were resuspended in 2 ml of NaCl-EDTA, aliquoted in 1-mL fractions and stored at -20 °C until used for DNA extraction. DNA was recovered using the FastDNA SPIN kit for soil (MP Biomedicals, Inc., Carlsbad, CA) following the manufacturer's recommendations. Subsequently,

DNA preparations that originated from the same experiment were pooled together for further processing.

The primer pair B7-27F and 534R (Muyzer *et al.*, 1993, Grigoryan *et al.*, 2008) were used to amplify the V3-V5 region of the 16S rRNA gene. For DGGE, a second round of PCR was conducted using a forward primer modified to include an extra 40 bp length GC clamp attached to its 5' end. The PCR reactions were performed in 50 µl volumes containing 1-2 µl of template DNA, 0.5 µmol of reverse and forward primers, 25 µl Taq 2X Master Mix (New England Bio Labs, Ipswich, MA) and 22 µl of molecular grade water (Mo-Bio Laboratories, Carlsbad, CA). PCR cycle conditions were set at an initial 5 min denaturing step at 94 °C, followed by 35 cycles of 45 s at 95 °C, 45 s at 60 °C, and 45 s at 72 °C, and a final elongation step of 5 min at 72 °C on a Techne TC-412 thermal cycler (Techne Inc., Burlington, NJ). PCR products were electrophoresed on 1.5 % agarose (Invitrogen, Carlsbad, CA), stained with ethidium bromide (Sigma–Aldrich, St. Louis, MO) and photographed.

For DGGE analysis, ~400 bp bacterial 16S rDNA PCR product was separated by DGGE (Muyzer *et al.*, 1993) using the DCODE system (Bio-Rad, Hercules, CA). A 10 µl aliquot of PCR product was mixed with 10 µl of loading dye buffer and resolved on an 8 % w/v polyacrylamide gel in 1× TAE buffer using denaturing gradients from 35 to 60 % (where 100 % denaturant contains 7 M urea and 40 % deionized formamide). DGGE was carried out at 70 V for 14 h at 60 °C. After electrophoresis, gels were stained with SYBR Green I (1:10,000 dilution in 1× TAE buffer) for 20–30 min and photographed. Detectable bands on the DGGE gels were picked and DNA was extracted. Extracted DNA was re-amplified using the B7-27F and 534R primers and sequenced at the Plant Biotechnology Institute (PBI, NRC, Saskatoon, SK). The sequences obtained were edited using Sequence Scanner software v1.0 (Applied Biosystems). Homologous sequences were retrieved from GenBank using BLAST software (Altschul *et al.*, 1990) and within Ribosomal Database Project database (Cole *et al.*, 2005). Sequences of 16S rRNA were verified using Chromas Explorer Lite software, version 3.3.0 (Heracle Biosoft S.R.L., Romania). The sequences were also edited manually using Chromas Explorer Lite, and alignments were edited manually using the Clustal Omega (EMBL-EBI; <http://www.ebi.ac.uk/Tools/webservices/>) (McWilliam *et al.*, 2013). Identification of distinct bacterial isolates was based on the percent identity, where 99-100 % identical sequences were associated with the same taxon. Thereafter, the sequences obtained were compared with the

closest type-strain16S rRNA gene relatives and assembled into a phylogenetic tree using the software program MEGA6 (Tamura *et al.*, 2013).

6.4 Results and Discussion

6.4.1 Evaluation of analytical assays for sulfate and sulfide determination

To evaluate the potential impact of sulfide production in a DGR, determination of appropriate protocols to analyze sulfate and sulfide was important. The adsorption or precipitation of sulfide within the clay materials and inclusion of clay materials in the assay was also considered since it could hinder collection of reproducible and unbiased data. Stone *et al.* (2016a) showed that sulfide adsorption in the clay is not an isolated incident. Accordingly, when the authors added bentonite to a sulfide standard curve, the slope decreased from 0.059 to 0.0006 (slope closer to zero hence line starts to flatten) indicating adsorption of sulfide in bentonite. It can be argued that the bentonite surface chemistry and its overall permanent surface charge may have contributed to the sulfide (HS^{2-}) adsorption phenomenon (Nguyen-Thanh *et al.*, 2005; Ismadji *et al.*, 2015). For instance, it is plausible that HS^{2-} will interact with the AlOH and/or SiOH edge sites on the surface of montmorillonite to counterbalance the charges. The possibility of HS^{2-} reacting with Na^+ and H^+ at exchange sites (within the interlayer space) of montmorillonite is also a possibility (Tetre *et al.*, 2006), although, this is improbable because HS^{2-} is about 0.32 nm in diameter, while montmorillonite interlayer spacing is only 0.2 nm (Nguyen-Thanh and Bandosz, 2003). In accordance with these considerations, the sulfide clay adsorption issue was resolved by soft centrifuging the sample at 14000 rpm for 90 s after the addition of pPDA reagent (or 0.2 % w/v of N, N-dimethyl-p-phenylenediamine sulfate in 20 % w/v H_2SO_4), by using the turbid-free supernatant in the final stage of the procedure (i.e., mixing with 10 % $\text{FeNH}_4(\text{SO}_4)_2 \cdot 10\text{H}_2\text{O}$ (in 2 % w/v H_2SO_4)) and by trapping the sample in zinc. However, it is possible that not all adsorbed sulfide was released from the clay after these additional steps. Appendix 10.12 illustrates how centrifuging the sample prior to addition of ferric iron affected methylene blue production.

Sulfate reduction results in the formation of hydrogen sulfide (Graham *et al.*, 2003), as seen in equation 6.3.



Therefore, accurate quantification of the remaining sulfate in the enrichment is vital in predicting how much sulfide has been generated over time. For sulfate analysis, it was determined that

using dissolved sulfate (section 6.3.3), which forms an insoluble salt with barium after addition of BaCl_2 , yielded reproducible results. Dissolved sulfate analysis was, therefore, selected as a routine laboratory method due to its simple, consistent and wide-spread use in SRB studies (Cypionka and Pfennig, 1986). Adjustments to these protocols have been made to reduce biases and receive optimized standard curves with different types of clays. For instance, in Figure 6.2, sulfate standard curves were produced using Zhilina medium (with 10 g/L NaCl) with MX-80, Canaprill or National Standard bentonite with varying sulfate concentrations (1, 2.5, 5, 10 and 20 mM). Also, the amount of dissolved sulfate that would become available for bacterial reduction was estimated, at either low or neutral pH values, using MP-AES. It was assumed initially that lower pH would facilitate clay dissolution and leaching of sulfate; however, preliminary data indicated that the optimum sulfate leaching conditions are likely at pH 7 and higher. The results of Darbi *et al.* (2003) are in agreement with the outcomes of the current study since the authors observed sulfate leaching from bentonite into water at $\text{pH } 7.5 \pm 0.2$. As a result, 0.124 mg and 0.297 mg sulfate were leached per gram of MX-80 at pH 2 and pH 7, respectively. On the other hand, around 0.096 mg sulfate was leached per gram of Canaprill at pH 2. In relation to the present study, this means that the enrichments containing 10 g of clay will originally have at least 0.09-0.16 mM of accessible sulfate readily available for SRB metabolism.

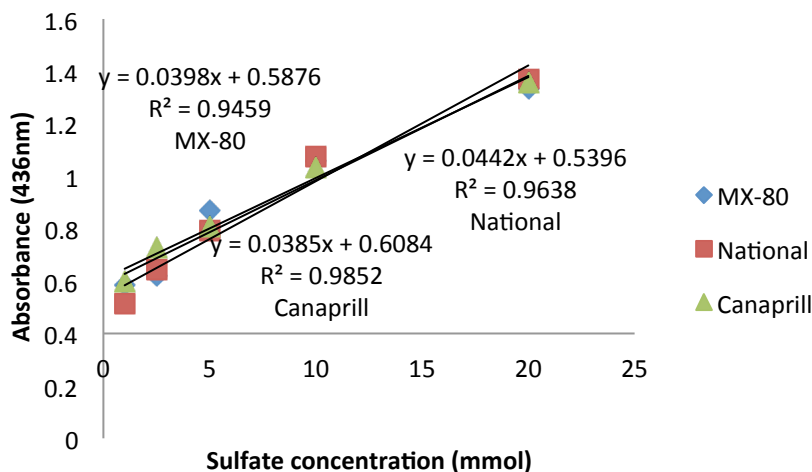


Figure 6.2. Sulfate standard curve produced for MX-80, Canaprill and National Standard bentonite. The sulfate standard curves were produced using SRB medium (with 10 g/L NaCl) with clay at varying concentrations of sulfate (1, 2.5, 5, 10 and 20 mM). The barium chloride turbidimetric method was used to determine sulfate concentration.

6.4.2 SRB10 Enrichments

Microcosm enrichment studies are controlled ecological systems that contain a portion of a natural environment (Drake and Kramer, 2011). Using enrichments allows for the evaluation of biogeochemical processes that would likely have occurred in natural environments. In this study, the allochthonous microbial activity was investigated using 10 % clay in water (wt/vol) to form slurry inside laboratory-prepared enrichments. About 20 mM of accessible sulfate was added to the Zhilina medium on top of the 0.09-0.16 mM sulfate already in the bentonite added (section 6.4.1), which may be utilized as electron acceptor for SRB activity. Subsequently, lactate or acetate (organic acids) was also added in order to enhance sulfate reduction. These organic acids served as electron donors and selected based on their ability to support SRB growth (equations 6.4 and 6.5) (Oren, 1999).



Bentonite contains organic carbon (~0.19% in MX-80; Sauzeat *et al.*, 2001) but does not naturally contain lactate. Therefore, lactate addition in the enrichment would stimulate bacterial activity. Aliphatic acid anions (e.g., acetate, butyrate and propionate), on the other hand, can occur naturally in the subsurface as products of fermentation (Fisher, 1987) and, acetate could accumulate in geological formations as it is a common end-product of microbial fermentation. However, in a DGR system where microbial fermentation in 100 % highly compacted bentonite would likely be inhibited, these end-products would be generated in minor quantities or be absent. It is actually more probable that higher amounts of fermentation end-products would be produced within the less-densed backfill regions of the repository (Wolfaardt and Korber, 2012). The pH of the Zhilina media with lactate and acetate was pre-adjusted to 7.2 or 8.5 prior to overnight equilibration at 4 °C. Thereafter, the pH eventually stabilized at 7.5 and 7.9, respectively. The MX-80 enrichment experiment was terminated after 70 days of incubation at 15 °C and 37 °C, while Canapril and National Standard bentonite enrichments went on for 120 days of incubation. The lower temperature (15 °C) is relevant to the *in situ* subsurface conditions at depth of 600 m, in accordance with the framework of the Canadian DGR concept. The 37 °C incubation temperature permits proliferation of a broader range of SRB and may be more appropriate if mimicking a younger repository. Throughout the course of the experiments, any

changes in the consistency and colour of the clay slurry were documented. The amount of sulfate consumed or sulfide produced was also monitored. Approximately 1 mL sample from each bottle was kept and stored at -80 °C for subsequent assay for organic acids by ion chromatography.

Sulfate in the enrichments served as the electron acceptor for SRB activity, while lactate or acetate oxidation occurred. The Small *et al.* (2008) sulfate reduction model predicts that sulfate concentrations decrease as sulfide is formed. This model is further explained by the sulfate reduction stoichiometry (equation 6.3), wherein one mole of sulfate generates one mole of sulfide. Hence, when 20 mM of sulfate is used, it is expected that 20 mM of sulfide will be produced. However, when sulfate and sulfide were assayed, the results were not consistent with the stoichiometry. First, at the outset of the experiment, less sulfate (~15-18 mM) was already detected than expected (i.e., ~20 mM was added) while no sulfide had been generated. Furthermore, an obvious fluctuation in sulfate was observed over time in all samples without affecting sulfide. No plausible explanation is available to explain these observations. It is possible that sulfate adsorption/desorption processes onto clay minerals took place, while the remainder was accessible for reduction. For instance, the presence of sodium-enriched montmorillonite in the clays could have slightly enhanced adsorption of sulfate onto the clay minerals. Perdrial *et al.* (2009) suggested that monovalent and divalent cations could serve as bridges to bind anions to the clay mineral surface. However, Alves and Lavorenti (2004) disagreed with this notion and argued that the iron and aluminium oxide content of clay minerals have a much greater impact on sulfate adsorption than clay mineralogy and cation bridges. Pecini and Avena (2013) further explained that the aluminium edges of clay minerals exposing $\text{OH}_2^{+/-}$ are reactive groups that have adsorptive properties. Therefore, at specific pH values, especially below the point of zero charge of edges ($\text{pH}_{\text{PZC,edge}}$ or isoelectric point of the edges), the edges would have positive or negative charges that could bind with cations or anions. However, in the case of MX-80 bentonite, the lower limit of $\text{pH}_{\text{PZC,edge}}$ is 4.0 ± 0.2 , which means that the clay minerals in the present study would not have adsorbed sulfate onto its edges or surface because it had more negative charges exposed since the pH was between 7.0 to 7.9. In the case of Canapril microcosms without supplemented organics (i.e., no sulfate reduction), the observation of no decrease in sulfate indicated no adsorption of the sulfate which may perhaps be attributed to its lower montmorillonite content (Nguyen-Thanh *et al.*, 2005). Finally, the Small *et al.* (2008) sulfate reduction model suggested that the subsequent decline in sulfide is possibly due to sulfide

precipitating as FeS (black precipitates) in the mixture. This was, in fact, one of the visual indications of SRB activity observed in the present study and predominantly seen in Canapril enrichments amended with lactate (both temperatures). Despite the apparent variability in sulfate concentrations in the enrichments, it is more important to focus on how much sulfide is generated over time, since sulfide could directly affect the metal canister in the DGR. It is also relevant to identify that no abiotic sulfide is present within the bentonite as it could affect the metal containers in a DGR.

In the case of sulfide production, it has been noted that it did not follow the sulfate reduction stoichiometry (equation 6.3) since < 20 mM sulfide were produced in ~ 20 mM sulfate-containing enrichments likely due to precipitation of FeS. However, lactate and acetate oxidations may have also influenced the unexpected sulfide concentrations in the enrichments. In the present study, sulfide production in MX-80 enrichments containing lactate (pH 7.5 and 7.9) at 37 °C is approximately 2.8 and 4.3 mM (Figure 6.3 A, B). But based on the HPLC organic acid data (Figure 6.4 A, B), lactate was completely oxidized within the first 20 days of incubation at 37 °C. Lactate oxidation occurred simultaneously with the production of acetate, which thereafter became partially degraded (Figure 6.4 A, B). Such a biogeochemical process has been observed in *Desulfovibrio* species (Postgate, 1984) when grown in media supplied with lactate. On the other hand, no detectable increase was observed in sulfide in the aqueous phase of enrichments amended with acetate and incubated at 37 °C (Figure 6.3 C, D). However, analysis of organic acids revealed a 50 % reduction in acetate (Figure 6.4 C, D). The disappearance of acetate could be prompted either by acetate oxidation of organisms other than SRB, or by acetate sorption onto clay particles. Oremland and Polcin (1982) explained that when acetate is readily available as electron donor, SRB would outcompete microorganisms such as methanogens for acetate utilization in sulfate-containing sediments. However, since it is possible that acetate is bound within the clay matrix (Wang and Lee, 1993), SRB would have difficulty utilizing acetate for growth once the available supplies have been exhausted. At 15 °C, sulfide accumulation was observed in the aqueous phase of enrichments amended with both lactate and acetate after 44 days. By the 65th day, 12.4 mM and 4.8 mM of sulfide was produced in lactate- and acetate-amended media, respectively (Figures 6.5 A-D). Lactate in these enrichments was completely oxidized around 25-30 days, with the transient production of 6-8 mM acetate (Figures 6.6 A, B), which was later degraded. In enrichments where acetate served as primary source of carbon,

acetate was partially (3 mM of 4 mM, pH 7.9) or completely (5 mM in about 48 days, pH 7.5) degraded (Figures 6.6 C, D).

Enrichments containing Canapryll clay generated sulfidogenic activity earlier in comparison with MX-80 at both 37 °C and 15 °C (Figure 6.7; data for first 30 days not available due to analytical assay error, but analysis was corrected and applied for the remainder of the analysis period). In enrichments containing lactate at 37 °C, sulfide production was first detected at 7-8 days of incubation. Thereafter, more than 20 mM of sulfide has accumulated by ~120 and 60 days at pH 7.2 and 7.5, respectively (Figures 6.7 A, B). For medium augmented with acetate at 37 °C, sulfide was only detected after 20 days and accumulated at ~2.1-2.2 mM by 40 days (Figures 6.7 C, D). No further production of sulfide was observed over the remainder of the 120-day incubation. Sulfide generation was lower in enrichments stored at 15 °C (Figure 6.8). Approximately 3.7-3.9 mM sulfide was produced in media containing lactate over the first 25 days; while, about 1.0 mM of sulfide was generated in media containing acetate at either pH 7.2 or 7.5. Canapryll clay, from badland sediments of Avonlea, SK, contains ~ 2.3% montmorillonite (Khan, 2012); hence, lower AlOH and/or SiOH edge sites on the surface of montmorillonite for H₂S to interact with as they were adsorbed (Nguyen-Thanh *et al.*, 2005). Therefore, the possibility that a lower montmorillonite ratio in the overall mineralogy of Canapryll clay played a role in preventing adsorption of sulfide to clay minerals, allowing easier detection of sulfide, must also be considered.

Enrichments with National Standard clay at 37 °C showed some increase in sulfide after 80 and 50 days of incubation with lactate. It produced around 0.7 mM and 2.1 mM of sulfide at pH 7.8 and 7.9, respectively (Figure 6.9). In contrast, earlier and more extensive production of sulfide occurred at 15 °C than that at 37 °C (Figure 6.10). About 3.8 mM of sulfide was generated by 60 days in media with lactate, and 2.5 mM by 85 days in media enriched with acetate. Subsequently, about 6 mM and 4 mM were produced in total in media with lactate and acetate, respectively (Figures 6.10 A-D).

Sulfidogenesis half-time ($t_{1/2}$) and relative activity (RA) indices were introduced to systematically compare sulfide production rates in the different microcosms used in this study. The half-time represents the amount of time required for an increase in sulfide concentration of 50 %, while RA relates to the rate at which concentrations of sulfide are detected in the microcosms. Based on the calculations, Canapryll has the highest sulfide production rates followed by National Standard

and then MX-80 in microcosms incubated at 37 °C and supplemented with lactate (Figure 6.11). Similar sulfide accumulation rates occurred in MX-80 and National Standard clays when lactate was added. No sulfide was detected in acetate-amended media incubated at 37 °C (Figures 6.8, 6.9 and 6.10).

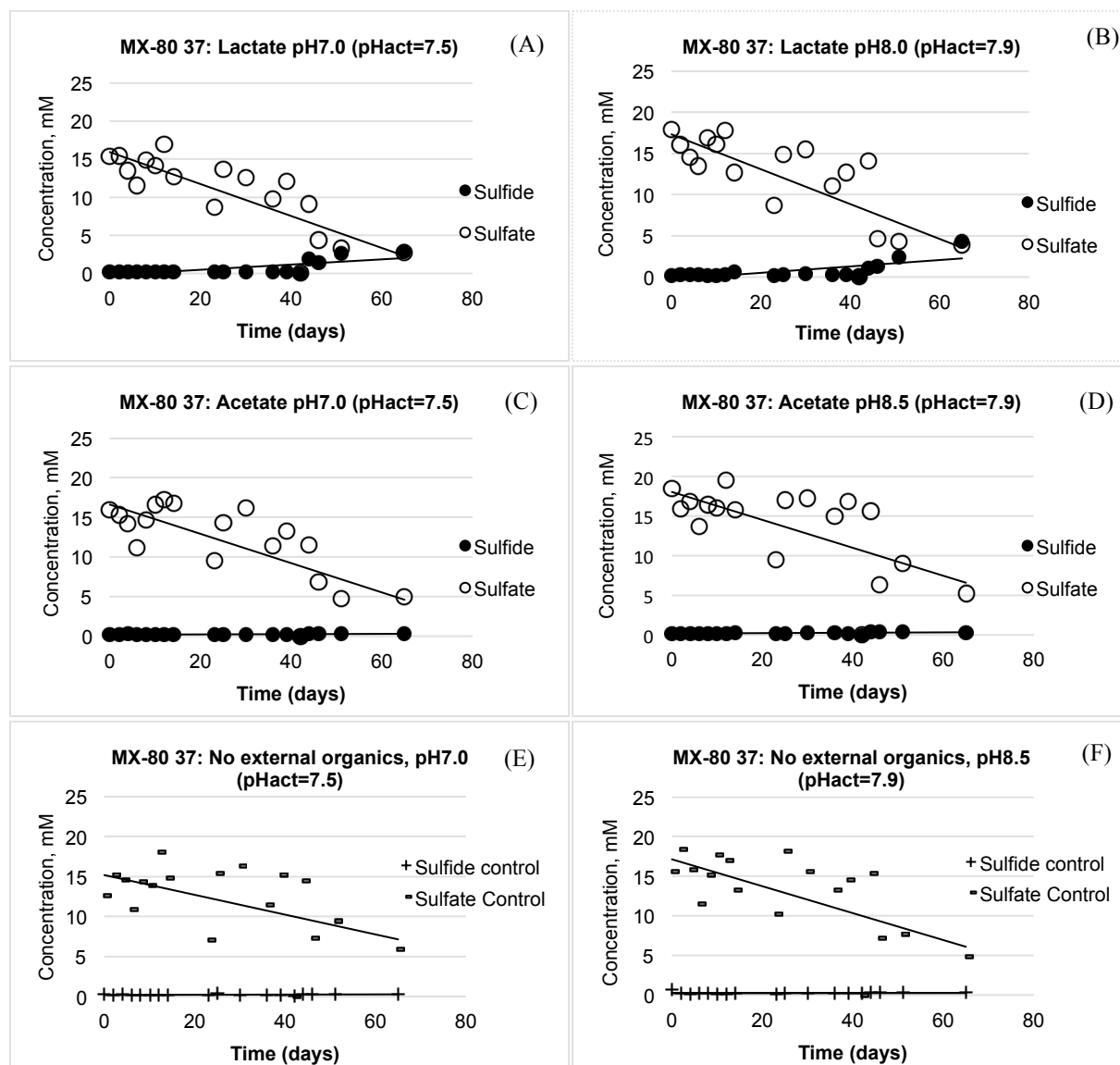


Figure 6.3. Average values of sulfide production and sulfate consumption in Zhilina's media containing 10% w/v MX-80 and supplemented with either 10 mM lactate (A, B) or 10 mM acetate (C, D) under different pH at 37 °C. No exogenous lactate or acetate was added for control samples (E, F). Anticipated and measured (act) pH values are indicated. Dashed lines specify moving average trend lines of concentrations. Concentrations of sulfide and sulfate are shown as a function of incubation time. Sulfate data shown for illustrative purposes only due to its unpredictable fluctuation in the enrichments.

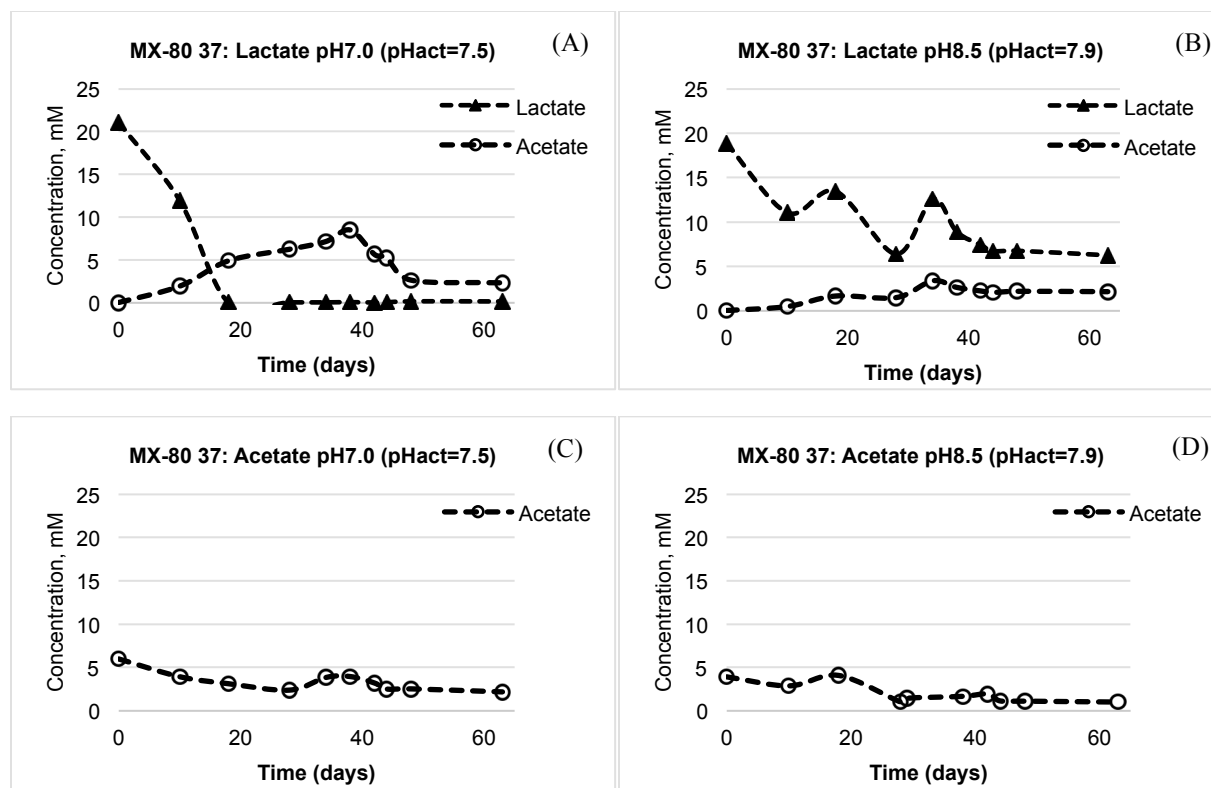


Figure 6.4. Concentration of acetic and lactic acids in Zhilina’s media containing 10% w/v MX-80 and supplemented with either 10 mM lactate (A, B) or 10 mM acetate (C, D) under different pH at 37 °C. Quantities of acetate and lactate are shown as a function of incubation time.

Sulfide generation was observed in all samples with acetate at 15 °C. Based on Figure 6.11, sulfide accumulation with acetate occurred gradually and to a lesser degree than in microcosms augmented with lactate. The rate of sulfidogenesis activity with lactate as electron donor occurred as follows: Canaprill >> MX-80 > National Standard. The rate of sulfidogenesis activity for all clay types amended with acetate was quite similar. Canaprill samples demonstrated a relatively faster sulfide accrual. The difference in montmorillonite content in MX-80 and Canaprill (75 % and ~2.3 %, respectively) may have influenced SRB activity in the microcosms. Wong *et al.* (2004) showed a reduction of SRB activity by 61 % in *Desulfovibrio vulgaris* strain Hildenborough culture with bentonite as inoculum. Hence, in a DGR setting, the likelihood of SRB activity being inhibited is higher since montmorillonite in HCB would extract microbially-accessible water (Motamedi *et al.*, 1996; Pedersen *et al.*, 2000b).

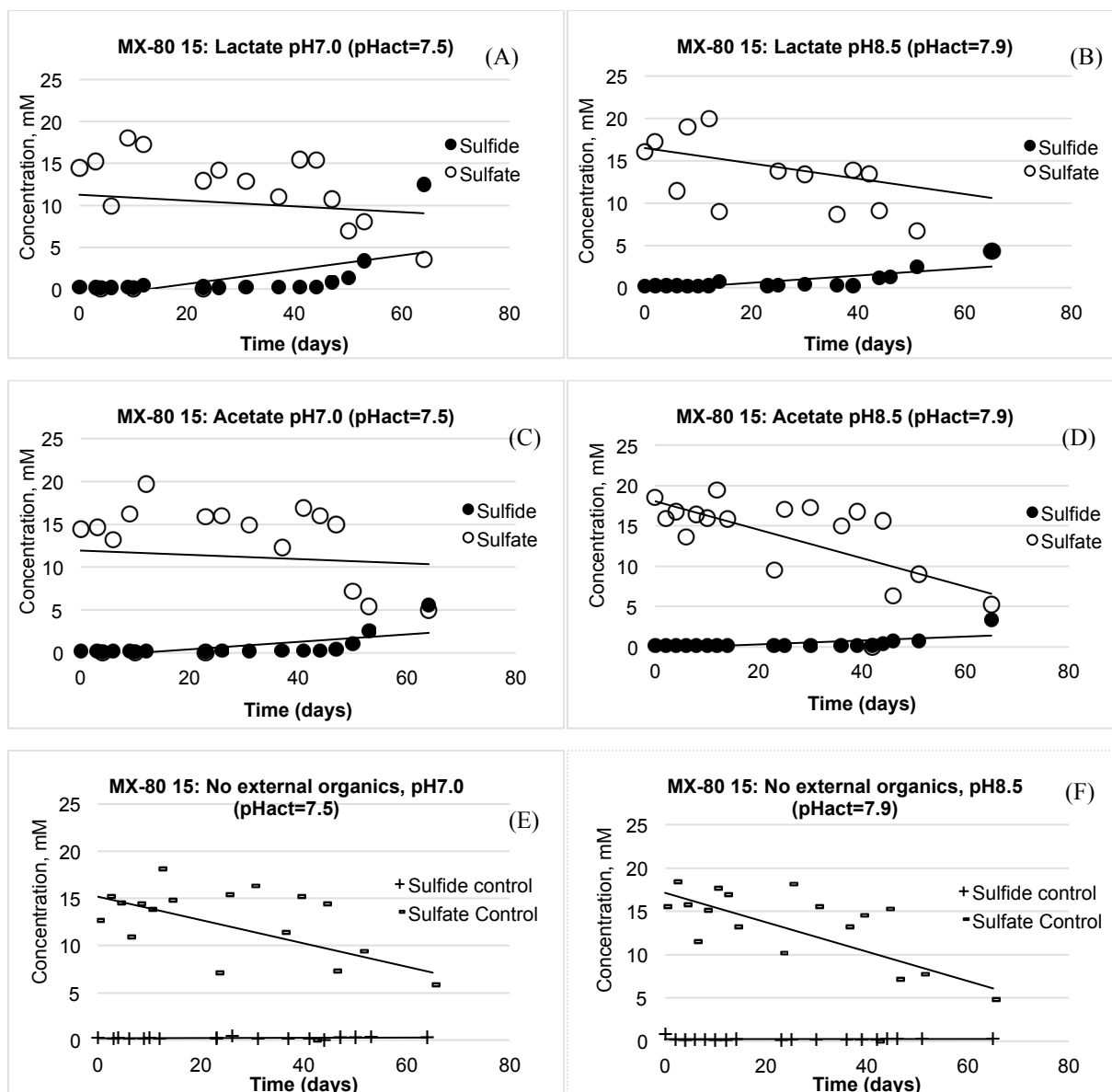


Figure 6.5. Average values of sulfide production and sulfate consumption in Zhilina's media containing 10% w/v MX-80 and supplemented with either 10 mM lactate (A, B) or 10 mM acetate (C, D) under different pH at 15 °C. No exogenous lactate or acetate were added into the control samples (E, F). Anticipated and actual pH values are indicated. Dashed lines specify moving average trend lines of concentrations. Concentrations of sulfide and sulfate are shown as a function of incubation time. Sulfate data shown for illustrative purposes only due to its unpredictable fluctuation in the microcosms.

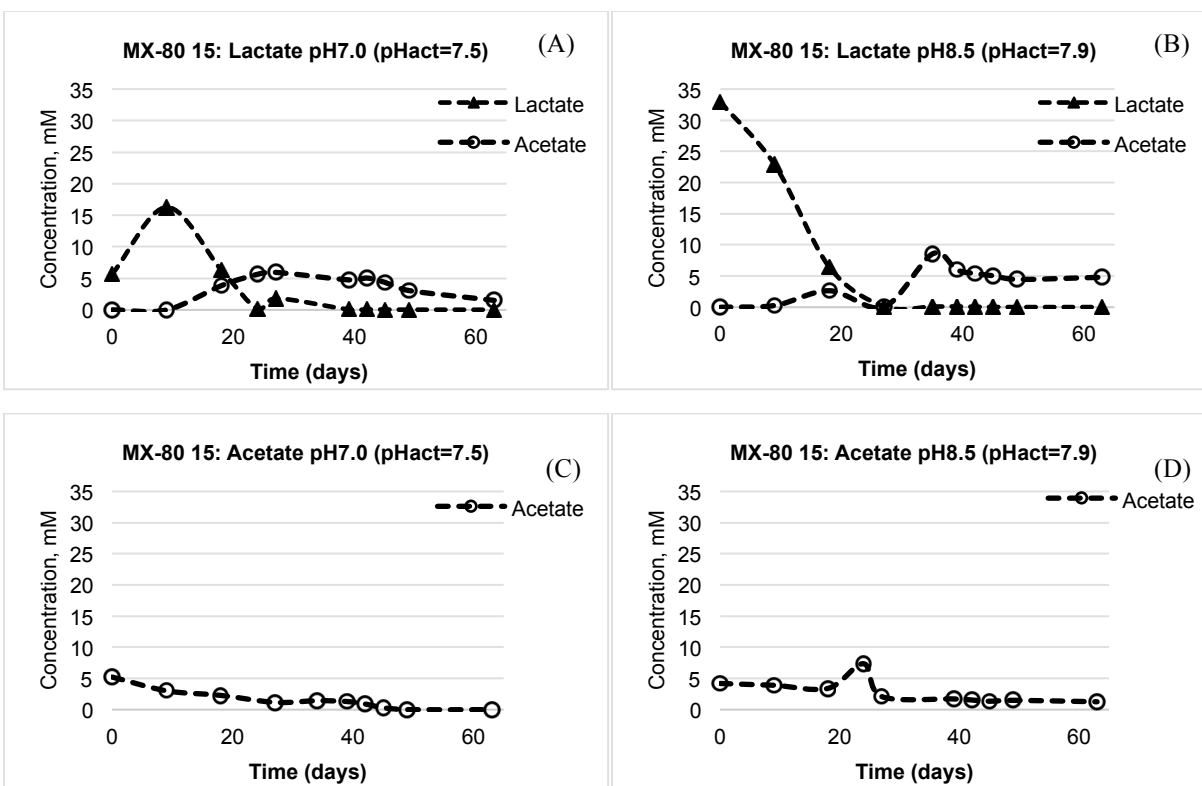


Figure 6.6. Concentration of acetic and lactic acids in Zhilina’s media containing 10% w/v MX-80 and supplemented with either 10 mM lactate (A, B) or 10 mM acetate (C, D) under different pH at 15 °C. Quantities of acetate and lactate are shown as a function of incubation time.

Another hypothesis could be that the interaction between aluminium (for aluminium content see Appendix 10.1) and the clay surface could inhibit SRB activity (Wong *et al.*, 2004). Inhibition, however, could be alleviated when appropriate organic acids were present to stimulate SRB proliferation in microcosm and DGR settings. In a high level waste (HLW) DGR, organic materials could be present as a result of the construction of the EBS, and could include blasting residues, machine exhaust and drilling fluids.

Lactate oxidation is a more thermodynamically favourable process in sulfate reduction activity than acetate oxidation (Oren, 1999). Results from the present microcosm studies have shown that lactate-amended cultures generated higher sulfide concentrations than acetate-amended cultures. Sulfate reduction coupled with lactate oxidation produced -160.1 kJ of free energy (sulfate), while acetate oxidation coupled to sulfate reduction could only generate -47.7 kJ (Oren, 1999). Therefore, it is apparent that indigenous SRB surviving within the microcosms would select

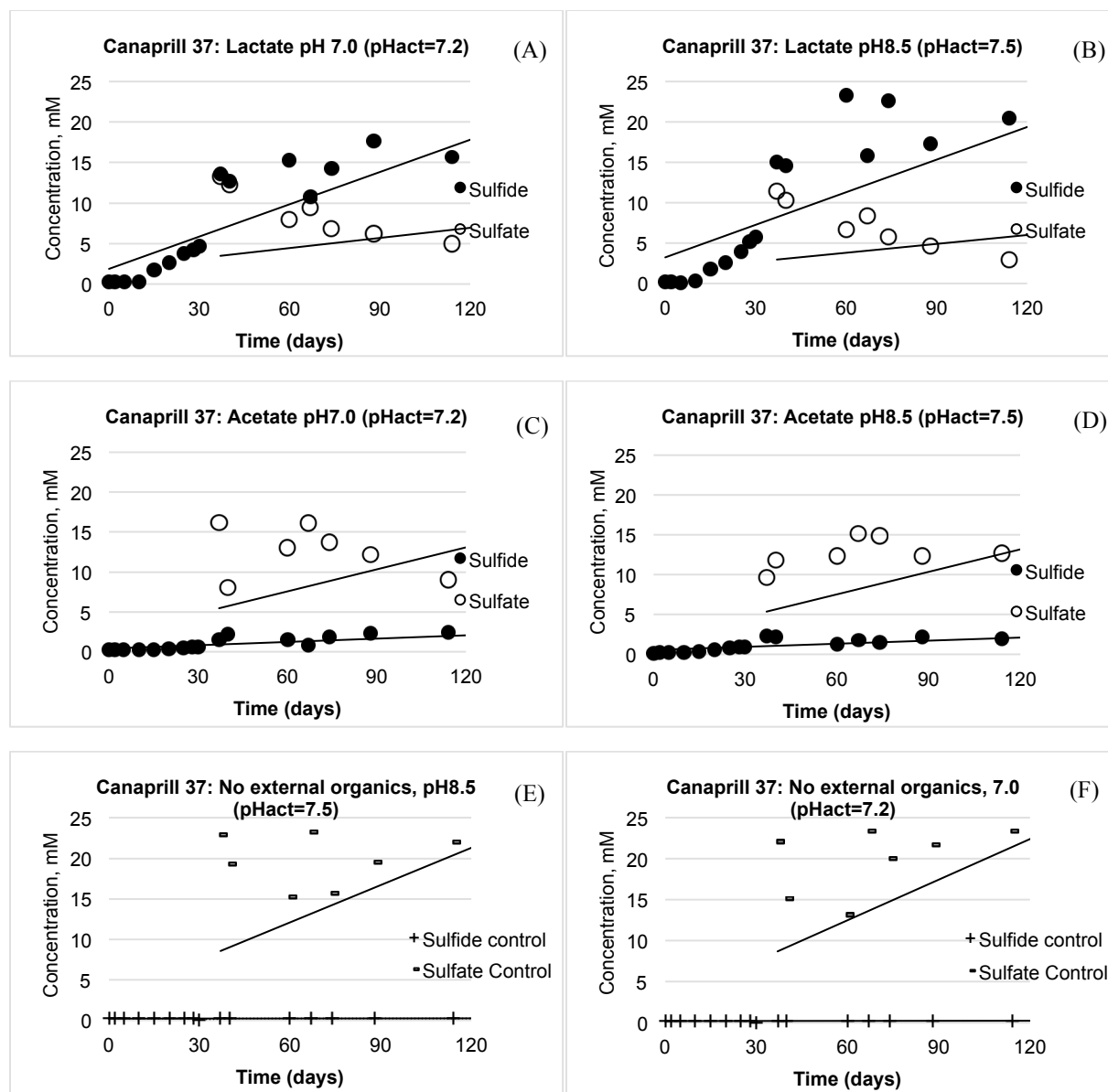


Figure 6.7. Average values of sulfide production and sulfate consumption in Zhilina's media containing 10% w/v Canaprill and supplemented with either 10 mM lactate (A, B) or 10 mM acetate (C, D) under different pH at 37 °C. No exogenous lactate or acetate was added into the control samples (E, F). Anticipated and actual pH values are indicated. Dashed lines specify moving average trend lines of concentrations. Concentrations of sulfide and sulfate are shown as a function of incubation time. Sulfate data shown for illustrative purposes only due to its unpredictable fluctuation in the microcosms.

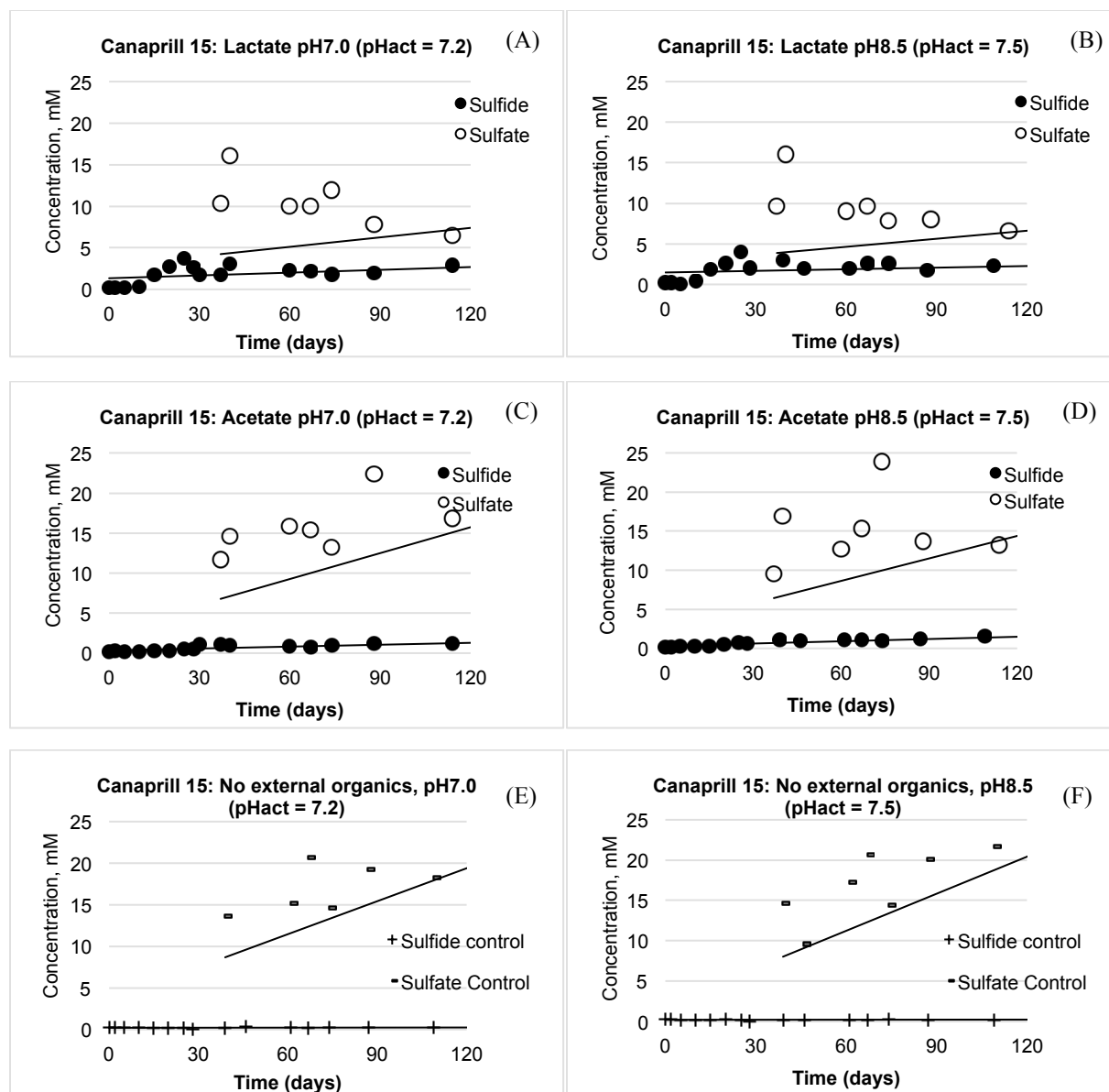


Figure 6.8. Average values of sulfide production and sulfate consumption in Zhilina's media containing 10% w/v Canapriil and supplemented with either 10 mM lactate (A, B) or 10 mM acetate (C, D) under different pH at 15 °C. No exogenous lactate or acetate was added into the control samples (E, F). Anticipated and actual pH values are indicated. Dashed lines specify moving average trend lines of concentrations. Concentrations of sulfide and sulfate are shown as a function of incubation time. Sulfate data shown for illustrative purposes only due to its unpredictable fluctuation in the microcosms.

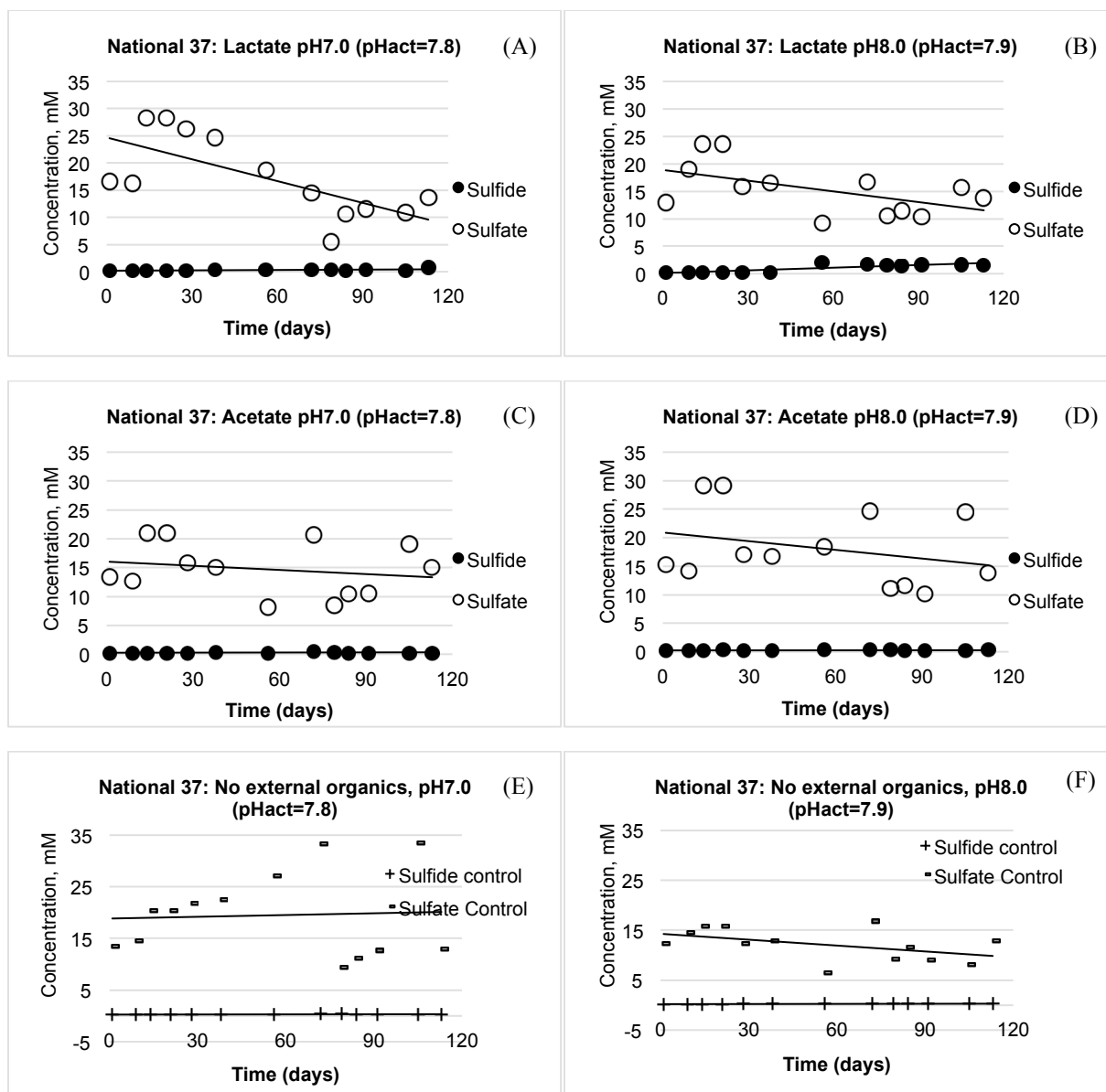


Figure 6.9. Average values of sulfide production and sulfate consumption in Zhilina's media containing 10% w/v National Standard and supplemented with either 10 mM lactate (A, B) or 10 mM acetate (C, D) under different pH at 37 °C. No exogenous lactate or acetate was added into the control samples (E, F). Anticipated and actual pH values are indicated. Dashed lines specify moving average trend lines of concentrations. Concentrations of sulfide and sulfate are shown as a function of incubation time. Sulfate data shown for illustrative purposes only due to its unpredictable fluctuation in the microcosms.

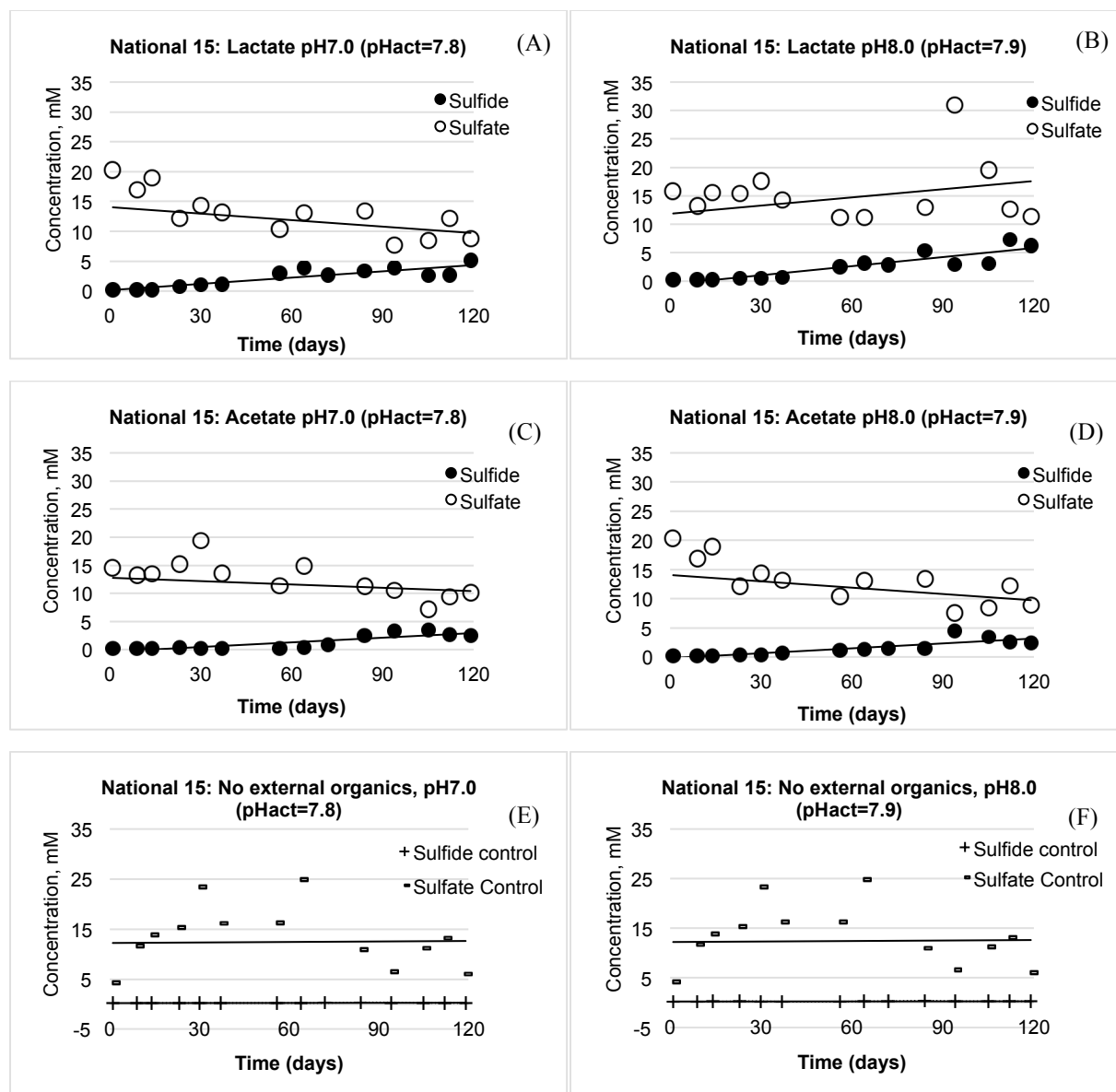


Figure 6.10. Average values of sulfide production and sulfate consumption in Zhilina's media containing 10% w/v National Standard and supplemented with either 10 mM lactate (A, B) or 10 mM acetate (C, D) under different pH at 15 °C. No exogenous lactate or acetate was added into the control samples (E, F). Anticipated and actual pH values are indicated. Dashed lines specify moving average trend lines of concentrations. Concentrations of sulfide and sulfate are shown as a function of incubation time. Sulfate data shown for illustrative purposes only due to its unpredictable fluctuation in the microcosms.

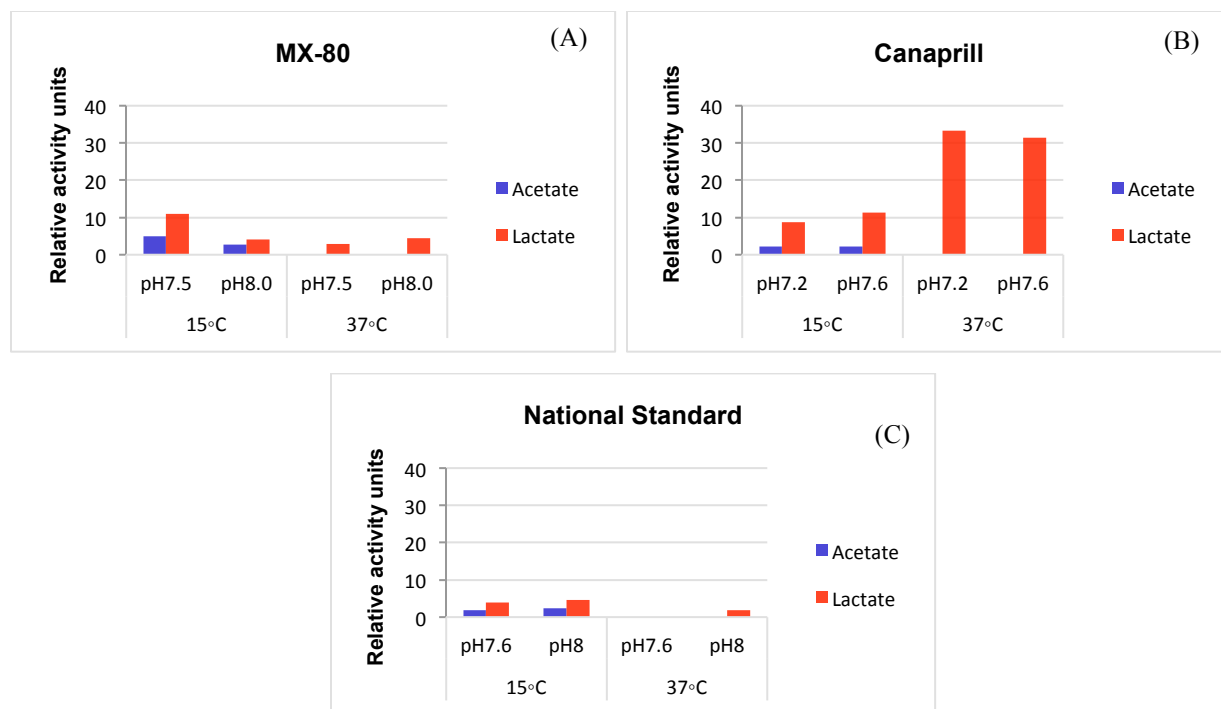
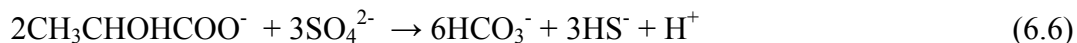


Figure 6.11. Relative sulfide-producing activities (RA) in clay enrichments under different conditions: (A) MX-80, (B) Canapril and (C) National Standard.

lactate over acetate upon revival. When Masurat *et al.* (2010a,b) isolated SRB from MX-80 bentonite, lactate was chosen to stimulate bacterial activity in their cultures. When it comes to utilization of organic acids to produce sulfide, microorganisms such as *Desulfovibrio* species can incompletely oxidize lactate to produce sulfide (equation 6.4; Pankhania *et al.*, 1988). Complete lactate oxidation can also occur, for instance, in *Desulfosarcina* species (equation 6.6; reviewed by Hamilton, 1985). There are also SRB that can grow solely on acetate (e.g. *Desulfobacter*, *Desulfotomaculum*, *Desulfuromonas* species) as in equation 6.5 (reviewed by Hamilton, 1985; Sorokin *et al.*, 2015).



Therefore, as long as sufficient amounts of organic acids and electron acceptors are available, SRB proliferation and sulfide production are likely to occur. Moreover, the ability for SRB to oxidize acetate and their adaptability to varying temperature, pH, salinity and high concentrations of sulfide, must also be taken in to consideration for their likelihood of growth as the genes for these bacterial functionalities are activated under specific conditions. In the case of this study, it is possible that the different clay types are inhabited by physiologically-heterogeneous sulfide-producing microbial consortia, consisting of incomplete- and complete-

organic acid oxidizers. Hence, while subject to conjecture, the complete-oxidizers, including SRB that consumed acetate, might have been active only at 15 °C; while incomplete-oxidizers could have metabolized at both 15 °C and 37 °C. In terms of pH effects, alkaline conditions were found to lessen SRB activity (i.e., pH ~ 8.6 and 9; Guitierrez *et al.*, 2009). However, in this study, pH changes did not show any significant difference in sulfide production when pH values were stabilized between 7.2 to 7.9. A possible explanation for this occurrence would be the fairly small differences in pH values after overnight equilibration, or the fact that the pH values are not sufficiently high. Changes in pH, however, could contribute to the varying amount of detected sulfide in each microcosm because of the changes in FeS solubility at different pH (pH = 3 to 10 (Small *et al.*, 2008, Rickard, 2006). These explanations would also indicate that fluctuations in the rates of sulfide production in enrichments with different clays, particularly in the subsurface, might be due to difference in abundance and diversity of SRB in clays (Masurat *et al.*, 2010b; Hamilton, 1985).

Following DNA extraction from SRB10 enrichments, only 12 out of 24 enrichments produced amplifiable PCR products for DGGE analysis, and of these, only five yielded DGGE bands (Figure 6.12). These bands represent the mixed microbial community found in the microcosms (Medihala *et al.*, 2013), and are identified in Table 6.1. It has been hypothesized that the bentonite will be dominated by Gram-positive spore-forming microorganisms (Stroes-Gascoyne *et al.*, 2010b). The phylogenetic analysis indicated that sequences belonged to the phyla Proteobacteria (class α and γ) and Firmicutes. No archaea were detected. The microbial communities identified in this study are similar to those found in the Opalinus clay formation in Mont Terri (Switzerland) and Toarcian argillite formation at Tournemire (France) (Moll *et al.*, 2013; Urios *et al.*, 2012), which are also dominated by Gram-positive bacteria. With respect to DGR microbiology, the strains isolated were mostly involved in anaerobic degradation processes. While most were isolated under anaerobic conditions, some of these microorganisms belonged to facultative anaerobic groups (e.g., *Bacillus*). The presence of *Pseudomonas* would suggest possible reduction of nitrate or oxygen (remaining oxygen that were not completely eliminated during degassing of the microcosm) in the microcosm. *Pseudomonas* sp. was isolated from a nitrate reduction system wherein oxidation of U (IV) occurred as nitrate was reduced (Wilkins *et al.*, 2007). Uranium bioaccumulation and adsorption by *Pseudomonas* has also been observed (VanEngelen *et al.*, 2010; Lloyd *et al.*, 2002). *Desulfobulbus* sp. and *Desulfuromonas*

sp. have the ability to reduce sulfate and sulfur, respectively (Hamilton, 1985), and thus could contribute to container corrosion in a DGR. These species were also among the SRB found in groundwater from boreholes of Olkiluoto Island where a Finnish nuclear power plant is located (Itävaara *et al.*, 2008). The presence of the *Pelobacter* group in SRB10 microcosms (Table 6.1) could potentially lead to reduction of ferric iron and sulfur disproportionation in the clay. The existence of these microorganisms in the microcosms would explain why lactate and acetate are being completely and incompletely oxidized, and how variations in detected sulfide occurred. Moreover, the survival of these microorganisms in bentonite is believed to have been due to the presence of spores from spore-forming SRB, which were dehydrated by clay minerals by pulling the water out of the SRB (Masurat *et al.*, 2010b).

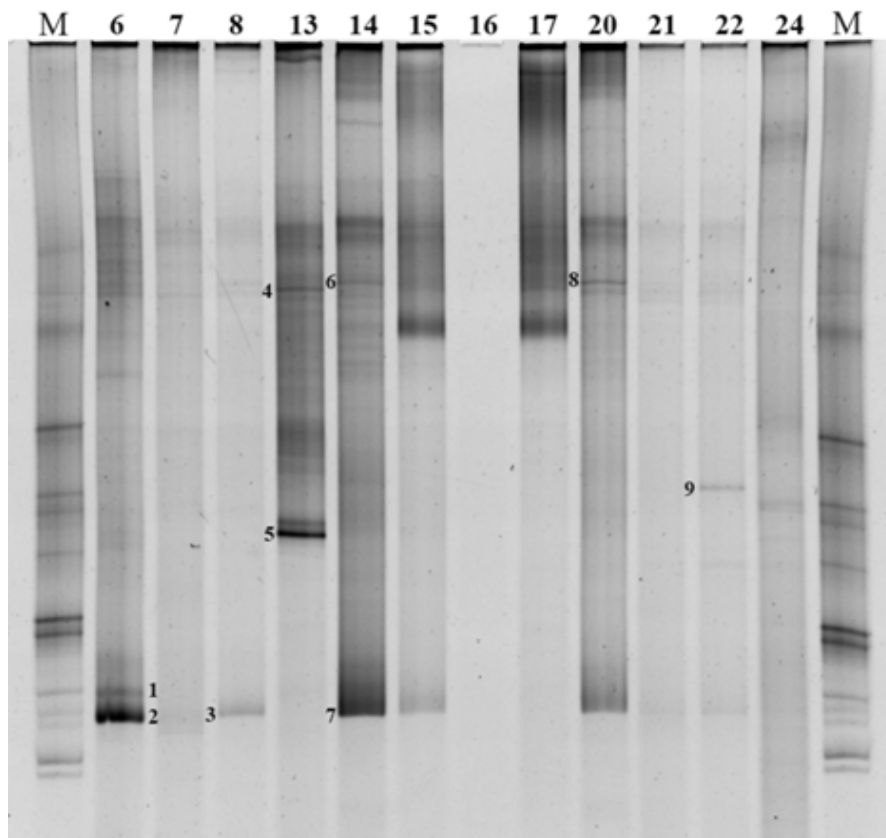


Figure 6.12. Denaturing gradient gel electrophoresis (DGGE) analysis of the dominant bacterial communities in SRB10 microcosms. M represents a marker constructed in this study which indicates known bacterial isolates from the subsurface. Lanes 6-8 and 17 contain National Standard bentonite samples. Lanes 13, 14 and 20 contain MX-80 bentonite. Lanes 15, 16, 21, 22 and 24 contains Canapril clay. Lanes with distinguishable bands are Lane 6 – National (lactate, 15 °C), 8 – National (acetate, 15 °C), 13 – MX-80 (no additional organics, 37 °C), and 14 - MX-80 (no additional organics, 15 °C). Numbers on the gel indicate the DGGE bands which were selected for sequence analysis (Table 6.1).

Table 6.1. Sequence identities (closest match) of the bands excised from DGGE gels as determined using NCBI (nucleotide database) and the BLASTn algorithm.

Band Number	Closest match	% Identity (bp)	Phylum/Class
1	<i>Desulfuromonas michiganensis</i>	97 % (370/382)	δ-proteobacteria
2	<i>Desulfuromonas michiganensis</i>	94 % (477/504)	δ-proteobacteria
3	<i>Pelobacter acetylenicus</i>	91 % (460/505)	δ-proteobacteria
4	<i>Pseudomonas alkaligenes</i>	99 % (420/423)	γ-proteobacteria
5	<i>Pseudomonas alkaligenes</i>	99 % (480/484)	γ-proteobacteria
6	<i>Desulfobulbus propionicus</i>	87 % (435/501)	δ-proteobacteria
7	<i>Desulfobulbus propionicus</i>	87 % (435/501)	δ-proteobacteria
8	<i>Desulfuromonadales sp</i>	96 % (527/549)	δ-proteobacteria
9	<i>Pseudomonas sp</i>	99 % (467/472)	γ-proteobacteria

6.4.3 SRB50 enrichments

It has been reported that granitic rocks (potential DGR site) in Canada may contain Na-Ca-Cl and Ca-Na-Cl brine solutions with total dissolved solids of more than 300 g/L (Gascoyne *et al.*, 1987). Accordingly, this research sought to investigate the effect of elevated salinity on SRB growth and activity. In enrichments containing 50 g/L NaCl (SRB50), no significant increase in sulfide was seen in any sample except for MX-80, National Standard and Canapril enriched with lactate and stored at 15 °C (Figures 6.13, 6.14 and 6.15).

The production of sulfide in these samples only started after the 88th day for MX-80 (~0.43 mM sulfide), the 91st day in National Standard (~1.089 mM sulfide) and the 98th day for Canapril (~0.68 mM sulfide). However, the sulfide production remained at low levels, and only reached 2.33 mM after 130 days of incubation at 15 °C. Figure 6.16 shows the rate of accumulation of sulfide in the enrichments and revealed that lactate was the preferred electron donor in all clays. The order of sulfidogenic activity was thus National > MX-80 ≈ Canapril for lactate (15 and 37 °C); MX-80 ≈ Canapril > National for acetate (15 °C); and MX-80 ≈ National > Canapril for acetate (37 °C). No further explanation is currently available as to why the order of sulfidogenic activity appeared as it did. It is, however, a more important observation that lactate was preferably utilized (over acetate) in SRB50 enrichments, a feature presumably due to the fact that lactate oxidation (equation 6.4) by SRB produces more free energy than acetate (equation 6.5) (Oren, 1999). Furthermore, the slow and low sulfide generation in SRB50 enrichments could be attributed to the high salinity in Zhilina media. It has been suggested that high salt concentrations are a limiting factor affecting sulfidogenesis (Sorokin *et al.*, 2010) and that it could lower water activity to less than 0.96 (Stroes-Gascoyne *et al.*, 2010b). A similar conclusion was derived by

Kulp *et al.* (2007) from their study. The result from the present study also agreed with Oren's (1999) hypothesis, which states that SRB, in general, generate insufficient metabolic energy to support cellular adaptation to osmotic stress. If osmotic solutes were available (e.g., glycerol, sucrose) in SRB cells, then there would exist the possibility for these cells to grow in high salinity media. In a DGR scenario, Stroes-Gascoyne and Hamon (2008) stressed that high salinity (≥ 60 g/L NaCl) would inhibit microbial activity and would perhaps induce spore germination due to the salt effect on water activity. Stroes-Gascoyne *et al.* (2010b) showed that water activity was approximately ≤ 0.94 when NaCl concentration of at least 50 g/L is present and when dry density is ≥ 1.6 g/cm³.

Vilks (2009) discussed that natural groundwater salinity (at 50 g/L and 250 g/L) in combination with HCB, low water activity and high swelling pressure inhibited microbial proliferation and that indigenous microorganisms were maintained at background levels. During the anoxic, drying phase within a DGR, further microbial inhibition would be expected since salt concentrations in the bulk and interface regions would also increase (Stone *et al.*, 2016b). Lastly, the presence of high salinity groundwater could also affect chemical sorption of radionuclides onto the host rock and clay minerals. Vilks (2009) and Vilks *et al.* (2011) showed that Na-Ca-Cl brine-saturated bentonite was effective at sorbing transition metals, such as U (VI) and Eu (III).

The presence of high salinity groundwater could cause irregularities in the functionality of bentonite in a DGR. Previous studies have shown that highly saline solutions decreased the swelling capacity of bentonite (Karnland, 2010; Foster, 1954; Marshall and Simpson, 2014; Mata and Ledesma, 2005). In the SRB50 enrichments used in this section, visible reduction in swelling of bentonite was also observed (data not shown). It has been suggested that high salinity could weaken and eventually deform the clay minerals, which leads to increased void spaces within the bulk bentonite (Börgesson *et al.* 1995, 1996; Baumgartner *et al.*, 2008).

In terms of sulfate dynamics, it was observed that the trends in the SRB50 enrichments were not consistent with that seen in SRB10 enrichments (Figures 6.3, 6.5 and 6.7), wherein sulfate decreased over time. In the SRB50 enrichments, sulfate concentrations fluctuated over the course of the experiment. A number of attempts were made to isolate the cause of these fluctuations, including variations in analytical technique. It was concluded that differences in technique did not influence the results (a standard error of 0.0009 was seen between technicians); however, the use of > 2-week old barium chloride reagent might have caused such differences. It is possible

that the difference in absorbance between old and fresh barium chloride reagent contributed to the error, yet this is highly unlikely since similar reagents were used for SRB10. A theory was offered by Blumenthal and Guernsey (1915) when they discovered an anomaly in their sulfate concentration values at high sodium chloride concentrations. The conjecture is that introducing higher amount of NaCl to the media (e.g., three times the original amount) would cause the sulfate in the solution to bind more strongly to sodium ions rather than barium ions since more sodium ions are present. As a result, more Na_2SO_4 would be produced and less BaSO_4 would be detected yielding lower and non-reproducible sulfate concentration values. This hypothesis has yet to be validated.

Finally, it would be interesting to compare the microbial community present in enrichments SRB50 and SRB10 to see which microorganisms predominate in the respective conditions. Unfortunately, no phylogenetic tree for the SRB50 study is currently available. Indeed, these studies are still ongoing and will be completed by others.

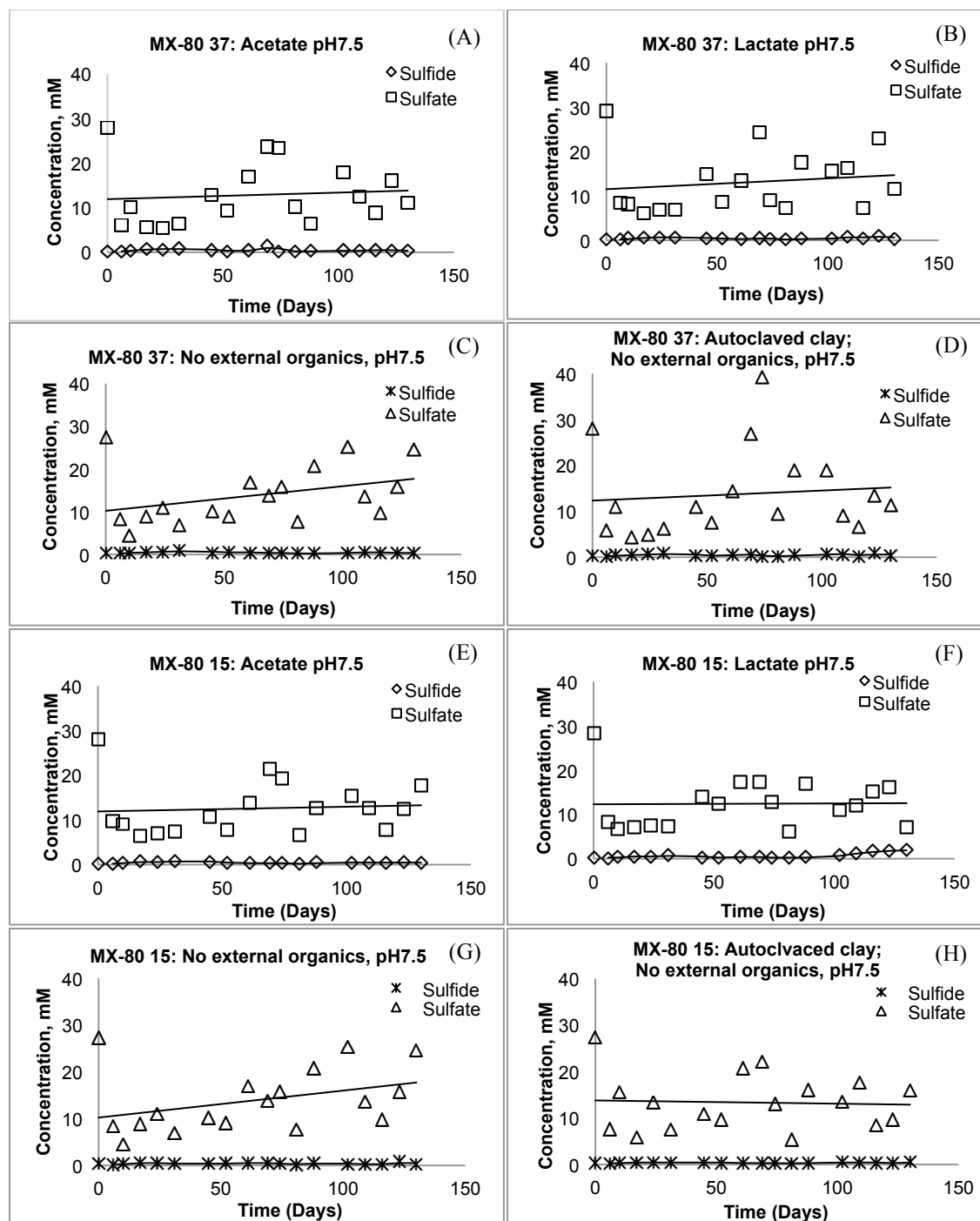


Figure 6.13. Average values of sulfide production and sulfate consumption in Zhilina's media containing 50 g/L NaCl and 10 % w/v MX-80 and supplemented with either 10 mM lactate (A, E) or 10 mM acetate (B, F) under pH = 7.5 at 15 and 37 °C. No exogenous lactate or acetate was added to the control samples (C, D, G, H). Solid lines specify moving average trend lines of concentrations. Concentrations of sulfide and sulfate are shown as a function of incubation time.

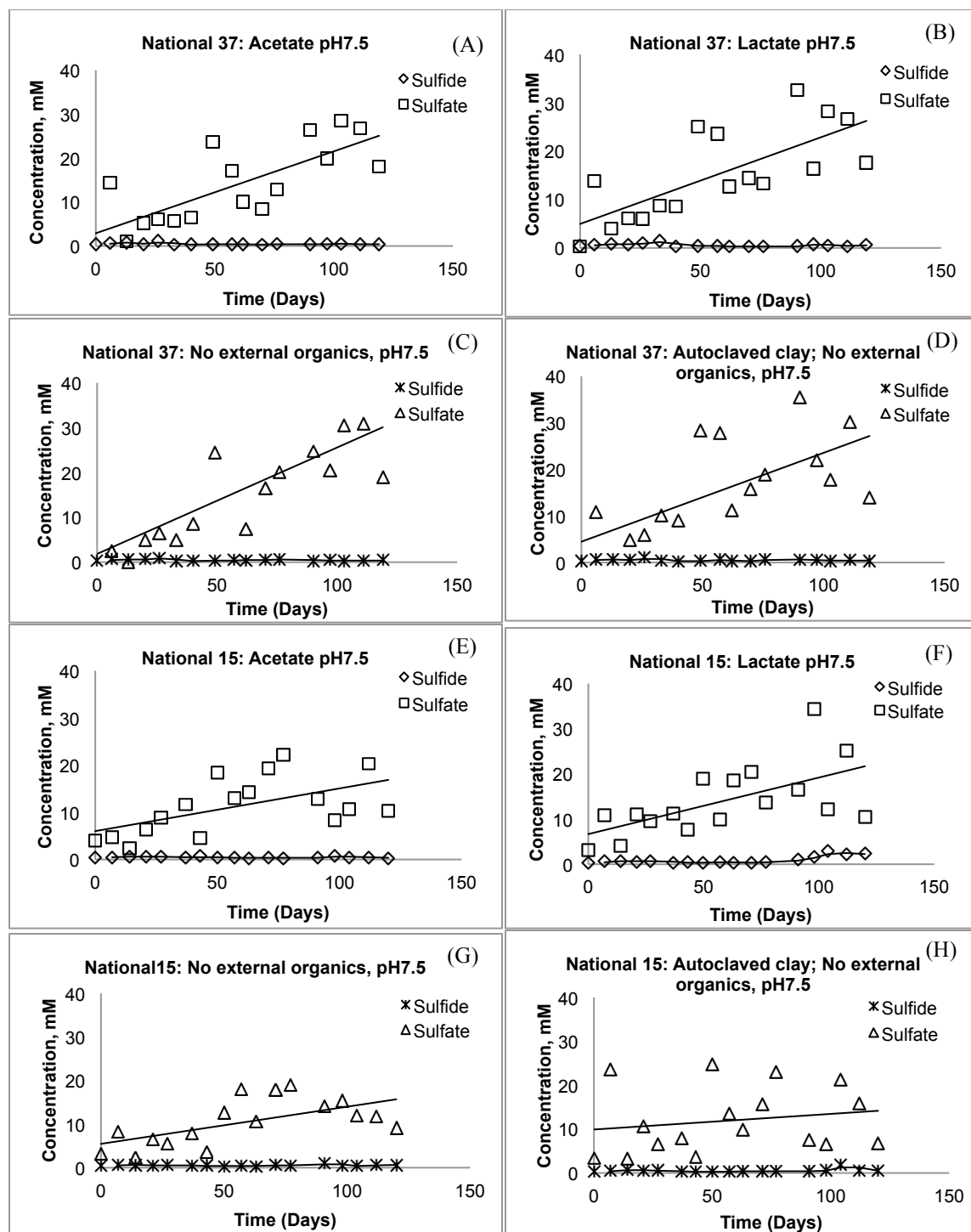


Figure 6.14. Average values of sulfide production and sulfate consumption in Zhilina's media containing 50 g/L NaCl and 10 % w/v National Standard and supplemented with either 10 mM lactate (A, E) or 10 mM acetate (B, F) under pH = 7.5 at 15 and 37 °C. No exogenous lactate or acetate was added into the control samples (C, D, G, H). Solid lines specify moving average trend lines of concentrations. Concentrations of sulfide and sulfate are shown as a function of incubation time.

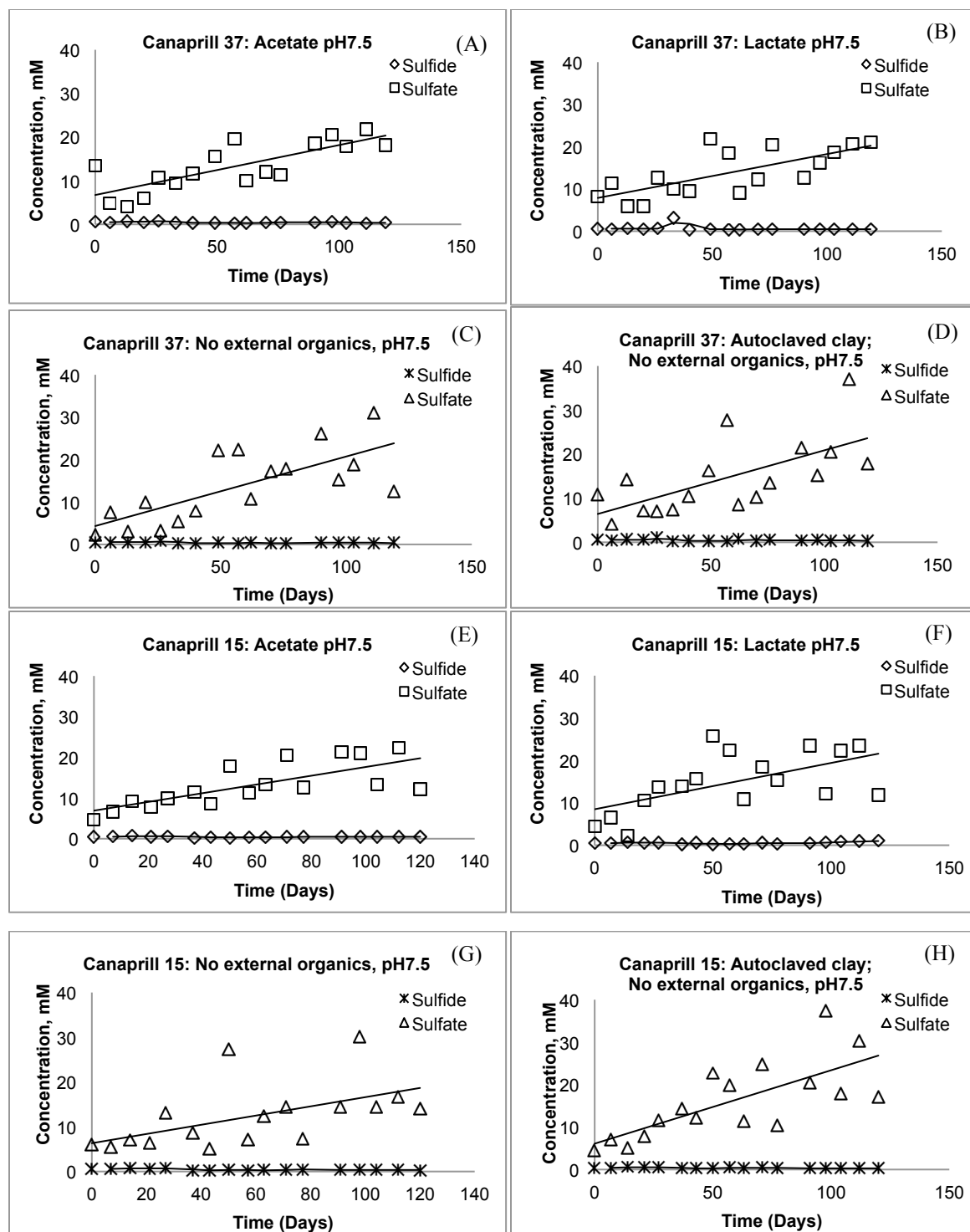


Figure 6.15. Average values of sulfide production and sulfate consumption in Zhilina's media containing 50 g/L NaCl and 10 % w/v Canapril and supplemented with either 10 mM lactate (A, E) or 10 mM acetate (B, F) under pH = 7.5 at 15 and 37 °C. No exogenous lactate or acetate was added into the control samples (C, D, G, H). Solid lines specify moving average trend lines of concentrations. Concentrations of sulfide and sulfate are shown as a function of incubation time.

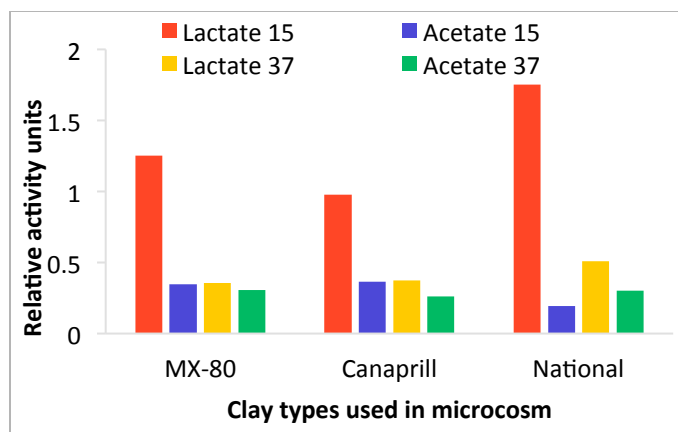


Figure 6.16. Relative sulfide-producing activities (*RA*) in clay enrichments under different conditions in SRB50 enrichment.

6.5 Conclusions

Sulfide production in the enrichments is temperature- and bentonite clay type-dependent. Higher sulfide concentrations were observed at 15 °C than 37 °C for SRB10 and SRB50 enrichments. Lactate was the preferred substrate for sulfidogenesis over acetate. However, increased salinity delayed and impeded sulfide production. Nonetheless, the overall observation on sulfide production from the different enrichments provided relevant information on the possible microbial communities using the available nutrients. The difference in the amount of sulfide produced could have been affected by the difference in the initial number of microorganisms inhabiting each clay type. From a DGR perspective, the possibility exists that microbes located at interfacial regions may produce corrosive-aggressive end products transiently, until such time that full swelling pressure in the EBS is achieved. However, these microbial products compounds would still need to diffuse through the high-density clay buffer in order to reach the used nuclear fuel container, and thus microbial activity at rock-clay interfaces is not likely to present a concern in terms of overall UFC integrity.

6.6 Connection to Next Section

The previous section discussed the potential of SRB to generate sulfide in enriched clay media (enrichments). It was determined that substrate type, temperature and clay type were key determinants affecting sulfide generation. Lactate was the preferred substrate in all enrichments with incubation at 15 °C yielding the most sulfide. High salinity impeded enrichment

sulfidogenesis, as evidenced in MX-80 SRB50. Overall, the enrichment model system provided an understanding of how SRB could perhaps behave when a DGR's target parameters of swelling pressure, dry density and water activity were not attained. However, it has to be taken in to account that there are no natural lactate occurring nor a lot of acetate present in such a DGR. Moreover, if fermentation were to occur, insufficient amounts of fermentation products would be produced and unlikely to enhance sulfide production. Autotrophic sulfate reduction is, however, a possibility. The next section examines the activity and potential for floc formation in HAB and two lab strain bacterial isolates at the water-bentonite interface in two model systems. These studies focus on the role of nutrients and bentonite concentration in microbe-bentonite aggregation.

7. AGGREGATE FORMATION AT WATER-CLAY INTERFACES AND MICROBE-MX-80 BENTONITE AGGREGATION UNDER CONTRASTING NUTRIENT REGIMES

Interface:

Daphne Jalique (DJ) designed and performed the experiment. DJ also conducted the preparations, tests, analyses and result interpretation for this section.

Dr. Alexander Grigoryan contributed to the design and interpretations of the data.

Hayley Kumitch provided technical assistance to this work.

Dr. Darren Korber provided guidance and assisted with experimental design, provided editing of the section, and held the research grant that supported this work.

7.1 Abstract

Interfaces and transition zones in a DGR may support biofilm formation or microbial activity due to the presence of sorbed nutrients and available space. As a result, microbial diversity and metabolic activity are may be higher, at least temporarily, at these locations in a DGR. In the present study, the bentonite-microbe interaction, microbial growth, and extracellular polymer substance (EPS) production were investigated using enrichment model systems. To examine clay-microbe interactions in more detail, an aggregation study was performed using confocal laser scanning microscopy (CLSM) and a Coulter counter particle sizing device under oligotrophic and copiotrophic conditions. In these experiments, the role of bacterial EPS during

clay-microbe aggregate formation was examined. M-salt medium was selected initially to mimic porewater, and this medium was later modified by manipulating nutrient ratios (C:N:P) to increase EPS production in the selected organotrophic bacteria (*Pseudomonas aeruginosa* and *Bacillus simplex*). Several quantitative and qualitative tests, including EPS abundance, particle sizing, and microscopy, were performed to delineate whether clay-microbe aggregation was likely to occur at interfaces under the correct nutrient regime. *P. aeruginosa* PAO-1 and *B. simplex* grew and produced EPS abundantly in treatment C containing 1x carbon (TC^{1x}), leading to higher clay-microbe aggregation. When nutrients and MX-80 bentonite contents were varied, microbial growth, EPS generation and clay-microbe aggregation were affected significantly. Given the wide diversity of indigenous organisms expected to be present in a DGR, a wide range of clay-aggregation potential would exist if the DGR system were to allow microbial activity at interfaces and transition zones.

7.2 Introduction

Clay-microbe interactions are of particular interest in a DGR because these reactions may compromise the functionality of the bentonite. Several biogeochemical processes have been observed to negatively-impact bentonite as clay-microbe interactions occur. Among these are the dissolution of clay minerals through redox reactions, discharge of metabolic by-products that clog spores (Dong, 2012), and bioconversion of montmorillonite to illite (SKB, 2006). The tendency for these reactions to occur would be quite insignificant considering the microbially-inhibiting parameters of HCB discussed in Stroes-Gascoyne *et al.* (2010b) and in Section 4 of this thesis. However, a DGR may contain regions of lesser dry density such as those associated with the inevitable fissures, cracks, and voids, as well as interfaces. These areas may be susceptible to increased activity of microorganisms, especially from spore-forming bacteria that are expected to survive over long periods (Chi Fru and Athar, 2008; Stroes-Gascoyne *et al.*, 2010b). Therefore, it is important to consider the possible impact of these microorganisms (e.g., the mode of growth, and potential effects on the clay properties) within the lower-density conditions associated with the abovementioned regions.

Adhesion of microorganisms to inanimate surfaces has been studied widely (Marshall *et al.*, 1971; Stotzky, 1985, 1986; Kjelleberg, 1984; Perdrial *et al.*, 2009; Dong, 2012). Previous investigations have shown that bacteria can have distinct effects on clay mineral dissolution (Meleshyn, 2014), growth (Ehrlich, 1999) and aggregation (Jaisi *et al.* 2008). Under oligotrophic

(nutrient poor) or copiotrophic (nutrient rich) conditions, bacteria have evolved various tactics to accumulate or utilize nutrients (Kjelleberg and Hermansson, 1984). Extracellular polymeric substances (EPS) has long been recognized to assist in many cellular associations (homogeneous and heterogeneous cell organization) (Wolfaardt *et al.*, 1999). It is also involved in the adhesion process of biofilm formation to maintain overall structural integrity. MacLeod *et al.* (1995) showed that EPS played a role in establishing stability in granular sludge in the presence of bacteria. Hence, EPS is believed to assist bacterial adhesion to mineral surfaces (Welch and Vandevivere, 1994), and could potentially alter or mask clay minerals and their chemistries (Kostka *et al.*, 1999; SKB, 2006; Mulligan *et al.*, 2009; Kim *et al.*, 2004).

The purpose of this study was to develop appropriate model systems to evaluate the potential of microbes to affect bentonite at regions with more available space such as the DGR interfaces. To examine this, EPS was presumed to mediate attachment of bacteria to the clay surface, which could contribute to clay-microbe aggregation. As a result, EPS production from the bacterial strains isolated in Section 3 were examined. Moreover, the effect of nutrient on EPS production and the effects of bentonite concentrations and nutrients on clay-microbe aggregation were also investigated.

To achieve this, a minimal salt media was varied and tested for its ability to support bacterial growth and EPS production. Afterwards, bacterial strains with the highest concentration of EPS produced were selected. Aggregate size was analyzed using Coulter counter and confocal laser scanning microscope (CLSM).

7.3 Materials and Methods

7.3.1 Selection of bacterial isolates and growth media for clay-microbe aggregation study

In order to determine whether EPS influences clay-microbe aggregation, it was necessary to identify the EPS-producing microorganism as well as optimize the growth media to generate higher concentrations of EPS. Minimal salts medium (a defined, basal salt solution; Appendix 10.13) was selected to mimic the low-nutrient porewater that would eventually come to saturate the MX-80 bentonite blocks in a DGR. The minimal medium was predominantly a phosphate medium without any carbon source. For the purpose of the study, three different carbon sources (i.e., sodium lactate, sodium acetate and glucose) at varying concentrations were added to M-salt medium in order to evaluate their respective effects on microbial growth and EPS production of

the bacteria tested (Table 7.2). The optimal medium formulation and bacterial strain were selected for use in clay aggregation investigations (section 7.3.2).

7.3.1.1 MX-80 bentonite bacterial isolates and culture conditions

A panel of bacterial isolates (*B. simplex*, *P. alginolyticus*, *A. oxydans*, *O. pekingese*, *K. polaris*, *S. kurssanovii* and *P. aeruginosa* PAO-1 (laboratory strain; positive control) isolated from MX-80 bentonite (section 3.3.3)) were evaluated during this study. These isolates were grown in full-strength tryptic soy broth (TSB) (for all isolates except for *P. aeruginosa* PAO-1, which was grown in Luria-Bertani (LB)) and incubated for 24 h (time of incubation was based on previous incubation trials and spectroscopic analysis) at 30 °C (no shaking). The isolates grown were enumerated subsequently using standard plate counts.

7.3.1.2 Optimization of M-salt components

M-salt medium optimization for EPS production was performed using a Plackett-Burman (PB) statistical design. The original M-salt medium composition is given in Appendix 10.13. The PB design screened the main effects of the important components of the M-salt medium through a two-factorial analysis by screening n variables (media components) in $n + 1$ experiments with each variable examined at two levels (Plackett and Burman, 1946). Table 7.1 shows the variables under investigation and the two levels of each variable that was tested. Table 7.2 shows the combination of ingredients calculated for each treatment (A to H) – phosphates are a combination of KH_2PO_4 , K_2HPO_4 and MgSO_4 (anhydrous) (Table 7.1). Design Expert software (Minitab package version 17.0) was used to conduct the PB analysis.

Two percent (Manivasagan *et al.*, 2013) of each of the bacterial inocula prepared in section 7.3.1.1 was added to 15 mL of each treatment in Table 7.2. Growth was monitored for five days (incubation period selected based on previous trials and spectroscopic analysis) using the microplate reader with absorbance measured at OD_{520} . Afterwards, 1 mL of each treatment was taken and stored at -80 °C for EPS production analysis. For EPS analysis, only samples with bacterial growth were analyzed.

Table 7.1. Levels of different experimental variables used for Plackett-Burman determination of EPS production by selected microorganisms.

Variables	Coded Symbol	Units	Lower value	Higher Value
Glucose	X_1	g/L	2.5	5.0
Acetate	X_2	mM	30.51	91.53
Lactate	X_3	mM	20	60
NH_4NO_3	X_4	g/L	0.1	1.0
Trace Element	X_5	mL/L	0.1	1
Phosphates: where 1X is $\text{KH}_2\text{PO}_4 = 2.7$ g/L; $\text{K}_2\text{HPO}_4 = 5.2$ g/L; MgSO_4 (anhydrous) = 0.12 g/L	X_6	x	1x	5x
NaCl	X_7	g/L	7	20

Table 7.2. Eight-Plackett-Burman design matrix for seven variables at pH = 7.2 ± 0.2. All treatments were applied individually to *B. simplex*, *P. alginolyticus*, *A. oxydans*, *O. pekingese*, *K. polaris*, *S. kurssanovii* and *P. aeruginosa* PAO-1.

Treatment	Treatment Abbreviation	Glucose (g/l)	Acetate (mM)	Lactate (mM)	NH_4NO_3 (g/L)	Trace element (mL/L)	Phosphates (x)	NaCl (g/L)
A	TA	5	91.53	60	0.1	1	1	7
B	TB	5	91.53	60	1	0.1	5	7
C 1x	TC ^{1x}	5	30.51	20	1	1	5	7
D	TD	2.5	30.51	60	1	1	1	15
E	TE	2.5	30.51	20	0.1	0.1	1	7
F	TF	5	91.53	20	1	0.1	1	15
G	TG	2.5	91.53	20	0.1	1	5	15
H	TH	5	30.51	60	0.1	0.1	5	15

7.3.1.3 EPS extraction and analysis

For EPS extraction, a modified protocol from Subramanian *et al.* (2010) and Manisavagan *et al.* (2013) was used. Briefly, 1 mL of bacterial culture was centrifuged at 10,000 rpm for 10 min at 4 °C. The supernatant was transferred to a sterile 1.5 mL Eppendorf tube. Thereafter, 2.2 volume of absolute ethanol was added to the supernatant and incubated for 1 hr at -20 °C. The EPS pellet was collected thereafter through centrifugation (10,000 rpm for 10 min at 4 °C), air-dried, and dissolved in sterile distilled water. The EPS concentrations were determined by the phenol-sulfuric acid method using glucose as standard (described below).

7.3.1.4 EPS determination protocol

The phenol-sulfuric acid colourimetric method was used to measure bacterial EPS (Dubois *et al.*, 1956; Liu *et al.*, 1973). Briefly, 426 µl of sample, 50 µl of 80 % w/w phenol and 1.064 mL of concentrated sulfuric acid were combined and incubated. A glucose standard curve was prepared with concentrations of 5, 10, 20, 40, 60, 80 and 100 µg/mL. Fifty µl of 80 % phenol (Sigma-

Aldrich Canada Co., ON, Canada) was added to every sample analyzed before concentrated sulfuric acid. Afterwards, the mixtures were vortexed for 10 s. Thereafter, 250 µl of each mixture was transferred to a 96-well plate to measure absorbance at 485 nm using a Packard microplate reader (RX-309, Packard BS10000 Spectracount Plate Reader Photometer; Packard, Meriden, CT, USA).

7.3.2 Effect of bentonite and nutrients on clay-microbe aggregation

7.3.2.1 TC^{1x} with variations in carbon and clay percentage (TC-CC enrichments)

The TC-CC enrichment study aimed to determine the effect of carbon substrates on EPS production and the effect of varying bentonite concentration on EPS production and clay-microbe aggregation. It was initially assumed that EPS production and clay-microbe aggregation worked in parallel with each other meaning that when EPS production decreases, clay-microbe aggregation would also decrease. In this section, the effect of carbon substrates on EPS production was examined first. Briefly, TC^{1x} was selected as the modified M-salt medium (Table 7.2) because it permitted higher *P. aeruginosa* PAO-1 growth, which is one of the best-studied model organisms for biofilm formation and excellent at forming aggregates in liquid batch cultures (Schlebeck *et al.*, 2009). Thereafter, the collective amount of glucose, sodium lactate and sodium acetate was varied from TC^{1x} to TC^{0x} (with 0x containing no carbon) and TC^{0.005x} (containing 0.005x carbon relative to 1x) (see Table 7.2 for TC^{1x} ingredients). The remainder of the ingredients retained their original concentrations (see Table 7.2).

Afterwards, an overnight culture of *P. aeruginosa* PAO-1 was prepared in LB broth and incubated at 30 °C. The culture was enumerated on plates prior to the experiment. After enumeration, the CFU/mL was calculated at 2.9×10^9 . Two percent of the pure culture was added to 40 mL of total volume of medium and MX-80 bentonite (added at 10, 5, 1 and 0.01 %) to examine the effect of bentonite on clay-microbe aggregation. Figure 7.1 shows a schematic of components that were combined in each enrichment (in duplicate). The enrichments were prepared aerobically to encourage bacterial aggregation (Schlebeck *et al.*, 2009) in the biosafety cabinet in sterile serum bottles sealed with sterile 20 mm Wheaton rubber flange, snap-on stoppers (Wheaton Industries Inc., NJ, USA). The medium, clay and culture were aseptically added to the serum bottles. The serum bottles were then placed sideways on a shaker (75 rpm) maintained at room temperature. The samples were analyzed every 0, 16, 40, 64, 112 and 160

hours for five days for growth (absorbance measured at 520 nm), EPS production, aggregate formation (CLSM) and particle sizing.

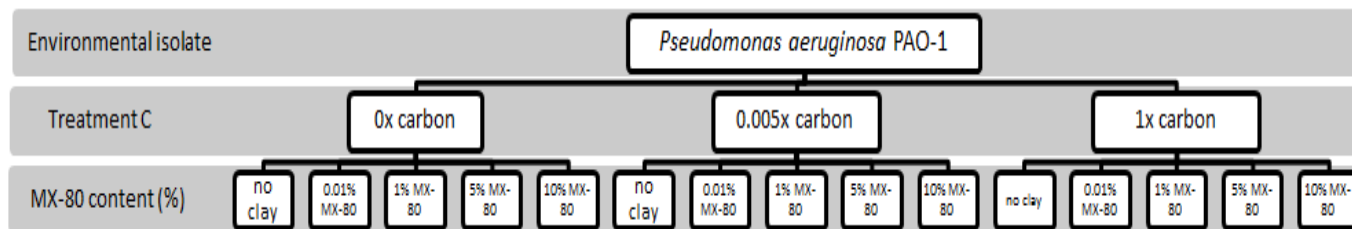


Figure 7.1. Schematic diagram of the TC-CC enrichment study. The diagram illustrates the components combined in the serum bottles. A total of 30 bottles, including duplicates, were prepared. The control contained inoculum but no clay. The serum bottles were shaken at 75 rpm for five days.

7.3.2.2 TC^{1x} with variations in C:N:P ratio (TC-CNP enrichments)

A C:N:P ratio experiment was carried out to further analyze the effect of low nutrient media to clay-microbe aggregation in the presence or absence of bentonite clay. This time, however, glucose concentration remained constant while nitrogen and phosphate concentrations varied. In this study, *B. simplex* was used instead of *P. aeruginosa* PAO-1, since *B. simplex* was directly isolated from MX-80 bentonite (see section 3.3.3). Table 7.3 shows media formulation variables based on the original M-salt medium (Appendix 10.13). The balanced treatment C:N:P ratio (to be referred to as BAL) was based on the study of Evans *et al.* (2012). The other ratios tested were based on Evans (2013). Accordingly, five different treatments were prepared with differences in the concentrations of carbon (glucose) (constant but at low concentration), nitrogen (NH₄NO₃) and phosphorus (KH₂PO₄). The concentrations of the remaining ingredients were kept constant (i.e., NaCl, MgSO₄ and trace elements). Figure 7.2 illustrates the different enrichment treatments. Each treatment, conducted in duplicate, contains 0.01 % MX-80 bentonite and a control (no bentonite). The addition of medium, clay and culture to the serum bottles was performed aseptically. Serum bottles were then placed sideways on a shaker at room temperature at 75 rpm. The samples were analyzed every hour after 8 hours of incubation up until the 17th hour. Samples were observed for aggregate formation under CLSM and were analyzed for particle sizing using a Coulter Counter.

Table 7.3. Treatment C modification - C:N:P ratio and other components for the enrichments containing *B. simplex* and MX-80 bentonite.

C:N:P ratio (Description)	100:10:1 (Balanced)	100:1:1 (N-limited)	100:10:0.1 (P-limited)	100:30:1 (N-excess)	100:10:2 (P-excess)
Media alias	BAL	NL	PL	NE	PE
Ingredients	Amount				
NaCl (g/L)	7	7	7	7	7
Glucose (mg/L)	50	50	50	50	50
MgSO ₄ (anhydrous) (mg/L)	120	120	120	120	120
NH ₄ NO ₃ (mg/L)	6.7	0.67	6.7	20	6.7
KH ₂ PO ₄ (mg/L)	2.2	2.2	0.22	2.2	4.4
Trace element (mL/L)	1	1	1	1	1
Final pH	7.5	7.5	7.5	7.3	7.3

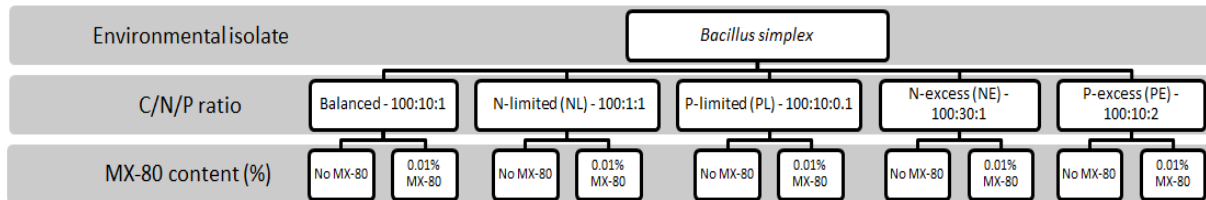


Figure 7.2. Schematic diagram of the C:N:P ratio enrichments study and other components combined in the enrichment bottles. A total of 20 bottles in duplicates were per bentonite sample were incubated including controls (no clay).

7.3.3 Particle Size Analysis

Cells and particles were suspended in PBS previously passed through a 0.2 µm pore size sterile filter. The liquid sample from each enrichment was analyzed using a MultisizerTM 3 Coulter Counter[®] (Beckman Instruments Inc., USA) using an aperture diameter of 100-µm. Particles passed through the Coulter orifice wherein volumes of cells and cell aggregates were measured electronically. The coincidence count, or the abnormal electric pulses generated by high concentrations of particles passing simultaneously through the aperture, was maintained below 10% in order to obtain concentrations and size distributions. Data were analyzed using Beckman Coulter Particle Characterization software (version 3.33).

7.3.4 CLSM analysis of fluor-stained aggregates

Samples from enrichments were diluted ten-fold in PBS prior to CLSM analysis. Thereafter, 2 µl of concanavalin A (lectin specific for α-mannopyranosyl and α-glucopyranosyl moieties;) conjugated with Alexa 647 dye (far red dye; excitation 543 nm, LP 560 nm; emission 650 nm)

was added to 1 mL sample. The samples were restained with nucleic acid stain SYTO[®] 9 (1.67 mM in DMSO; Invitrogen, Molecular Probes, USA; excitation wavelength = 488 nm; bandwidth of filter set = 514/30) to enhance bacterial fluorescence, and propidium iodide (1.67 mM in DMSO; Invitrogen, Molecular Probes, USA; excitation wavelength = 543 nm; bandwidth of filter set = 593/40) to identify dead cells after Alexa 647-stained cells have been quantified. Qualitative analysis of stained samples was performed using a Nikon C2+ confocal microscope system (Nikon, Japan) mounted on a Nikon Eclipse LV100D-U microscope (Nikon, Japan).

The CLSM was integrated with a Nikon remote focus accessory (Product no. 79586; Nikon Inc., USA) for vertical positioning during laser sectioning of clay sample with *GFP-PFE*. Optical thin sections were obtained at different depths depending on where the clusters of *P. aeruginosa* PAO-1, *B. simplex* and MX-80 bentonite were found. Digital image analysis of the optical thin sections was performed using the image analysis software (NIS Elements version 4.1 LO, 64-bit), to determine biofilm and aggregate depth and to confirm the formation of biofilm on the surface of each type of clay.

7.3.5 Statistical Analysis

For statistical analysis, one-way ANOVA was used to determine the effects of different carbon concentrations on the growth of *P. aeruginosa* PAO-1. The two-way ANOVA analysis was performed to identify the influence of clay content and carbon concentrations on EPS production. Minitab 17 was used for the statistical analyses.

7.4 Results and Discussion

7.4.1 Clay aggregation in TC-CC enrichments

Based on the energy-generating reactions, glucose theoretically provides more energy followed by lactate and acetate (Roden and Jin, 2011). In TC^{1x}, the ratio of energy generation between the carbon sources, under equivalent concentrations of oxygen, was calculated. TC^{1x} has similar amounts of lactate (20 mM) and acetate (31.53 mM) to provide - similar amount of energy, but glucose was provided (5 g/L or 27.75 mM) at 2.23 times higher energy levels than lactate. This suggests that the glucose present in TC^{1x} is abundant and provided copious amounts of energy for EPS production. EPS production was investigated when glucose: acetate: lactate concentrations were proportionally reduced from original levels of 27.75 mM: 31.53 mM: 20 mM (representing TC^{1x}) to 3:64 mM: 0.16 mM: 0.1 mM (represents TC^{0.005x}) or no organics at all (represents TC^{0x}).

As expected, *P. aeruginosa* PAO-1 grew to higher final optical densities in TC^{1x} than in TC^{0x} and TC^{0.005x} (Figure 7.3) in the absence of MX-80 bentonite. One-way ANOVA analysis was conducted to determine the influence of carbon concentrations on the growth of *P. aeruginosa* PAO-1. The carbon contents in TC were 0x, 0.005x and 1x. The analysis for carbon yielded $F(2,15) = 11.27$, $p < 0.001$, indicating that the effect of carbon on *P. aeruginosa* PAO-1 growth was significant. The results suggested that high carbon concentrations would allow more bacterial growth and once carbon sources were limited, bacterial growth would decrease.

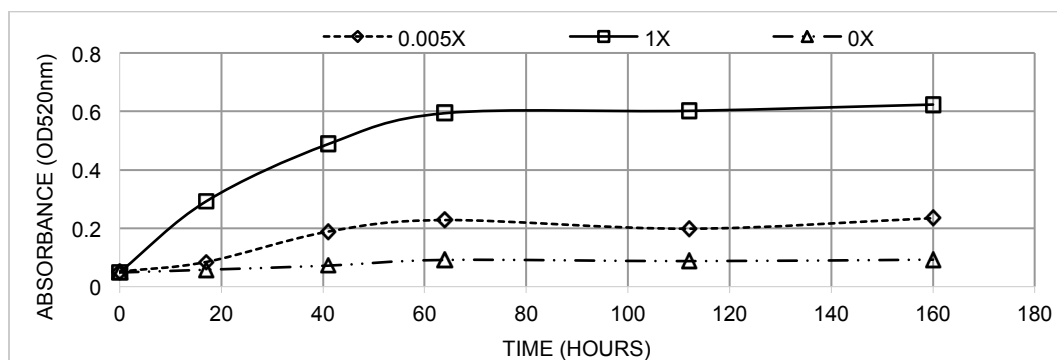


Figure 7.3. Growth curve of *P. aeruginosa* PAO-1 grown in TC-CC enrichments. Culture grown in TC^{1x} showed higher absorbance followed by TC^{0.005x} and TC^{0x}. Absorbance was measured at 520 nm using a microplate reader.

When it comes to EPS production, it has been observed that ~105 µg/mL EPS was generated by *P. aeruginosa* PAO-1 in TC^{1x} in the absence of clay and much lower when clay is present (Figure 7.4). The exuberant generation of EPS could be due to the ability of *Pseudomonas* strains to synthesize and degrade the carbon and energy reserved within the EPS (Schiller *et al.*, 1993). In contrast, < 3 µg/mL of EPS was produced in both TC^{0x} and TC^{0.005x} (Figure 7.4 A,B) in the absence and presence of clay. Based on the two-way ANOVA analysis on the influence of carbon concentrations and clay contents on EPS production, it was determined that all effects are significant ($p < 0.001$). The carbon concentrations yielded $F(2,75) = 46.85$, $p < 0.001$, while clay content produced $F(4,75) = 7.28$, $p < 0.001$. Moreover, the interaction effect was also significant yielding $F(8,89) = 6.95$, $p < 0.001$. The main effects plot for EPS generating (Figure 7.5) visually indicated how each factor affects EPS production. The overall result suggested that in high nutrient media such as TC^{1x}, EPS production would be high (i.e., the steeper slope the greater the effect on EPS production). However, as the content of clay increase from 0 to 10 %, the production of EPS would be affected and would decrease as the clay concentration increases. On

the other hand, if carbon sources would remain low (i.e., TC^{0x} or $0.005x$), EPS production would remain low regardless of clay content.

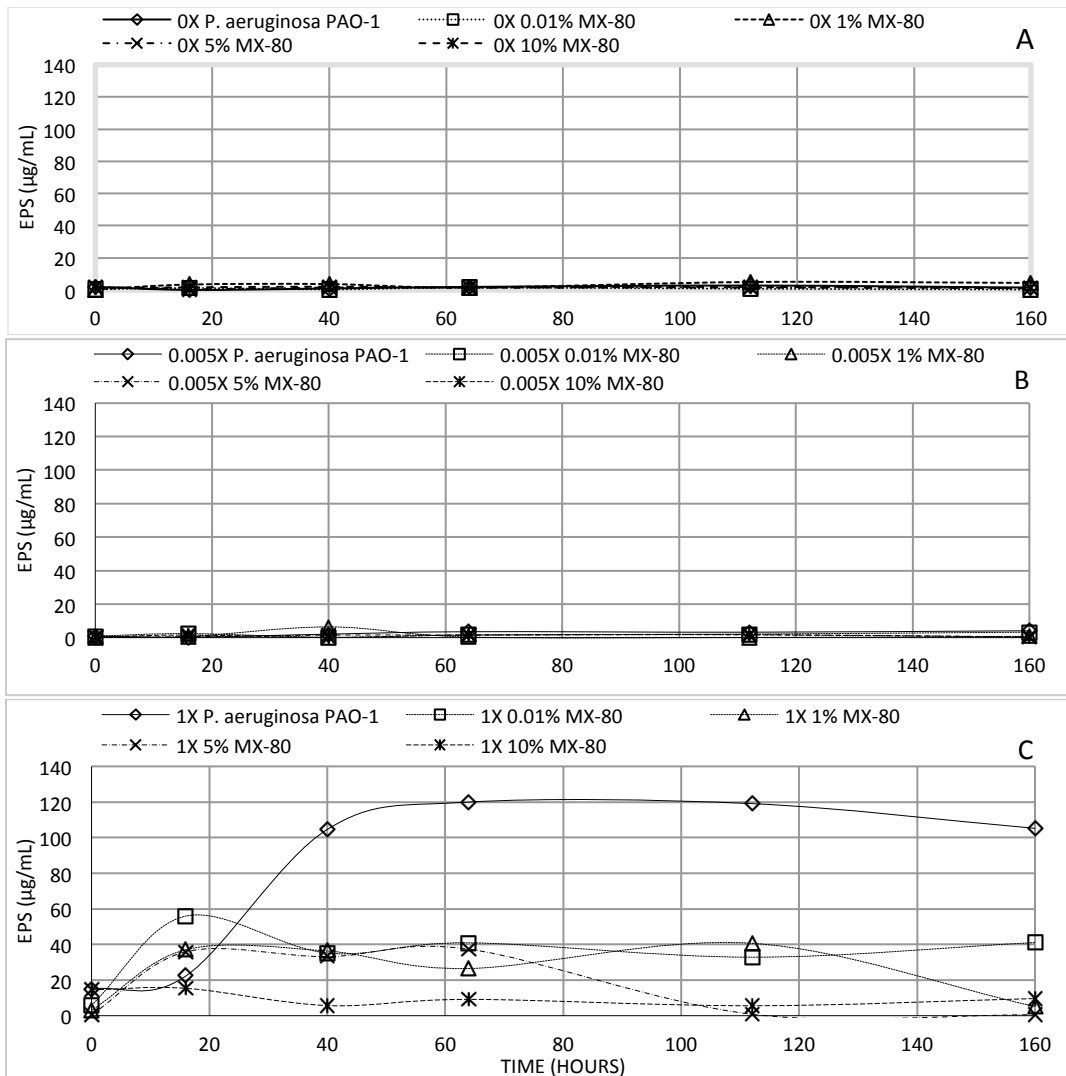


Figure 7.4. EPS production in *P. aeruginosa* PAO-1 grown using treatment C with different clay percentages (0, 0.01, 1, 5 and 10 %) and a) 0X, b) 0.005X and c) 1X carbon (TC-CC enrichments). EPS was extracted and quantified using the phenol-sulfuric acid colourimetric method after 0, 16, 40, 64, 112 and 160 h of incubation.

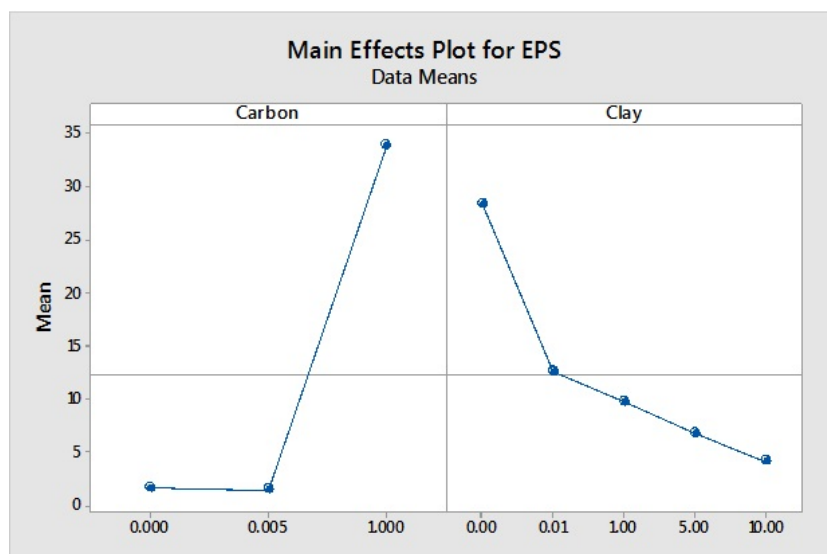


Figure 7.5. Main effect plot showing the influence of carbon concentration and clay content on EPS production in *P. aeruginosa* PAO-1. The longer the distance between the dots, the greater the effect of the factors on growth.

The growth curve and EPS production results were, hence, in agreement and verified assumed direct relationships since at low growth, little to no EPS would be produced, and vice versa. It was also convenient to discover that the presence of clay could counteract EPS production in TC^{1x} since it has been hypothesized that EPS production contributes to clay-microbe aggregation. Such finding is relevant to a DGR because the possible cell-to-cell or clay-microbe aggregation could negatively affect its overall functionality. Therefore, in the particle size analysis, it would be excellent to observe no aggregate formation in the presence of clay at TC^{1x}. The particle size analysis has confirmed the aforementioned assumption that high bentonite content would limit aggregation in high carbon media since aggregates were only formed in TC^{1x} without MX-80. More favourably, little to no aggregates was formed in those samples with 0.01, 1, 5 and 10 % MX-80 bentonite. Figure 7.6 shows the aggregate diameter size changing from 0.2 μm to 60 μm after 160 h in TC^{1x} pure cultures (no MX-80 added) of *P. aeruginosa* PAO-1. The data indicated that particle diameters are > 20 μm after 40 hours (T40), an increase from the particle diameter baseline of 0.2 μm , which suggest aggregate formation and that they are clearly larger than unicells. Figure 7.7 shows a decline and detection of periodic spikes in bacterial aggregation when MX-80 is present in the medium. Figures 7.8 A to E show the fluorescent-stained images of clay and bacteria in the TC-CC enrichments. Fluorescently-labelled lectins, such as concanavalin A, bind to glycoconjugate groups and proteins associated with cell walls,

thus a counterstain like Syto 9 and PI are used to distinguish EPS from cells. The EPS, in this case, will appear purple, while Syto 9 and PI will appear green and red, respectively. Blue shows overlap of PI and Alexa 647 stains (red and purple, accordingly). These images indicate presence of aggregation in TC^{1x} with 0 and 0.01 % MX-80 (Figure 7.8 A,B) and then decline as the MX-80 content increases (Figure 7.8 C to E).

7.4.2 Clay aggregation in TC-CNP enrichments

Figure 7.9 shows the growth curves of *B. simplex* in TC-CNP enrichments under different C:N:P regimes (identified as PL (100:10:0.1), NL (100:1:1), NE (100:30:1), PE (100:10:2) and balanced (100:10:1)). In TC-CNP enrichments with 0 % MX-80 bentonite, the optical density measurements show that growth is occurring more slowly than those with 0.01 % MX-80. It also suggests that *B. simplex* growth patterns are similar regardless of the treatment, and that only the presence of clay affects the growth. However, the presence of clay is not indicative of higher growth since it is possible that 0.01% clay made the enrichment more turbid than those without.

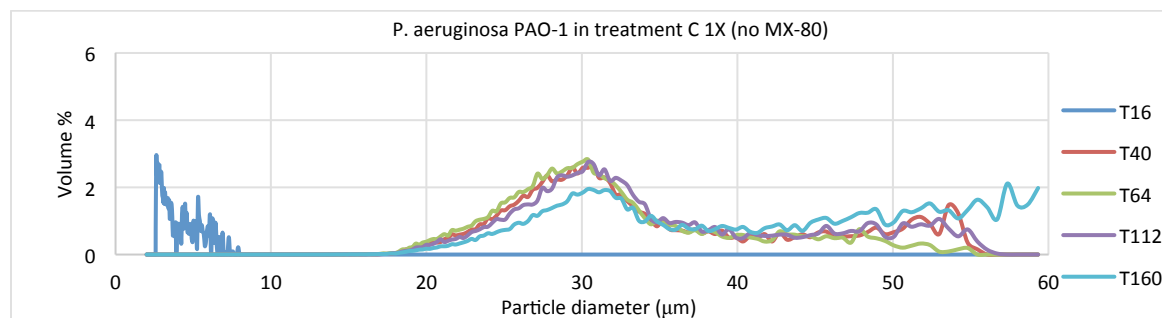


Figure 7.6. Particle size analysis (μm) of *P. aeruginosa* PAO-1 grown without MX-80 bentonite in TC^{1x}. Particle sizing was conducted at 16, 40, 64, 112 and 160 h.

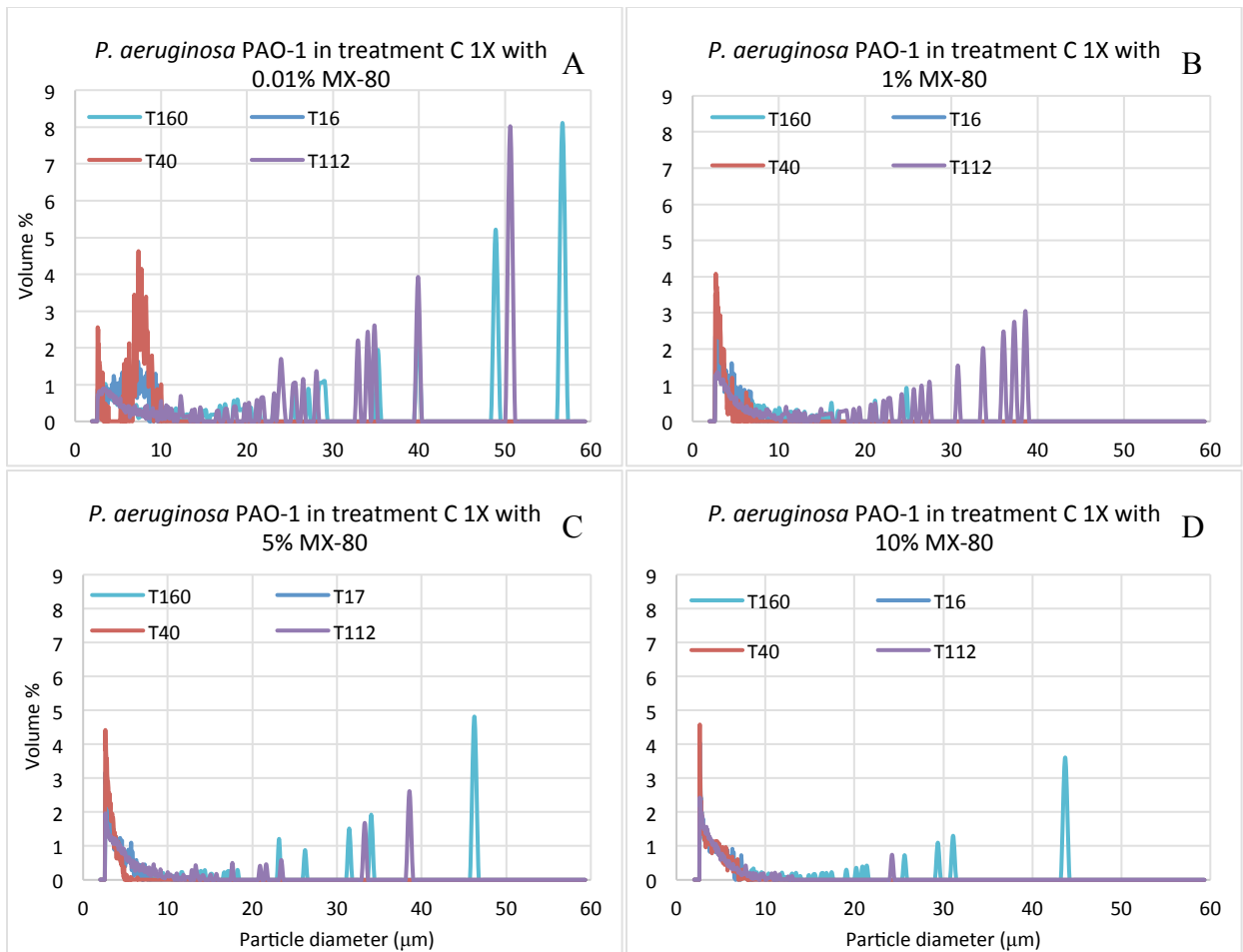
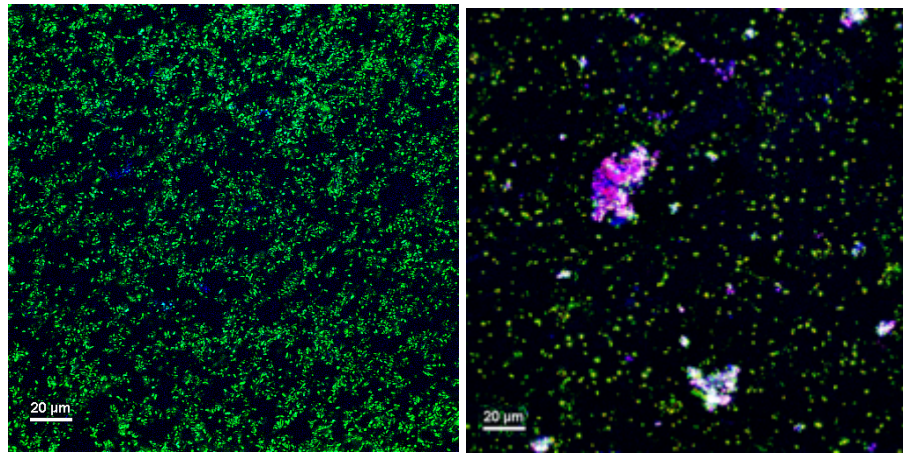
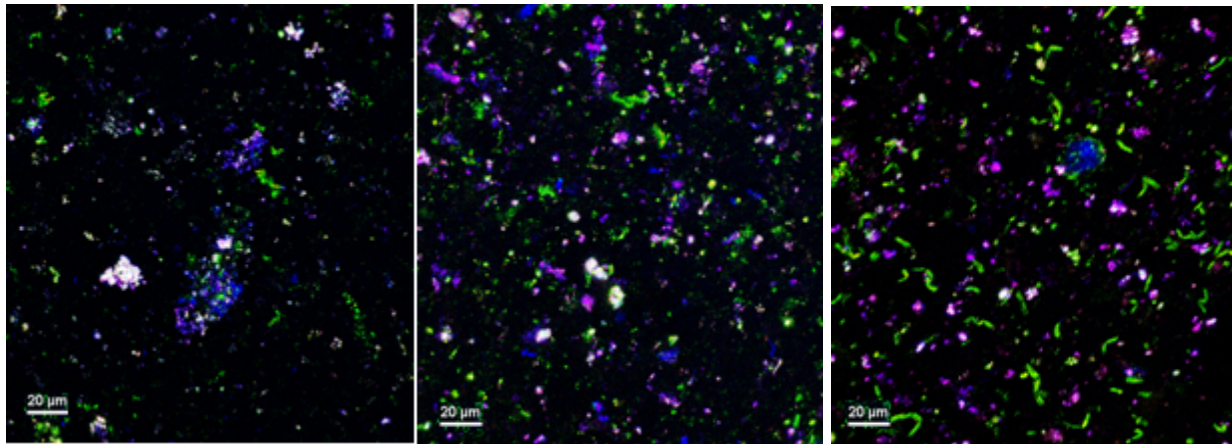


Figure 7.7. Particle size analysis (μm) of *P. aeruginosa* PAO-1 grown in TC^{1x} containing different percentages of MX-80 bentonite, A) 0.1 %, B) 1 %, C) 5 % and D) 10 % using Coulter Counter.



A

B



C

D

E

Figure 7.8. CLSM images of *P. aeruginosa* PAO-1 after 160 h in TC^{1x} containing A) 0 % MX-80, B) 0.01 %, C) 1 %, D) 5 % and E) 10 % MX-80. The samples were incubated at room temperature and shaken at 75 rpm. Samples were stained with concanavalin A-Alexa 647 lectin probe (purple), Syto 9 (green) and PI (red). Blue indicates overlap between Alexa 647 (purple) and PI (red).

What is occurring instead could be that in low-nutrient media, clay chemistry influenced bacterial attachment on the clay surface. Since *B. simplex* is a Gram-positive bacterium, it is possible that it would attach on the negatively-charged surface of montmorillonite, which is the main mineral component of MX-80 bentonite. Furthermore, since MX-80 bentonite contains 0.65 % CaO, it is possible that Ca²⁺ could serve as a divalent bridge between negatively charged bacteria, as in *B. simplex* (Jaisi *et al.*, 2008). On the other hand, the presence of more Na⁺ (2.57 % NaO) in the interlayer space of a negatively charged montmorillonite could reduce divalent bridging, thus leading to a reduced probability that *B. simplex* would attach to the MX-80 bentonite surface. It can be argued that the presence of montmorillonite may induce a “gelation layer” around the clay minerals upon water/media saturation resulting in little *B. simplex* attachment to the MX-80 bentonite surface (Perdrial *et al.*, 2009). Nutrients such as sugars may increase microbial adhesion to clay surfaces (Stotzky, 1985). Kjelleberg (1984) quoted in his review that a medium containing 1 g organic carbon per L increased adhesion of marine bacteria to hydrophobic surfaces. On the contrary, Marshall *et al.* (1971) reported repressed adhesion of *Pseudomonas* species to a glass surface when the glucose concentration was above 7 mg/L. In the present study where *B. simplex* was cultivated in variable concentrations of phosphate and nitrogen (fixed amount of carbon source – glucose at 50 mg/L), varying degrees of clay-microbe aggregation were observed. In an oligotrophic environment, as imposed by the low nutrient TC-CNP enrichment containing *B. simplex* culture with 0 % MX-80, aggregates with < 12 µm of mean diameter was observed in all treatments in comparison to the enrichment containing 0.01 % MX-80 bentonite (Table 7.6), where a mean aggregate diameter of < 21 µm was seen, suggesting that the presence of inorganic material induced aggregation.

The attachment of bacteria to clay surfaces could assist in their survival. In this case, Fe₂O₃ content in bentonite could play a facilitating role. For instance, *Pseudomonas fluorescens* could synthesize a pyoverdine-type siderophore that could complex with Fe(III), as well as with actinides (Moll *et al.*, 2008; Boukhalfa *et al.*, 2007). Such complexation could support bacterial growth as well as attachment to the surface of the clay (Perdrial *et al.*, 2009) - MX-80 bentonite contains 3.25 % Fe₂O₃. With *B. simplex*, Štyriaková *et al.* (2010) indicated that *Bacillus* spp. increased iron dissolution and reduction in quartz sands in the presence of glucose. Therefore, it is possible that similar reaction could have happened in the TC-CNP enrichment with glucose as main carbon source. As seen in Table 7.4, the particle size of TC-CNP enrichments with 0.01 %

MX-80 inoculated with *B. simplex* increased compared to the enrichments with 0 % bentonite. The data show larger aggregates (> 10 µm) in those enrichments with 0.01 % MX-80 than in those with 0 % MX-80. For instance, 20.73 µm mean aggregate diameter in PL 0.01 % and 18.32 µm for NL 0.01 %, while only 11.81 µm and 7.99 µm mean aggregate diameter for PL and NL, respectively, without clay (0 % MX-80). The CLSM images also showed the aggregation between treatments (Figures 7.10 and 7.11).

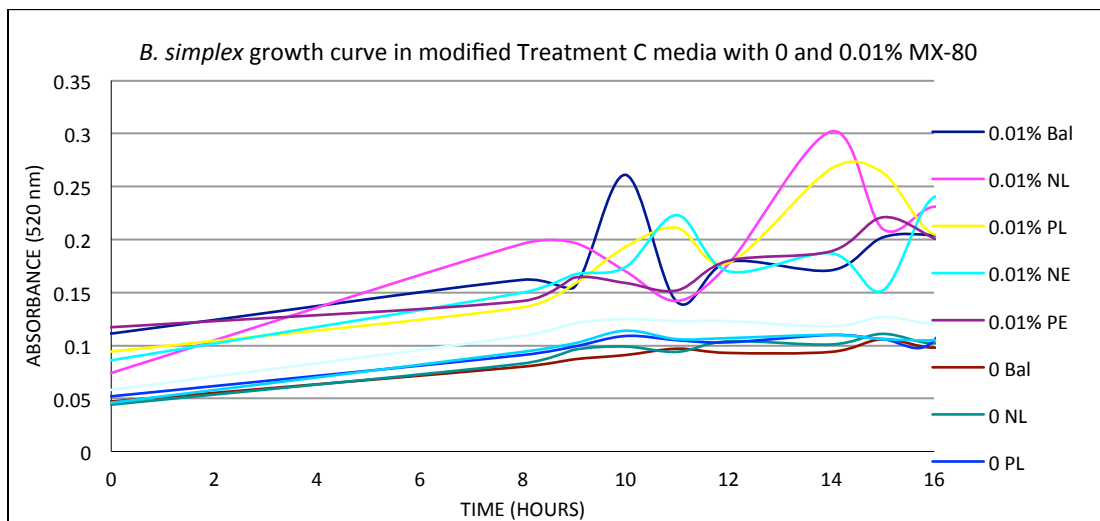


Figure 7.9. Growth curve of *B. simplex* grown in treatment C with varying C:N:P ratios. Balanced (BAL) has a ratio of 100:10:1, NE (N-excess) - 100:30:1, NL (N-limited) - 100:1:1, PE (P-excess) - 100:10:2 and PL (P-limited) - 100:10:0.1 (TC-CNP enrichments). Cultures grown in these treatments contained 0.01 % MX-80 or no MX-80. Absorbance was measured at 520 nm using a microplate reader for all samples.

Table 7.4 Summary of volume distribution as the result of Coulter Counter particle sizing of the TC-CNP treatments with 0 and 0.01 % MX-80 and *B. simplex*.

Treatment	Clay %	Mean (μm)	Standard Deviation (μm)	Median (μm)	Specific Surface Area (cm^2/cm^3)
Balanced (100:10:1)	0	6.77	7.45	3.17	16264.7
NL (100:1:1)	0	7.99	7.80	3.66	14610.1
PL (100:10:0.1)	0	11.81	11.14	5.64	12820.8
NE (100:30:1)	0	5.41	7.38	2.72	19315.7
PE (100:10:2)	0	6.34	7.90	3.15	17011.7
Balanced (100:10:1)	0.01	8.56	6.55	6.64	12999.4
NL (100:1:1)	0.01	18.32	11.48	20.21	7859.16
PL (100:10:0.1)	0.01	20.73	12.33	22.30	6896.12
NE (100:30:1)	0.01	14.60	10.16	15.29	9973.11
PE (100:10:2)	0.01	9.56	5.48	9.14	9600.99

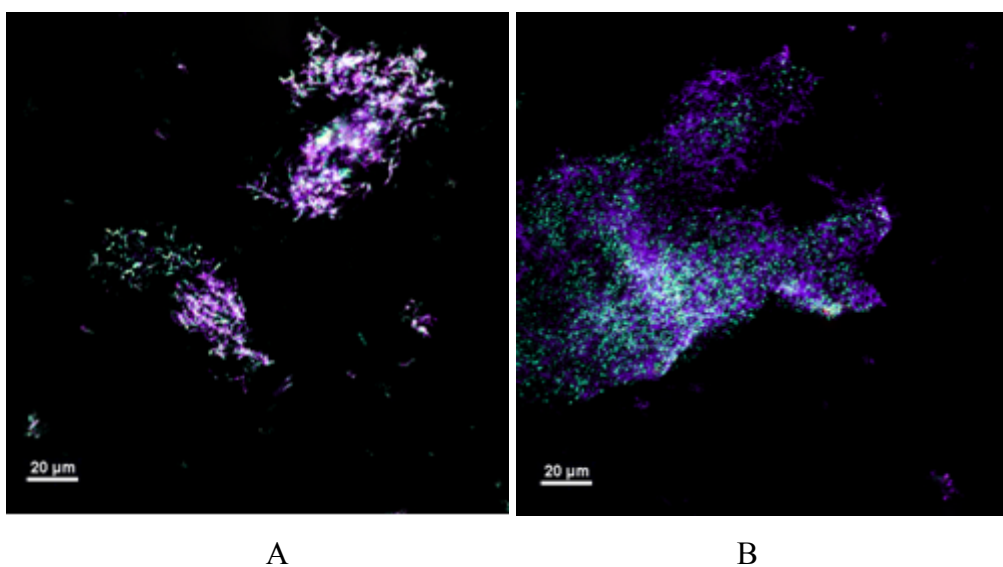
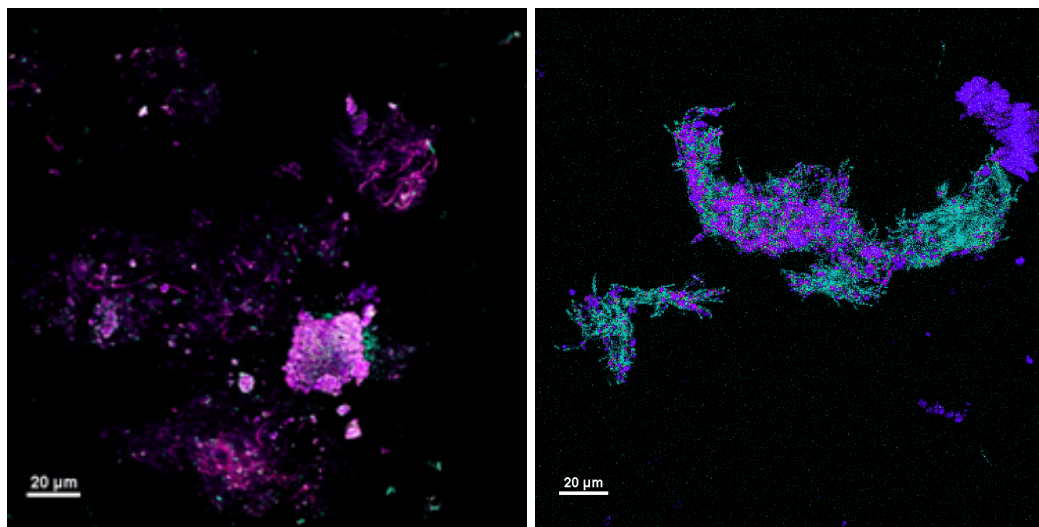


Figure 7.10. CLSM images of *B. simplex* in treatment C having a C:N:P ratio of A) 100:1:1 - NL (N-limited) after 17 hrs and B) 100:10:2 - PE (P-excess) after 17 hours without MX-80 bentonite. The samples were taken from enrichment that were incubated at room temperature for 15 hrs while shaken at 75 rpm. The samples were stained with ConA-Alexa 647 lectin probe (purple = EPS), Syto 9 (green = live cells) and PI (red = dead cells).



A

B

Figure 7.11. CLSM images of *B. simplex* in treatment C having a C:N:P ratio of A) 100:30:1 - NE (N-excess) after 17 hrs and B) 100:10:1 - Balanced after 17 hours with 0.01 % MX-80 bentonite. The samples were taken from enrichment that were incubated at room temperature for 15 hrs while shaken at 75 rpm. The samples were stained with ConA-Alexa 647 lectin probe (purple = EPS), Syto 9 (green = live cells) and PI (red = dead cells).

Such an occurrence could mean that the low-nutrient environment allowed microorganisms to scavenge nutrients, such as Fe (III), from the MX-80 bentonite to support growth (e.g., Perdril *et al.*, 2009 and Jaisi *et al.*, 2008). On the other hand, it could serve as a survival mechanism in order to establish a niche to stabilize growth by either trapping nutrients or utilizing their own EPS (Wolfaardt *et al.*, 1999). In terms of nutrients, in the TC-CNP enrichments with 0.01 % MX-80 bentonite, the mean diameters were higher in PL (20.73 μm) followed by NL (18.32 μm) and NE (14.60 μm), while PE (9.56 μm) and BAL (8.56 μm) mean diameters were quite similar. The enrichments without clay also produced similar ranking of treatment with it comes to mean diameters, however, the values are smaller – PL (11.81 μm) > NL (7.99 μm) > NE (5.41 μm) > PE (6.34 μm) \approx BAL (6.77 μm). The limitations in phosphorus and nitrogen supplies showed an increase in aggregation in both PL and NL media containing 0 and 0.01 % MX-80. In these cases, EPS may perhaps be secreted at high concentrations with both cells and clay particles becoming incorporated into the EPS matrix to form aggregates. Evans (2013) (in his review) and Bragadeeswaran *et al.* (2011) observed that nutrient limiting enrichments increased EPS production, and hence, aggregation in *Bradyrhizobium japonicum* and *Bacillus cereus*. Their

observations further suggested that firm adhesion to surfaces is not only affected by nutrients, but also by the type of bacteria.

Stotzky (1986) has provided an extensive physico-chemical explanation of the mechanisms of clay-microbe surface interaction wherein the author considered H-bonding (due to protonation between clay and microbes), water bridging (similar to H-bonding but for anionic groups of a biological entity), cation bridging (multivalent exchangeable cations on the surface of the clay causing adhesion of anionic biological entities), van der Waals interaction (Hendricks, 1940; Kozak and Mandeville, 1995), and coordination (ligand exchange), depending on the environmental conditions. Stotzky (1986) also stated that pH might be a factor that affects clay-microbe interaction wherein the pH would define the net charge on the surface of the microbes and of some pH-dependent clay samples. The pH of the entire system in this study was around 7.3 so this factor might have caused some changes in the net charges of the microbial cells and the clay sample (Cai *et al.*, 2013) allowing *B. simplex* and montmorillonite surfaces to interact.

While the experiments conducted in this thesis were not representative of those in a DGR, there are possible locations within a repository where low-density clays would be anticipated, such as interfaces between the host rock and the clay buffer and in the early clay buffer saturation phase. Nonetheless, a DGR would contain mainly highly-compacted clay minerals and would, therefore, be a diffusion-dominated system (King *et al.*, 1996). As a result, the mechanisms discussed earlier would be insignificant because as a DGR became saturated with groundwater, the clays would swell. The hydration and water competition events would limit mobilization and restrict activity of the microorganisms. Perdrial *et al.* (2009) also emphasized that the additional porosity generated as a result of microbial structural changes in MX-80 bentonite would not increase interconnected porosity in HCB. This could be due to water gels being accommodated within the additional pore space and then resealing the spaces.

7.4.4 Role of nutrient in EPS production

Under growth favourable conditions, as in TC^{1x}, *P. aeruginosa* PAO-1 and *B. simplex* produced generous amounts of EPS, which later led to microbial aggregation in the media (both without bentonite and with 0.01 % MX-80). It was concluded from the present study that carbon (mainly glucose) and phosphates affected EPS production predominantly in these microorganisms. Therefore, when carbon concentrations were depleted or removed (as in TC^{0.005x} and TC^{0x}), microbial growth, EPS production and aggregation declined significantly in cultures containing

P. aeruginosa PAO-1 and *B. simplex* (both in the presence and absence of MX-80 bentonite). The end results further confirms that nutrient content affects EPS production (Sheng *et al.*, 2010) as well as the extent of aggregation in the system.

Other factors affecting EPS production and clay-microbe aggregation are the carbon substrate and growth phase of the organism (Sheng *et al.*, 2010). Carbon substrate type has considerable effect on EPS production. When *P. aeruginosa* PAO-1 was grown in TC^{1x} containing 5 g/L glucose, 30.51 mM acetate and 5x phosphates, EPS production was at its highest. The amount of EPS was reduced in other treatments (TA to TH) for *P. aeruginosa* PAO-1, especially when acetate and glucose concentrations were decreased. *B. simplex* EPS production was affected mainly by glucose, acetate and NaCl. The results from the present study suggest that in a low-carbon environment, such as a HLW DGR where an average of 0.5 g/L organic carbon is available (Sauzeat *et al.*, 2001), the growth of perhaps *Pseudomonas* and *Bacillus* species would be inhibited. However, other oligotrophic and copiotrophic microorganisms could adapt to a low nutrient environment through sporulation, adhesion to inorganic material (e.g. clay) or reduction in cell size (Koch, 2001; Marshall *et al.*, 1971).

7.4.5 Role of EPS in microbe-bentonite aggregation

EPS production by the bacterial strains isolated from MX-80 bentonite was investigated to determine if their presence would influence aggregation of microbes and bentonite in the model system. Jaisi *et al.* (2008) demonstrated that the rate of nontronite particle aggregation increased when *Shewanella putrifaciens* CN32-extracted EPS was added to unreduced nontronite, which further confirmed the role of EPS on microbe-mineral aggregation. Therefore, in the present study, the increase in aggregate size of *P. aeruginosa* PAO-1 observed in EPS-stimulating media (i.e., TC^{1x}), detected under CLSM similarly suggests a close association of EPS to aggregate formation. According to Chenu (1995), for aggregation to occur through EPS, the polysaccharide or polymer must be adsorbed at the surface of the clay mineral. When adsorption is attained, it will lead to inter-particle bridging of clay minerals, extending from one particle to the bulk solution (Harris and Mitchell, 1973). The resulting networks would lead to the entanglement of clay minerals within the EPS. Relevant reactions that would make use of this mechanism would be those related to bioreduction (Jaisi *et al.*, 2008; Perdrial *et al.*, 2009), dissolution of clay minerals (Dong, 2012) and bacterial respiration (Stotzky and Rem, 1967).

7.4.5 Role of MX-80 bentonite in clay-microbe aggregation: relevance for a DGR

Based on the results obtained from the TC-CC and TC-CNP enrichments with *P. aeruginosa* PAO-1 and *B. simplex* with MX-80 bentonite, clay-microbe aggregation was affected by the concentration of bentonite. In solution-dominated enrichments, where 0.01 % MX-80 was added, aggregate size diameters increased and aggregates were present more often for both microbial species regardless of the nutrient concentration. Earlier onset of aggregation was also observed (after a couple of hours) in these enrichments in comparison to those without MX-80 bentonite. These observations are in agreement with Alimova *et al.* (2006) who observed smectite clay promoting aggregation. However, in the present study, when 1 %, 5 % or 10 % of MX-80 bentonite was present, aggregation declined or not detected at all. Therefore, the presence of higher concentrations of MX-80 seemed to have affected the ability of microbial isolates to form aggregates negatively.

The addition of 0.01 % MX-80 may have encouraged aggregation because of the slight attractive forces and surface charges that the microorganisms were able to utilize to adsorb onto MX-80 bentonite surfaces (Stotzky, 1986). It is, therefore, very likely that when particles or inanimate surfaces are available, microorganisms will be attracted to these as long as conditions are favourable. Since the bulk concentration of microorganisms was higher than MX-80 in TC-CC, the microbes may have been able to overcome the distance-dependent energy barriers (involving Derjaguin-Landau-Verwey-Overbeek (DLVO) or extended DLVO theory) around the clay mineral (i.e., montmorillonite) (Sheng *et al.*, 2010; Leech and Hefford, 1980). Huang *et al.* (2015) demonstrated that the energy barrier for montmorillonite is high and that overcoming this energy barrier is bacterial-strain specific. If the microorganism is able to overcome such energy barrier, then bacterial adhesion followed by aggregation may occur. Huang *et al.* (2015) have shown that *Bacillus subtilis* (Gram-positive) adhesion to montmorillonite surfaces occurred but was less favourable than for *Pseudomonas putida* (Gram-negative). Similar observations were observed in the present study, i.e., *B. simplex* showed more favourable clay-microbe aggregation in 0.01 % MX-80 in TC^{1x} than *P. aeruginosa* PAO-1. Moreover, the production of EPS may also contribute to clay-microbe aggregation because it can penetrate the energy barriers presented by MX-80 bentonite (Huang *et al.*, 2015). Harris and Mitchell (1973) also suggested that an increase in the quantity of EPS in the presence of clay minerals resulted in a maximum degree of aggregation. However, when constant amount of EPS is

present in the system with an increasing volume of clay minerals as in 5 and 10 % MX-80 bentonite in the present study, the effect of EPS on aggregation would become insignificant. Only when the quantity of EPS was increased could complete re-stabilization of aggregation in the same system proceed, due to the now much higher number of available reactive sites on which adhesion could occur.

However, even though clay-microbe interaction was possible in 0.01 % MX-80, the aggregates may be weak and could, therefore, lead to microbial desorption from the clay mineral surfaces (Rong *et al.*, 2008). At pH 7, Rong *et al.* (2008) showed the exothermic adsorption of *Pseudomonas putida* to montmorillonite surfaces because of hydrogen bonding. When the pH increased (more alkaline), repulsive electrostatic forces became dominant causing a reduction of microbial adsorption to the clay surface. In the present study, the pH was at 7.2 ± 0.2 , which could mean that similar non-electrostatic forces were involved. Lastly, Rong *et al.* (2008) reported the possible correlation of surface area with the affinity of microorganisms for the montmorillonite surface. Montmorillonite has a high surface area, but the tendency for bacteria (regardless of Gram classification) to access the inner surface of the clay minerals is likely improbable due to steric hindrance.

Because of the unstable adsorption of microorganisms to montmorillonite caused by many physical and chemical factors, it can be assumed that higher concentrations of MX-80 bentonite would further restrict microbial adhesion to the surface, which is the key to generate clay-microbe aggregation. As seen in this study, the presence of 1, 5 and 10 % MX-80 bentonite diminish aggregation in the enrichments. It is probable that in a clay-dominated system, cell attachment to the clay mineral surface would become negligible (Perdrial *et al.*, 2009) and that no aggregation would occur. Perdrial *et al.* (2009), however, reported on the activity of *Shewanella putrefaciens* in a clay-dominated system and indicated that the microorganism was able to slightly alter the structure of the MX-80 bentonite leading to an increase in interconnected porosity. But because of the swelling pressure present in the compacted system and the ability of MX-80 to form gels upon saturation, the bacteria did not cause further damage to the compacted MX-80. Instead, water penetrated the pores and sealed the newly formed pores caused by microbial aggregation. Nonetheless, as long as the overall integrity of the actual DGR environment is maintained, the likelihood of microbial-clay aggregation would be low or non-existent.

7.5 Conclusions

EPS was found to be the causal agent of microbe and microbe-bentonite aggregation. Hence, when EPS production was minimized, aggregation was also diminished. In nutrient-rich media, such as TC^{1x} carbon, EPS production was abundant in both *P. aeruginosa* PAO-1 and *B. simplex*. When the carbon supply was decreased to 0.005x and even 0x, EPS production, microbial growth and aggregation were reduced. These results indicate that copiotrophic conditions are required for *P. aeruginosa* and *B. simplex* to produce high concentrations of EPS. When *B. simplex* was exposed to lower glucose concentrations (50 mg/L) and lower and varying amounts of phosphate and nitrogen, clay-microbe aggregation also varied. It was discovered that limitations in nitrogen and phosphate increased aggregation while in excess conditions, *B. simplex*'s ability to aggregate diminished.

Higher concentrations of MX-80 seemed to have affected the ability of microbial isolates to form aggregates. When 0.01 % of MX-80 was added in nutrient-rich media, aggregation with both *P. aeruginosa* PAO-1 and *B. simplex* was detected. However, when 1, 5 and 10 % MX-80 was present, little to no aggregation was observed. This suggested that the microbe:bentonite ratio affected the adhesion of microbes to the bentonite surface. When the microbe:bentonite ratio was high (solution-dominated system), the slight repulsive forces and energy barriers could be overcome by bacteria and EPS, thereby allowing aggregation. However, when the microbe:bentonite ratio was low (clay-dominated system), microorganism and EPS were not able to overcome the repulsing forces and energy barriers induced by bentonite. The results suggested that low nutrient systems encourage microorganisms to adhere to surfaces to scavenge nutrients or establish a niche to further support growth.

Based on the results obtained from these studies, it is unlikely that extensive microbial or clay-microbes aggregates would occur at water-bentonite interfaces or in high dry density regions because these locations are usually low in nutrients. These results, therefore, are favourable since a DGR is designed in such a way that these phenomena would not occur. For future work, the study of components of clay that might influence the decline in microbial aggregates would be useful. Moreover, applying the same study to a different microbial strain isolated from MX-80 individually or as a consortium would adapt in these treatments.

8. GENERAL CONCLUSIONS

The purpose of the work in this thesis was to study the microbial effects that could negatively affect a DGR, with the microbial source being the bentonite buffer material selected. The study involved: i) the identification of microorganisms inhabiting natural and commercial bentonite materials through culture-dependent or –independent methods, ii) the confirmation of effectiveness of microbially-inhibiting parameters on microbial culturability and activity, iii) the assessment of the rate of sulfide generation in a bentonite inoculum as a result of SRB activity, and iv) the development of model systems to examine microbial behaviour at nutrient-rich/-poor water-bentonite interfaces.

Results obtained from the extraction of DNA directly from bentonite (Avonlea (or CCP), and MX-80 Wyoming) confirmed the difficulty in attaining amplifiable products from clays. Although the Swiss protocol worked when products were diluted to a certain degree, the protocol was not successful for extended use. As such, culture-dependent techniques were used to obtain DNA from bentonite samples. Thereafter, through 16S rRNA sequencing, it was determined that Gram-positive and Gram-negative spore-forming bacteria dominate the bulk MX-80 bentonite. From these, 22 identified isolates belonged to phylum *Actinobacteria* while six belonged to *Firmicutes*. Their ability to form spores or transform to VBNC state could be the reason why these microorganisms were able to withstand the harsh processing conditions MX-80 bentonite have gone through.

The selection and use of culture-dependent techniques proved amenable to the HCB MX-80 plug studies, and confirmed the presence of primarily Gram-positive spore-forming microorganisms within and on the outside of the bentonite plugs. The same study also demonstrated and confirmed the effectiveness of having a dry density of $\geq 1.6 \text{ g/cm}^3$, a swelling pressure of $> 2,000 \text{ KPa}$, and an a_w of < 0.96 at suppressing microbial culturability to at or below background levels, which is relevant for a future DGR. The HCB environment appeared to be selective to Gram-positive bacteria which became metabolically active when the inhibitory

conditions imposed by the HCB were eliminated (i.e., during culturing). Moreover, it was also determined that using 50 g/L NaCl in the infiltration solution further suppressed microbial culturability in the HCB plugs. Hence, if these inhibitory conditions were maintained in a DGR, the viability of indigenous organisms would be expected to decline over DGR-relevant time-scales, ultimately reaching a point where viable cells may no longer be recovered.

Since the parameters affecting the microbial culturability to remain at or below background in the HCB MX-80 could be manipulated, it was also of interest to determine what these indigenous microorganisms are capable of doing when conditions become conducive for growth. In the enrichment study with SRB media, sulfide production assessment was performed and it was determined that sulfide production was temperature- and bentonite clay type-dependent. Higher sulfide concentrations were observed at 15°C than at 37°C for SRB10 and SRB50 enrichments. Lactate was the preferable substrate for sulfidogenesis; however, increased salinity delayed and impeded sulfide production.

The ability of a HAB isolated from MX-80 bentonite and of a selected lab strain (*P. aeruginosa* PAO-1), to form aggregates at water-bentonite interfaces was investigated using CLSM and Coulter counter particle sizing equipment. EPS was found to contribute to microbe and microbe-bentonite aggregation. Nutrient content, bacterial content and substrate type were factors identified to affect aggregation and EPS production in *B. simplex* and *P. aeruginosa* PAO-1.

Based on the results obtained from these studies, it is unlikely that microbes would become significantly metabolically active within the EBS in a future DGR, either at the water-bentonite interface or in regions of lower dry density, so long as microbially inhibiting parameters are maintained, as they would be in a properly designed and constructed DGR. For future work, the evolution of culture-independent techniques, such as MiSeq high throughput sequencing, on MX-80 bentonite, the preferred buffer material for the Canadian DGR, would help eliminate the difficulty of labour consuming and less than fully representative results from culture-dependent methods. Related to this, the advancement of DNA extraction protocols to obtain higher concentrations of amplifiable PCR products remains a challenge. Furthermore, it would be interesting to compare the microbial community present in enrichments SRB50 and SRB10 to see which microorganisms predominate under the imposed conditions. Duplication of the SRB50 study could be used to generate a phylogenetic tree needed for such a comparison. Lastly, an investigation of the components in clay that might influence the decline in microbial aggregation

would be useful. Applying the same study to a different microbial strain isolated from MX-80 individually or to a consortium would be useful in order to see how other microbes or a consortium would adapt in these treatments.

The work presented in this thesis provides insights in the activity and culturability of the microorganisms present in MX-80 bentonite, as part of the safety assessment of a DGR for long-term storage and isolation of used nuclear fuel. The research also confirms existing knowledge on microbial regulation within highly compacted MX-80 bentonite, and provides scenarios of what may occur when such conditions are compromised. Finally, the work provided many opportunities to evaluate relevant microbiological techniques for use in, for instance, DNA extraction (culture-dependent and -independent techniques), polymer analysis, CLSM and staining, and nutrient optimization for EPS production.

9. REFERENCES

- Aller, R. C. (1982). The Effects of Macrobenthos on Chemical Properties of Marine Sediment and Overlying Water. In *Animal-Sediment Relations*, McCall, P. L. & P. J. S. Tevesz (eds). Plenum, New York: 53–102.
- Allison V.J., Z. Yermakov, R.M. Miller, J.D. Jastrow, and R. Matamala. (2007). Assessing Soil Microbial Community Composition Across Landscapes: Do Surface Soils Reveal Patterns? *Soil Science Society of America Journal* 71: 730-734.
- Alimova, A., M. Roberts, A. Katz, E. Rudolph, J.C. Steiner, R.R. Alfano and P. Gottlieb. (2006). Effects of Smectite Clay on Biofilm Formation by Microorganisms. *Biofilms* 3: 47-54.
- Altschul, S.F., W. Gish, W. Miller, E.W. Myers and D.J. Lipman. (1990). Basic Local Alignment Search Tool. *Journal of Molecular Biology* 215(3): 403-410.
- Alves, M.E. and A. Lavorenti. (2004). Sulfate Adsorption and its Relationships with Properties of Representative Soils of São Paulo State, Brazil. *Geoderma* 118: 89-99.
- Amend, J.P. and E.L. Shock. (2001). Energetics of Overall Metabolic Reactions of Thermophilic and Hyperthermophilic Archaea and Bacteria. *FEMS Microbiology Reviews* 25(2): 175-243.
- Amend, J.P. and A. Teske. (2005). Expanding Frontiers in Deep Subsurface Microbiology. *Palaeogeography, Palaeoclimatology Palaeoecology*. 219: 131-155.
- Amann, R.I., L. Krumholz and D.A. Stahl. (1990). Fluorescent-Oligonucleotide Probing of Whole Cells for Determinative, Phylogenetic, and Environmental Studies. *Journal of Bacteriology* 172:762-770.
- Amann, R.I., W. Ludwig and K.H. Schleifer. (1995). Phylogenetic Identification and *In situ* Detection of Individual Microbial Cells Without Cultivation. *Microbiology Reviews* 59: 143-169.
- APHA. (1992). Standard Methods for the Examination of Water and Wastewater (Eaton. A.D., Clesceri, L.S., Rice, E.W. and Greenberg A.E., Eds) American Public Health Association, Washington DC, USA.
- APHA 9215 C. (2005). Heterotrophic Plate Count. The Spread Plate Method. In “APHA, AWWA, and WEF. 2005. Standard Methods for the Examination of Water and Wasterwater”, 21st edition, APHA, Washington, DC.
- ASTM Standard D2216-10. (2011). Standard Test Methods for Laboratory Determination of Water (Moisture) Content of Soil and Rock by Mass. ASTM International, West Conshohocken, PA. DOI: 10.1520éD2216-10, www.astm.org.

- Baker, B.J., D.B. Moser, B.J. MacGregor, S. Fishbain, M. Wagner, N.K. Fry, B. Jackson, N. Speolstra, S. Loos, K. Takai, B. Sherwood Lollar, J. Fredrickson, D. Balkwill, T.C. Onstott, C.F. Wimpee, and D.A. Stahl. (2003). Related Assemblages of Sulphate-Reducing Bacteria Associated with Ultra-deep Gold Mines of South Africa and Deep Basalt Aquifers of Washington State. *Environmental Microbiology* 5:267-277.
- Barton, L.L. and D.E. Northup. (2011). *Microbial Habitat: an Ecological Perspective in Microbial Ecology*. John Wiley & Sons, Inc. New Jersey, USA. p. 120.
- Baumgartner, P., D. Priyanto, J.R. Baldwin, J.A. Blatz, B.H. Kjartanson and H. Batenipour. (2008). Preliminary Results of One-Dimensional Consolidation Testing on Bentonite Clay-Based Sealing Components Subjected to Two Pore-Fluid Chemistry Conditions. Nuclear Waste Management Organization, Toronto, Ontario, Canada NWMO TR-2008-04.
- Beech, I., A. Bergel, A. Mollica, H-C Flemming, V. Sotto and W. Sand. (2000). Microbially Influenced Corrosion of Industrial Materials – Biocorrosion Network. Brite Euram Thematic Network on MIC of Industrial Materials, Brite-Euram III Thematic Network N° ERB BRRT-CT98-5084 Biocorrosion 00-02.
- Beller, H. R., V. Madrid, G.B. Hudson, W.W. McNab, and T. Carlsen. (2004). Biogeochemistry and Natural Attenuation of Nitrate in Groundwater at an Explosives Test Facility. *Applied Geochemistry* 19: 1483–1494.
- Bennett, D.G. and R. Gens. (2008). Overview of European Concepts for High-level Waste and Spent Fuel Disposal with Special Reference Waste Container Corrosion. *Journal of Nuclear Materials* 379:1-8.
- Bentley, D. R. (2006). Whole-Genome Re-Sequencing. *Current Opinion in Genetics and Development* 16:545–552.
- Blumenthal, P.L and S.C Guernsey. (September 1915). The Determination of Sulfur as Barium Sulfate. *Research Bulletin* 26.
- Börgesson, L., H. Hokmark H, and O. Karnland. (1988). Rheological Properties of Sodium Smectite Clay. SKB Technical Reports: TR-88-30
- Börgesson, L., L.-E. Johannesson, T. Sandén and J. Hernelind. (1995). Modelling of the Physical Behaviour of Water Saturated Clay Barriers. Laboratory Tests, Material Models and Finite Element Application. SKB Technical Report TR-95-20, Svensk Kärnbränslehantering AB.
- Börgesson, L., O. Karnland and L.-E. Johannesson. (1996). Modelling of the Physical Behaviour of Clay Barriers Close to Water Saturation. *Eng. Geol.* 41: 127-144.
- Börgesson, L., A. Dueck, and L.-E. Johannesson. (2010). Material Model for Shear of the Buffer – Evaluation of Laboratory Test Results. SKB Technical Report TR-10-31, Svensk Kärnbränslehantering AB.
- Borgonie, G., A. Garcia-Moyano, D. Litthauer, W. Bert, A. Bester, E. van Heerden, C. Möller, M. Erasmus and T.C. Onstott. (2011). Nematoda from the Terrestrial Deep Subsurface of South Africa. *Nature* 474: 79-82.

- Boukhalfa, H., G.A. Icopini, S.D. Reilly and M.P. Neu. (2007). Plutonium (IV) Reduction by the Metal-Reducing Bacteria *Geobacter metallireducens* GS15 and *Shewanella oneidensis* MR1. *Applied and Environmental Microbiology* 73(18): 5897-5903.
- Bradbury, M.H. and B. Baeyens. (2002). Porewater Chemistry in Compacted Re-saturated MX-80 Bentonite: Physico-chemical Characterisation and Geochemical Modelling. National Cooperative for the Disposal of Radioactive Waste, Switzerland, NAGRA Technical Report 01-08.
- Bragadeeswaran, S., R. Jeevapriya, K. Prabhu, S. Sophia Rani, S. Priyadharsini and T. Balasubramanian. (2011). Exopolysaccharide Production by *Bacillus cereus* GU812900, a Fouling Marine Bacterium. *African Journal of Microbiology Research* 5(24): 4124-4132.
- Bredemann, G., and W. Werner. (1933). Botanische Beschreibung Haufinger am Buttersaureabbau Beteiligter Sporenbildender Bakterienspezies. *Zentralb Bakteriol Parasitenkd Infektionskr Hyg Abt II* 87: 446-475.
- Brewer, J.H. (1942). A New Petri Dish Cover and Technique for Use in the Cultivation of Anaerobes and Micro-aerophiles. *Science* 95: 587.
- Brown, A.D. (1990). *Microbial Water Stress Physiology. Principles and Perspectives*. John Wiley and Sons, Chichester.
- Bull, T.A. (2004). *Microbial Diversity and Bioprospecting* (ed). American Society for Microbiology, ASM Press, Washington. DC.
- Cai, P. Q. Huang, and S.L. Walker. (2013). Deposition and Survival of *Escherichia coli* O157:H7 on Clay Minerals in a Parallel Plate Flow System. *Environmental Science and Technology* 47: 1896-1903.
- Capouet, M., U. Noseck, M. Navarro, A. Rüber, A. Van Luik and X. Sillen. (2015). Relevance of Gases in the Post-Closure Safety Case. Nuclear Energy Agency/ Radioactive Waste Management Committee/ Intergration Group for the Safety Case. IGSC Position Paper NEA/RWM/IGSC(2015)1.
- Chapelle, F.H. (1993). *Ground-Water Microbiology and Geochemistry*: New York, N.Y., JohnWiley and Sons, Inc., p. 424.
- Chautard, C., A. Ritt, M. Libert and L. De Windt. (2013). Influence of Hydrogen in the Presence of Organic Matter on Bacterial Activity under Radioactive Waste Disposal Conditions. *Procedia Earth And Planetary Science* 7: 147-150.
- Chenu, C. (1995). Extracellular Polysaccharides: An Interface Between Microorganisms and Soil Constituents. In *Environmental Impact of Soil Component Interactions: Natural and Anthropogenic Organics* Volume 1 (eds. Huang, P.M., J. Berthelin, J.-M. Bollag, W.B. McGill and A.L. Page). CRC Lewis Publishers, pp. 217-228.
- Chi Fru, E. and R. Athar. (2008). *In situ* Bacterial Colonization of Compacted Bentonite under Deep Geological High-Level Radioactive Waste Repository Conditions. *Applied Microbiology Biotechnology* 79: 499-510.
- Cline, J. D. (1969) Spectrophotometric Determination of Hydrogen Sulfide in Natural Waters. *Limnology and Oceanography* 14: 454-458.

- CNSC. (2015, March 2). The CNSC's early involvement in the NWMO's Adaptive Phased Management approach. Retrieved from <https://www.cnsccsn.gc.ca/eng/waste/high-level-waste/cnsc-role-in-nwmo-apm-project/index.cfm>
- Coenye, T. and P. Vandamme. (2003). Intragenomic Heterogeneity between Multiple 16S ribosomal RNA Operons in Sequenced Bacterial Genomes". *FEMS Microbiology Letters* 228(1): 45–49.
- Cole, J.R., B. Chai, R.J. Farris, Q. Wang, S.A. Kulam, D.M. McGarrell, G.M. Garrity and J.M. Tiedje (2005). The Ribosomal Database Project (RDP-II): Sequences and Tools for High-Throughput rRNA Analysis. *Nucleic Acid Research* 33: D294–D296.
- Costerton, J.W., P.S. Stewart and E.P. Greenberg. (1999). Bacterial Biofilms: A Common Cause of Persistent Infections. *Science* 284(5418): 1318-1322.
- Craig, R.F. (1987). *Soil Mechanics*, 4th Edition, Chapman and Hall, London, U.K.
- Cypionka, H., and N. Pfennig. (1986). Growth Yield of *Desulfotomaculum orientis* with Hydrogen in Chemostat Culture. *Archives of Microbiology* 143:396-399.
- Darbi, A., T. Viraraghavan, Y-C. Jin, L. Braul and D. Corkal. (2003). Sulfate Removal from Water. *Water Quality Research Journal Canada* 38(1): 169-182.
- De Hoon, M.J., R.J. Taft, T. Hashimoto, M. Kanamori-Katayama, H. Kawaji, M. Kawano, M. Kishima, T. Lassmann, G.J. Faulkner, J.S. Mattick, C.O. Daub, P. Carninci, J. Kawai, H. Suzuki and Y. Hayashizaki. (2010). Cross-Mapping and the Identification of Editing Sites in Mature MicroRNAs in High-Throughput Sequencing Libraries. *Genome Research* 20: 257–264
- Dixon, D.A. (2000). Porewater Salinity and the Development of Swelling Pressure in Bentonite-Based Buffer and Backfill Materials. POSIVA Report 2000-04, Finland.
- Dixon, D.A., N.A. Chandler and P. Baumgartner. (2002). The Influence of Groundwater Salinity and Influences on the Performance of Potential Backfill Materials. In *Proceedings of the 6th International Workshop on Design and Construction of Final Repositories, Backfilling in Radioactive Waste Disposal*, Brussels, Belgium, 11-13 March 2002.
- Dong, H. (2012). Clay-Microbe Interactions and Implications for Environmental Mitigation. *Elements* 8:113-118.
- Drake, J.M. and A.M. Kramer. (2011). Mechanistic Analogy: How Microcosms Explain Nature. *Theoretical Ecology* 5(3): 433-444.
- Duarte, S., F. Cassio, and C. Pascoal. (2012). Denaturing Gradient Gel Electrophoresis (DGGE) in Microbial Ecology – Insights from Freshwaters. In *Gel Electrophoresis – Principles and Basics*, S. Magdeldin (ed). InTech Chapters. <http://www.intechopen.com/books/gel-electrophoresis-principles-and-basics>.
- Dubois, M., K.A. Gilles, J.K. Hamilton, P.A. Rebers and F. Smith. (1956). Colorimetric Method for Determination of Sugars and Related Substances. *Analytical Chemistry* 28: 350-356.
- Dueck, A. (2010). Thermo-Mechanical Cementation Effects in Bentonite Investigated by Unconfined Compression Tests. SKB Technical Report TR-10-41, Svensk Kärnbränslehantering AB.

- EC. (2004). Geological Disposal of Radioactive Wastes Produced by Nuclear Power from Concept to Implementation. European Commission Community Research EUR-21224. European Commission.
- Ehrlich, H.L. (1999). Microbes as Geologic Agents: Their Role in Mineral Formation. *Geomicrobiology Journal* 16(2): 13153.
- Ehrlich, H.L. and D.K. Newman. (2009). *Geomicrobiology of Fossil Fuels in Geomicrobiology Fifth Edition*. Taylor and Francis Group, LLC. Florida, USA. p. 139-156.
- Ekendahl, S., J. Arlinger, and K. Pendersen. (1994). Characterization of Attached Bacterial Populations in Deep Granitic Groundwater from the Stripa Research Mine by 16s rRNA Gene Sequencing and Scanning Electron Microscopy. *Microbiology* 140:1575-1583.
- Elo, S., I. Suominen, P. Kampfer, J. Juhanaja, M. Salkinoja-Salonen and K. Haahtela. (2001). *Paenibacillus borealis* sp. nov., a Nitrogen-Fixing Species Isolated from Spruce Forest Humus in Finland. *International Journal of Systematic and Evolutionary Microbiology* 51: 535–545.
- Enning, D. and J. Garrelfs. (2014). Corrosion of Iron by Sulfate-Reducing Bacteria: New Views of an Old Problem. *Applied and Environmental Microbiology* 80(4): 1226-1236.
- Evans, P., J. Smith, M.W. LeChevallier, O.D. Schneider, L.A. Weinrich, and P.K. Jemba. (2012). Assessing and Enhancing Biological Filtration [Project #4231].
- Evans, A.N. (2013). The Effect of Nutrient Limitations on the Production of Extracellular Polymeric Substances by Drinking-Water Bacteria. Master's Thesis, University of Texas, Austin, TX, USA.
- Ferguson, R. L., E. N. Buckley, and A. V. Palumbo. (1984). Response of Marine Bacterioplankton to Differential Filtration and Confinement. *Applied Environmental Microbiology* 47:49–55.
- Fisher, J.B. (1987). Distribution and Occurrence of Aliphatic Acid Anions in Deep Subsurface waters. *Geochimica et Cosmochimica Acta* 51: 2459-2468.
- Foster, M.D. (1954). The Relation Between Composition and Swelling in Clays. *Clays and Clay Minerals* 3 (1): 205-220.
- Francis, A.J. (2006). Microbial Transformations of Radionuclides Released from Nuclear Fuel Reprocessing Plants. International Symposium on Environmental Modelling and Radioecology, October 18-20, Japan.
- Francis, A.J., J.B. Gillow, C.J. Dodge, R. Harris, T.J. Beveridge and H.W. Papenguth. (2004). Association of Uranium with Halophilic and Nonhalophilic Bacteria and Archaea. *Radiochimica Acta* 92: 481-488.
- Fredrickson, J.K. and D.L. Balkwill. (2006). Geomicrobial Processes and Biodiversity in the Deep Terrestrial Subsurface. *Geomicrobiology Journal* 23: 345-356.
- Fredrickson J.K., J.P. McKinley, B.N. Bjornstad, D.B. Ringelberg, D.C. White, L.R. Krumholz, J.M. Suflita, F.S. Colwell, R.M. Lehman and T.J. Phelps. (1997). Poresize Constraints on the Activity and Survival of Subsurface Bacteria in a Late Cretaceous Shale-Sandstone Sequence, Northwestern New Mexico. *Geomicrobiology Journal* 14:183–202.

- Fry, N.K., J.K. Fredrickson, S. Fishbain, M. Wagner and D.A. Stahl. (1997). Population Structure of Microbial Communities Associated with Two Deep, Anaerobic, Alkaline Aquifers. *Applied Environmental Microbiology* 80:1498-1504.
- Ganesh, R., K.G. Robinson, G.D. Reed, and G.S. Sayler. (1997). Reduction of Hexavalent Uranium from Organic Complexes by Sulfate and Iron-Reducing Bacteria. *Applied and Environmental Microbiology* 63: 4385-4391.
- Garisto, F., D.H. Barber, E. Chen, A. Inglot and C.A. Morrison. (2009). Alpha, Beta and Gamma Dose Rates in Water in Contact with Used CANDU Fuel. Nuclear Waste Management Organization, Toronto, Canada NWMO TR-2009-27.
- Garland, W.J. (2003). How and Why is CANDU Designed the Way It Is? CANDU Owners Group Inc. Retrieved from http://www.candu.org/pdf/how_reactor_works.pdf
- Gascoyne, M., C.C. Davison, J.D. Ross, and R. Pearson. (1987). Saline Groundwaters and Brines in Plutons in the Canadian Shield. In *Saline Water and Gases in Crystalline Rocks* (eds. P. Fritz and S.K. Frape). Geological Association of Canada Spec. Paper No. 33, p. 53-68.
- Gonzalez-Pastor, J.E., E.C. Hobbs, and R. Losick. (2003). Cannibalism by Sporulating Bacteria. *Science* 301: 510–513.
- Goto, K., R. Fujita, Y. Kato, M. Asahara and A. Yokota. (2004). Reclassification of *Brevibacillus brevis* Strains NCIMB 13288 and DSM 6472 (=NRRL NRS-887) as *Aneurinibacillus danicus* sp. nov. and *Brevibacillus limnophilus* sp. nov. *International Journal of Systematic and Evolutionary Microbiology* 54: 419–427.
- Graham, J., R. Plant, J. Small and D. Smalley. (2003). Program User's Guide for the code GRM, Version 4.1. BNFL Report 00/EN0127/7/1. 2002 Post Closure Safety Case Report DTP/150.
- Grigoryan, A.A., S.L. Cornish, B. Buziak, S. Lin, A. Cavallaro, J.J. Arensdorf, and G. Voordouw. (2008). Competitive Oxidation of Volatile Fatty Acids by Sulfate- and Nitrate-Reducing Bacteria from an Oil Field in Argentina. *Journal of Applied and Environmental Microbiology* 74: 4324-35.
- Guitierrez, O., D. Park, K.R. Sharma and Z. Yuan. (2009). Effects of Long-Term pH Elevation on the Sulfate-Reducing and Methanogenic Activities of Anaerobic Sewer Biofilms. *Water Research* 43(9): 2549-2557.
- Hahn, O. and F. Strassman. (1964). Über den Nachweis und das Verhalten der bei der Bestrahlung des Urans mittels Neutronen entstehenden Erdalkalimetalle. *Naturwissenschaften* 27, 11-15 (1939). An English translation appears in Graetzer, H.G.: Discovery of Nuclear Fission *American Journal of Physics*, 32:9-15. In English, the title of Hahn and Strassman's paper is "Concerning the Existence of Alkaline Earth Metals Resulting from Neutron Irradiation of Uranium".
- Hallbeck, L. (2009). Microbial Processes in Glaciers and Permafrost: A Literature Study on Microbiology Affecting Groundwater at Ice Sheet Melting. Swedish Nuclear Fuel Waste Management Company, Stockholm, Sweden. SKB TR-09-37
- Hallbeck, L. and K. Pedersen. (2008). Explorative Analyses of Microbes, Colloids and Gases. SDM-Site Forsmark. Swedish Nuclear Fuel and Waste Management Company (SKB), Stockholm, Sweden. SKB TR-08-85

- Hama, K., K. Bateman, O. Coombs, V.L. Hards, A.E. Milodowski, J.M. West JM, P.D. Wetton, H. Yoshida and K. Aoki. (2001). Influence of Bacteria on Rock-Water Interaction and Clay Mineral Formation in Subsurface Granitic Environments. *Clay Mineralogy* 36: 599–613.
- Hamilton, W.A. (1985). Sulfate-Reducing Bacteria and Anaerobic Corrosion. *Annual Reviews in Microbiology* 39: 195-217.
- Handelsman, J. (2004) Metagenomics: Application of Genomics to Uncultured Microorganisms. *Microbiology and Molecular Biology Reviews* 68: 669–685.
- Harris, R.H. and R. Mitchell. (1973). The Role of Polymers in Microbial Aggregation. *Annual Reviews in Microbiology* 27: 27-50.
- Haveman, S.A. and K. Pedersen. (2002). Microbially Mediated Redox Processes in Natural Analogues for Radioactive Waste. *Journal of Contaminant Hydrology* 55: 161-174.
- Haveman, S.A., K. Pedersen and P. Ruotsalainen. (1999). Distribution and Metabolic Diversity of Microorganisms in Deep Igneous Rock Aquifers of Finland. *Geomicrobiology Journal* 16: 277–294.
- Haydel, S.E., C.M. Remenih and L.B. Williams. (2008). Broad-Spectrum *In Vitro* Antibacterial Activities of Clay Minerals against Antibiotic-Susceptible and Antibiotic-Resistant Bacterial Pathogens. *The Journal of Antimicrobial Chemotherapy* 61(2): 353-361.
- Hedin, A. (2006). Safety Function Indicators in SKB's Safety Assessments of a KBS-3 Repository. Proc. International High-Level Radioactive Waste Management Conference, Las Vegas, NV, April 30–May 04, 2006.
- Hendricks S.B. (1940). Base Exchange of the Clay Mineral Montmorillonite for Organic Cations and its Dependence upon Adsorption due to van der Waals Forces. Presented at the Seventeenth Colloid Symposium, held at Ann Arbor, Michigan, June 6-8, 1940. Pp. 75-81.
- Hersman, L.E. (1997). Subsurface Microbiology: Effects on the Transport of Radioactive Wastes in the Vadose Zone. In *The Microbiology of the Terrestrial Deep Subsurface* (eds. P.S. Amy and D.L. Haldeman). CRC Lewis Publishers, New York, pp. 290–324.
- Heyrman, J., B. Vanparys, N.A. Logan, A. Balcaen, M. Rodriguez-Diaz, A. Felske, and P. De Vos. (2004). *Bacillus novalis* sp. nov., *Bacillus vireti* sp. nov., *Bacillus soli* sp. nov., *Bacillus bataviensis* sp. nov. and *Bacillus drentensis* sp. nov., from the Drentse A Grasslands. *International Journal of Systematic and Evolutionary Microbiology* 54(Pt 1): 47-57.
- Hirkala, D.L.M. and J.J. Germida. (2004). Field and Soil Microcosm Studies on the Survival and Conjugation of a *Pseudomonas putida* Strain Bearing a Recombination Plasmid, pADPTel. *Canadian Journal of Microbiology* 50: 595–604.
- Hoehler, T.M. and B. B. Jørgensen. (2013). Microbial Life Under Extreme Energy Limitation. *Nature Reviews Microbiology* 11: 83-94.
- Hofstetter, Th.B., Y. Sosedova, C. Gorski, A. Voegelin and M. Sander. (2014). Redox Properties of Iron-Bearing Clays and MX-80 Bentonite – Electrochemical and Spectroscopic Characterization. National Cooperative for the Disposal of Radioactive Waste, Switzerland. NAGRA Technical Report 13-03.

Huang, Q., H. Wu, P. Cai, J.B. Fein and W. Chen. (2015). Atomic Force Microscopy Measurements of Bacterial Adhesion and Biofilm Formation onto Clay-Sized Particles. *Nature Scientific Reports* 5(16857): 1-12.

Humphreys, P.N., J.M. West and R. Metcalfe. (2010). Microbial Effects on Repository Performance. Prepared by Quintessa Ltd. for the Nuclear Decommissioning Authority, Oxfordshire, United Kingdom. Report No. QRS-1378Q-1.

IAEA. (2001a). Monitoring of Geological Repositories for High Level Radioactive Waste. International Atomic Energy Agency, Vienna, Austria. IAEA-TECDOC-1208.

IAEA. (2001b). The Use of Scientific and Technical Results from Underground Research Laboratory Investigations for the Geological Disposal of Radioactive Waste. International Atomic Energy Agency, Vienna, Austria. IAEA-TECDOC-1243.

IAEA. (2006). Safety Standard Report for Geological Disposal: Geological Disposal of Radioactive Waste, Safety Requirements, IAEA Safety Standards Series No. WS-R-4, Vienna, Austria. International Atomic Energy Agency. Retrieved from http://www-pub.iaea.org/MTCD/publications/PDF/Pub1231_web.pdf

Ismadji, S. F.E. Soetaredjo and A. Ayucitra. (2015). Natural Clay Minerals as Environmental Cleaning Agents. In *Clay Materials for Environmental Remediation*, SpringerBriefs in Green Chemistry for Sustainability, pp 5-57.

Itävaara, M., M.-L. Vehkomäki and A. Nousiainen. (December 2008). Sulphate-Reducing Bacteria in Ground Water Samples from Olkiluoto – Analyzed by Quantitative PCR. Report POSIVA 2008-82.

Jaisi, D.P., H. Dong, J. Kim, Z. He and J.P. Morton. (2008). Nontronite Particle Aggregation Induced by Microbial Fe(III) Reduction and Exopolysaccharide Production. *Clay and Clay Minerals* 55(1): 96-107.

Janssen, P.H. (2006). Identifying the Dominant Soil Bacteria Taxa in Libraries of 16S rRNA Genes. *Applied Environmental Microbiology* 72(3): 1719-1728.

Jay, J.M. M.J. Loessner and D.A. Golden. (2005). *Modern Food Microbiology*. 7th Edition., Springer.

Jeffreys, A., V. Wilson, and S. Thein. (1985a). Hypervariable minisatellite Regions in Human DNA. *Nature* 314 (6006): 67-73.

Jeffreys, A., V. Wilson, and S. Thein. (1985b). Individual-Specific 'Fingerprints' of Human DNA. *Nature* 316 (6023): 76–79.

Jones, J.G. (1977). The Effect of Environmental Factors on Estimated Viable and Total Populations of Planktonic Bacteria in Lakes and Experimental Enclosures. *Freshwater Biology* 7: 67-91.

Karland, O., S. Olsson and U. Nilsson. (2006). Mineralogy and Sealing Properties of Various Bentonites and Smectite-Rich Clay Materials. . Swedish Nuclear Fuel and Waste Management Company Report, SKB TR 06-30.

Karland, O. (2010). Chemical and Mineralogical Characterization of the Bentonite Buffer for the Acceptance Control Procedure in a KBS-3 Repository. SKB Technical Report TR-10-60.

- Kelly, S.D., I.G. Parker, M. Sharman and M.J. Dennis. (1998). On-line Quantitative Determination of 2H/1H Isotope Ratios in Organic and Water Samples Using an Elemental Analyser Coupled to an Isotope Ratio Mass Spectrometer. *Journal of Mass Spectrometry* 33: 735-738.
- Kennedy, J., N.D. O'Leary, G.S. Kiran, J.P. Morrissey, F. O'Gara, J. Selvin, and A.D.W. Dobson. (2011). Functional Metagenomic Strategies for the Discovery of Novel Enzymes and Biosurfactants with Biotechnological Applications from Marine Ecosystems. *Journal of Applied Microbiology* 111(4): 787-799.
- Khan, F.W. (2012). Engineering Properties of Badlands in Semi-Arid Regions. Master's Thesis, University of Regina, Saskatchewan, Canada.
- Kieft, T.L., W.P. Kovacik, D.B. Ringelberg, D.C. White, D.L. Haldeman, P.S. Amy and L.E. Hersman. (1997). Factors Limiting Microbial Growth and Activity at a Proposed High-Level Nuclear Repository, Yucca Mountain, Nevada. *Applied and Environmental Microbiology* 63(8): 3128-3133.
- Kim, J., Y. Furukawa, T. Daulton and S.W. Newell. (2004). Characterization of Microbially Fe(III)-Reduced Nontronite: Environmental Cell-Transmission Electron Microscope Study. *Clay and Clay Minerals* 51(4): 382-389.
- King, F. (2006a). OPG's Deep Geologic Repository for Low and Intermediate Level Radioactive Waste: Recent Progress. Topseal. Lecture presented at Olkiluoto, Finland. Retrieved from <http://www.euronuclear.org/events/topseal/presentations/PP-Session-IV-King.pdf>
- King, F. (2006b). Review and Gap Analysis of the Corrosion of Copper Containers under Unsaturated Conditions. Ontario Power Generation, Toronto, Ontario, Canada Report No. 06819-REP-01300-10124-R00.
- King, F. (2007). Overview of a Carbon Steel Container Corrosion Model for a Deep Geological Repository in Sedimentary Rock. Nuclear Waste Management Organization, Toronto, Canada NWMO TR-2007-01.
- King, F., M. Kolar and D.W. Shoesmith. (1996). Modelling the Effects of Porous and Semi-Permeable Layers on Corrosion Processes. Corrosion/96, NACE International, Houston, TX, paper no. 380.
- King F., L. Ahonen, C. Taxen, U. Vuorinen and L. Werme. (2002). Copper Corrosion Under Expected Conditions in a Deep Geologic Repository. Svensk Kärnbränslehantering AB Technical Report TR-01-23.
- Kjelleberg, S. (1984). Adhesion to Inanimate Surfaces. In *Microbial Adhesion and Aggregation* (ed. K.C. Marshall). New York: Springer-Verlag, pp. 51-67.
- Kjelleberg, S. and M. Hermansson. (1984). Starvation-Induced Effects on Bacterial Surface Characteristics. *Applied and Environmental Microbiology* 48(3): 497-503.
- Klausen, M., A. Aaes-Jørgensen, S. Molin, and T. Tolker-Nielsen. (2003). Involvement of Bacterial Migration in the Development of Complex Multicellular Structures in *Pseudomonas aeruginosa* Biofilms. *Molecular Microbiology* 50(1): 61-68.

- Kleb, H. (2013, September). OPG's Deep Geologic Repository. Joint Panel Hearing on OPG's Proposed Deep Geologic Repository. Formal presentation presented at Canadian Nuclear Association, Canada. Retrieved from <http://www.cna.ca/2013/09/20/speaking-notes-heather-kleb-interim-president-ceo-joint-review-panel-hearing-opgs-proposed-deep-geologic-repository/>
- Koch, A.L. (1994). Growth measurement. In *Methods for General and Molecular Bacteriology* (eds. Gerhardt, P., Murray, R.G.E., Wood, W.A. and Krieg, N.R.). Washington, DC: American Society for Microbiology, pp. 248-277.
- Koch, A.L. (2001). Oligotrophs Versus Copiotrophs. *Bioessays* 23:657-661.
- Kostka, J.E., E. Haefele, R. Viehweger and J.W. Stucki. (1999). Respiration and Dissolution of Iron(III)-Containing Clay Minerals by Bacteria. *Environmental Science and Technology* 33: 3127-3133.
- Kotelnikova, S., J.L. Macario and K. Pedersen. (1998). *Methanobacterium subterraneum* sp. nov., A New Alkaliphilic, Eurythermic and Halotolerant Methanogen Isolated from Deep Granitic Groundwater. *International Journal of Systematic Bacteriology* 48:357-367.
- Kozak, J.J. and J.B. Mandeville. (1995). Influence of Swelling on Reaction Efficiency in Intercalated Clay Minerals. 3. Ion-Dipole Reactant Pairs. *Journal of Physical Chemistry* 99: 12933-12940.
- Kristensen, E. (2000). Organic Matter Diagenesis at the Oxic/Anoxic Interface in Coastal Marine Sediments, with Emphasis on the Role of Burrowing Animals. In *Life at Interfaces and Under Extreme Conditions* (eds. G. Liebezeit, S. Dittmann and I. Kröncke). Kluwer Academic Publishers, Netherlands.
- Kulp, T.R., S. Han, C.W. Saltikov, B.D. Lanoil, K. Zargar and R.S. Oremland. (2007). Effects of Imposed Salinity Gradients on Dissimilatory Arsenate Sulfate Reduction, and Other Microbial Processes in Sediments from Two California Soda Lakes. *Applied and Environmental Microbiology* 73 (16): 5130-5137.
- Kuramae, E.E., E. Yergeau, L.C. Wong, A.S. Pijl, J.A. van Veen and G.A. Kowalchuk. (2012). Soil Characteristics More Strongly Influence Soil Bacterial Communities than Land-use Type. *FEMS Microbiol. Ecol.* 79: 12-24.
- Kwong, G.M. (2011). Status of Corrosion Studies for Copper Used Fuel Containers Under Low Salinity Conditions. Nuclear Waste Management Organization, Ontario, Canada TR-2011-14
- Laloui, L. B. Francois, M. Nuth, H. Peron and A. Koliji. (2008). A Thermo-Hydro-Mechanical Stress-Strain Framework for Modelling the Performance of Clay Barriers in Deep Geological Repositories for Radioactive Waste. In *Unsaturated Soils: Advances in Geo-Engineering* (eds., Toll *et al.*). Taylor and Francis Group, London. Pp. 63-80.
- Lawrence, J.R., M.J. Hendry, L.I. Wassenaar, J.J. Germida, G.M. Wolfaardt, N. Fortin and C.W. Greer. (2000). Distribution and Biogeochemical Importance of Bacterial Populations in a Thick Clay-Rich Aquitard System. *Microbiology Ecology* 40:273-291.
- Lawrence, J.R., B. Zhu, G.D.W. Swerhone, E. Topp, J. Roy, L.I. Wassenaar, T. Rema and D.R. Korber. (2008). Community-Level Assessment of the Effects of the Broad-Spectrum Antimicrobial Chlorhexidine on the Outcome of River Microbial Biofilm Development. *Applied and Environmental Microbiology* 74(11): 3541-3550.

- Leech, R. and R.J.W. Hefford. (1980). The Observation of Bacterial Deposition from Flowing Suspension. In *Microbial Adhesion to Surfaces* (eds. Berkeley, R.C.W., et al.) Chichester, West Sussex: Horwood, pp. 544-545.
- Leff, L.G., J.R. Dana, J.V. McArthur, and L.J. Shimkets. (1995). Comparison of Methods of DNA Extraction from Stream Sediments. *Applied Environmental Microbiology* 61: 1141-1143.
- Libert, M., O. Bildstein, L. Esnault, M. Jullien and R. Sellier. (2011). Molecular Hydrogen: An Abundant Energy Source of Bacterial Activity in Nuclear Waste Repositories. *Physics and Chemistry of the Earth* 36: 1616-1623.
- Liu, D., P.T.S Wong, and B.J. Dutka. (1973). Determination of Carbohydrate in Lake Sediment by a Modified Phenol-Sulfuric Acid Method. *Water Research* 7:741-746.
- Liu, X-Y., B-J. Wang, C-Y. Jiang and S-J. Liu. (2008). *Ornithinimicrobium pekingense* sp. nov., Isolated from Activated Sludge. *International Journal of Systematic and Evolutionary Microbiology* 58: 116-119.
- Little, B. and P. Wagner. (1996). An Overview of Microbiologically Influenced Corrosion of Metals and Alloys Used in the Storage of Nuclear Wastes. *Canadian Journal of Microbiology*, 42: 367–374.
- Little, B.J. and J.S. Lee. (2007). Causative Organisms and Possible Mechanisms in Microbiologically Influenced Corrosion, R. Winston Review (Ed.). John Wiley & Sons, Inc. New Jersey, USA. Pp. 22-55.
- Little, B., P. Wagner and F. Mansfeld. (1991). Microbiologically Influenced Corrosion of Metals and Alloys. *International Materials Reviews* 36: 253-272.
- Lloyd, J.R., J. Chesnes, S. Glasauer, D.J. Bunker, F.R. Livens and D.R. Lovley. (2002) Reduction of Actinides and Fission Products by Fe(III) Reducing Bacteria. *Geomicrobiology Journal* 19: 103–120.
- LLRWMO. (2012). Inventory of Radioactive Waste in Canada. Low-Level Radioactive Waste Management Office, Ottawa, Canada LLRWMO-01613-041-10003, CC3-1/2012, 978-1-100-54191-4.
- López-Fernández, M., O. Fernández-Sanfrancisco, A. Moreno-García, I. Martín-Sánchez, I. Sánchez-Castro and M.L. Merroun. (2014). Microbial Communities in Bentonite Formations and their Interactions with Uranium. *Applied Geochemistry* 49: 77-86.
- Lovering, J.R. A. Yip and T. Nordhaus. (2016). Historical Construction Costs of Global Nuclear Power Reactors. *Energy Policy* 91: 371-382.
- Lovley, D.R., E.J.P. Philips, Y.A. Gorby, and E.R. Landa. (1991). Microbial Reduction of Uranium. *Nature* 350: 413-416.
- Lovley, D.R. and F.H. Chapelle. (1995). Deep Subsurface Microbial Processes. *Reviews of Geophysics* 33(3): 365-381.
- Luna, G.M., A. Dell’Anno, and R. Danovaro. (2006). DNA Extraction Procedure: A Critical Issue for Bacterial Diversity Assessment in Marine Sediments. *Environmental Microbiology* 8: 308-320.

- Lütke, L., H. Moll, V. Bachvarova, S. Selenska-Pobell and G. Bernhard. 2013. The U(VI) Speciation Influenced by a Novel *Paenibacillus* Isolate from Mont Terri Opalinus Clay. *Dalton Transactions* 42: 6979-6988.
- Maak, P. (1999). The Selection of a Corrosion-Barrier Primary Material for Used-Fuel Disposal containers. Ontario Power Generation, Nuclear Waste Management Division Report 06819-REP-01200-10020-R00.
- Maak, P., K. Birch and G.R. Simmons. (2010). Evaluation of Container Placement Methods for the Conceptual Design of a Deep Geological Repository. Nuclear Waste Management Organization Report, NWMO TR-2010-20, Toronto, ON.
- Maarit, N.R., I. Heiskanen, K. Wallenius, and K. Lindstrom. (2001). Extraction and Purification of DNA in Rhizosphere Soil Samples for PCR-DGGE Analysis of Bacterial Consortia. *Journal of Microbiological Methods* 45(3): 155-165.
- MacLeod, F.A., S.R. Guiot and J.W. Costerton. (1995). Electron Microscopic Examination of the Extracellular Polymeric Substances in Anaerobic Granular Biofilms. *World Journal Microbiology and Biotechnology* 11: 481-485.
- Manisavagan, P. P. Sivasankar, J. Venkatesan, K. Senthilkumar, K. Sivakumar and S-K. Kim. (2013). Production and Characterization of an Extracellular Polysaccharide from *Streptomyces violaceus* MM72. *International Journal of Biological Macromolecules* 59: 29-38.
- Marchandin, H., C. Teyssier, J. Campos, H. Jean-Pierre, F. Roger, B. Gay, J.-P. Carlier, E. Jumas-Bilak. (2009). *Negativicoccus succinicivorans* gen. Nov., sp. Nov., Isolated from Human Clinical Samples, Emended Description of the Family *Veillonellaceae* and Description of *Negativicutes* classis nov., *Selenomonadales* ord. Nov. and *Acidaminococcaceae* fam. Nov. In the Bacterial Phylum *Firmicutes*. *International Journal of Systematic and Evolutionary Microbiology* 60(6): 1271–1279.
- Margulies, M., M. Egholm, W.E. Altman, S. Attiya, J.S. Bader, L.A. Bemben, J. Berka, M.S. Braverman, Y.-J. Chen, Z. Chen, S.B. Dewell, L. Du, J.M. Fierro, X.V. Gomes, B.C. Godwin, W. He, S. Helgesen, C. He Ho, G.P. Irzyk, S.C. Jando, M.L.I. Alenquer, T.P. Jarvie, K.B. Jirage, J.-B. Kim, J.R. Knight, J.R. Lanza, J.H. Leamon, S.M. Lefkowitz, M. Lei, J. Li, K.L. Lohman, H. Lu, V.B. Makhijani, K.E. McDade, M.P. McKenna, E.W. Myers, E. Nickerson, J.R. Nobile, R. Plant, B.P. Puc, M.T. Ronan, G.T. Roth, G.J. Sarkis, J.F. Simons, J.W. Simpson, M. Srinivasan, K.R. Tartaro, A. Tomasz, K. A. Vogt, G.A. Volkmer, S.H. Wang, Y. Wang, M.P. Weiner, P. Yu, R.F. Begley and J.M. Rothberg. (2005). Genome Sequencing in Microfabricated High-Density Picolitre Reactors. *Nature* 437:376-380.
- Marmur, J. (1961). A Procedure for the Isolation of Deoxyribonucleic Acid from Microorganisms. *Journal of Molecular Biology* 3: 208-218.
- Marshall, K.C. (1975). Clay Mineralogy in Relation to Survival of Soil Bacteria. *Annual Review of Phytobiology* 13: 357-373.
- Marshall, M.H.M. and M.J. Simpson. (2014). Characterization of Natural Organic Matter in Bentonite Clays. NWMO TR-2014-10.
- Marshall, K.C., R. Stout and R. Mitchell. (1971). Mechanism of the Initial Events in the Sorption of Marine Bacteria to Surfaces. *Journal of Genetic Microbiology* 68:337-348.

- Masurat, P., S. Eriksson and K. Pedersen. (2010a). Evidence of Indigenous Sulfate-Reducing Bacteria in Commercial Wyoming bentonite MX-80. *Applied Clay Science* 47: 51-57.
- Masurat, P., S. Eriksson and K. Pedersen. (2010b). Microbial Sulphide Production in a Compacted Wyoming Bentonite MX-80 under *in situ* Conditions Relevant to a Repository for High-Level Radioactive Waste. *Applied Clay Science* 47: 58-64.
- Mata, C. and A. Ledesma. (2005). Hydro-chemical Behaviour of Bentonite – Based Mixtures in Engineered Barriers. In *Advances in Understanding Clay Barriers* (eds. E.E. Alonso and A. Ledesma). Taylor & Francis Group, London. p. 231-240.
- Mauclaire, L, J.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 Terry Rock Formation). *Physics and Chemistry of the Earth* 32:232-240.
- McCabe, A. (1990). The potential significance of microbial activity in radioactive wasdisposal. *Experientia* 46, 779.
- McMurry, J., D.A. Dixon, J.D. Garroni, B.M. Ikeda, S. Stroes-Gascoyne, P. Baumgartner and T.W. Melnyk. (2003). Evolution of Canadian Deep Geologic Repository: Base Scenario. AECL Report No.: 06819-REP-01200-100920R00.
- McWilliam, H., W. Li, M. Uludag, S. Squizzato, Y.M. Park, N. Buso, A.P. Cowley and R. Lopez. (2013). Analysis Tool Web Services from the EMBL-EBI. *Nucleic Acids Research* 41(W1): 597-600.
- Medihala, P.R., J.R. Lawrence, G.D.W. Swerhorne and D.R. Korber. (2012). Effect of Pumping on the Spatio-Temporal Distribution of Microbial Communities in a Water Well Field. *Water Research* 46(4): 1286-1300.
- Meleshyn, A. (2014). Microbial Processes Relevant for Long-Term Performance of Radioactive Waste Repositories in Clays. In *Clays in Natural and Engineered Barriers of Radioactive Waste Confinement*, Special Publication – Geological Society of London 400(1): 179-194.
- Michaelsen, A., F. Pinzari, K. Ripka, W. Lubitz, and G. Piňar. (2006). Application of Molecular Techniques for Identification of Fungal Communities Colonizing Paper Material. *International Biodeterioration and Biodegradation* 58: 133-141.
- Min, M., H. Xu, J. Chen and M. Fayek. (2005). Evidence of Uranium Biomineralization in Sandstone-Hosted Roll-Front Uranium Deposits, Northwestern China. *Ore Geology Reviews* 26(3-4): 198-206.
- Moll, H., A. Johnsson, M. Schäfer, K. Pedersen, H. Budzikiewicz and G. Bernhard. (2008). Curium (III) Complexation with Pyoverdins Secreted by a Groundwater Strain of *Pseudomonas fluorescens*. *Biometals* 21(2): 219-228.
- Moll, H., L. Lütke, V. Bachvarova, R. Steudner, A. Geißler, E. Krawczyk-Bärsch, S. Selenska-Pobell and G. Bernhardt. (2013). Microbial Diversity in Opalinus Clay and Interaction of Dominant Microbial Strains with Actinides. Helmholtz Zentrum Dresden Rossendorf, Wissenschaftlich-Technische Berichte, Final Report HZDR-036.
- Moll, H., L. Lütke and A. Cherkouk. (2015). Bacterial Diversity in Clay and Actinide Interactions with Bacterial Isolates in Relation to Nuclear Waste Disposal. In *Radionuclides in*

- the Environment* (eds. C. Walther and D.K. Gupta). Springer International Publishing, Switzerland, pp. 209-230.
- Mortland, M.M. (1986). Mechanism of Adsorption of Nonhumic Organic Species by Clays. In *Interactions of Soil Minerals with Natural Organics and Microbes SSSA Special Publication Number 17*, (eds. P.M. Huang and M. Schnitzer). Soil Science Society of America Inc., USA. Pp 59-76.
- Mostafa, R., M. Uhlen and P. Nyren. (1998). A Sequencing Method Based on Real-Time Pyrophosphate. *Science* 281(5375): 363-365.
- Motamedi, M., O. Karland and K. Pedersen. (1996). Survival of Sulfate Reducing Bacteria at Different Water Activities in Compacted Bentonite. *FEMS Microbiology Letters* 141: 83-87.
- Mulligan, C.N., R.N. Yong and M. Fukue. (2009). Some Effects of Microbial Activity on the Evolution of Clay-Based Buffer Properties in Underground Repositories. *Applied Clay Science* 42:331-335.
- Muyzer G, and K. Smalla. (1998). Application of Denaturing Gradient Gel Electrophoresis (DGGE) and Temperature Gradient Gel Electrophoresis (TGGE) in Microbial Ecology. *Antonie van Leeuwenhoek* 73:127-141
- Muyzer G, A.G. Uitterlinden, and E.C. De Waal. (1993). Profiling of Complex Microbial Populations by Denaturing Gradient Gel Electrophoresis Analysis of Polymerase Chain Reaction-Amplified Genes Coding for 16S rRNA. *Applied Environmental Microbiology* 59: 695-700.
- Muyzer ,G.; T. Brinkoff, U. Nübel, C. Santegoeds, H. Schäfer, and C. Wawer. (2004). Denaturing Gradient Gel Electrophoresis (DGGE) in Microbial Ecology. In *Molecular Microbial Ecology Manual* (2nd ed.) (eds. G.A. Kowalchuk, F.J. de Bruijn, I.M. Head, A.D.L. Akkermans, and J.D. van Elsas). Kluwer Academic Publishers, ISBN 978-1-4020-4860-9, Dordrecht, The Netherlands, pp. 743-770.
- Nakatsu, C.H., V. Torsvik, V. and L. Øvreas. (2000). Soil Community Analysis using DGGE of 16S rDNA Polymerase Chain Reaction Products. *Soil Science Society of America Journal* 64: 1382-1388.
- Nakamura, L.K. (1987). *Bacillus alginolyticus* sp. nov. and *Bacillus chondroitinus* sp. nov., Two Alginate-Degrading Species. *International Journal of Systematic Bacteriology* 37: 284-286.
- Neu, M.P., G.A. Icopini and H. Boukhalfa. (2005). Plutonium Speciation Affected by Environmental Bacteria. *Radiochimica Acta* 93: 705-714.
- Newby, D.T., D.W. Reed, L.M. Petzke, A.L. Igoe, M.E. Delwiche, F.F. Roberto, J.P. McKinley, M.J. Whiticar and F.S. Colwell. (2004). Diversity of Methanotroph Communities in a Basalt Aquifer. *FEMS Microbiology Ecology* 48: 333-344.
- Newsome, L., K. Morris and J.R. Lloyd. (2014). The Biogeochemistry and Bioremediation of Uranium and Other Priority Radionuclides. *Chemical Geology* 363: 164-184.
- Nicholas, K.B., H.B. Nicholas Jr. and D.W. Deerfield. (1997). GeneDoc: Analysis and Visualization of Genetic Variation, *EMBNEW.NEWS* 4,14.

- Nguyen-Thanh, D. and T.J. Bandosz. (2003). Effect of Transition-Metal Cations on the Adsorption of H₂S in Modified Pillared Clays. *Journal of Physical Chemistry B* 107: 5812-5817.
- Nguyen-Thanh, D., K. Block and T.J. Bandosz. (2005). Adsorption of Hydrogen Sulfide on Montmorillonites Modified with Iron. *Chemosphere* 59: 343-353.
- Nikolova, R., W. Powrie, P. Humphreys and D.J. Smallman. (2001). Performance of Leachate Drainage Systems, Proceedings Sardinia 2001, Eighth International Waste Management and Landfill Symposium., Margherita di Pula, Caligari, Italy, pp. 103-112.
- NWMO. (2005). Choosing a Way Forward. The Future Management of Canada's Used Nuclear Fuel. Nuclear Waste Management Organization, Toronto, Canada.
- NWMO. (2010). Multiple-Barrier System. Backgrounder, Nuclear Waste Management Organization, Toronto, Canada.
- NWMO. (2012). Adaptive Phased Management: Used Fuel Repository Conceptual Design and Postclosure Safety Assessment in Crystalline Rock – Pre-project Report. Nuclear Waste Management Organization, Toronto, Ontario. NWMO TR-2012-16
- Oblinger, J.L. and J.A. Koburger. (1975). Understanding and Teaching the Most Probable Number Technique. *Journal of Milk Food Technology* 38(9): 540-545.
- Ogram, A., G.S. Sayler and T. Barkay. (1987). The Extraction and Purification of Microbial DNA from Sediments. *Journal of Microbiological Methods* 7: 57-66.
- Ohnuki, T., N. Kozai, F. Sakamoto, T. Ozaki, T. Nankawa, Y. Suzuki and A.J. Francis. (2010). Association of Actinides with Microorganisms and Clay: Implications of Radionuclide Migration from Waste-Repository Sites. *Geomicrobiology Journal* 27:225-230.
- Onstott, T.C., E. van Heerden and L. Murdoch. (2010). Microbial Life in the Depths of the Earth. *Geosciences* 11: 52-59.
- OPG. (1981). Nuclear Definitions. Ontario Power Generation (Ontario Hydro) Public Brochure.
- Oremland, R.S. and S. Polcin. (1982). Methanogenesis and Sulfate Reduction: Competitive and Noncompetitive Substrates in Estuarine Sediments. *Applied Environmental Microbiology* 44(6): 1270-1276.
- Oren, A. (1999). Bioenergetic Aspects of Halophilism. *Microbiology and Molecular Biology Reviews* 63 (2): 334-348.
- Panak, P.J., C.H. Booth, D.L. Caulder, J.J. Bucher, D.K. Shuh and H. Nitsche. (2002). X-ray Absorption Fine Structure Spectroscopy of Plutonium Complexes with *Bacillus sphaericus*. *Radiochimica Acta* 90: 315-321.
- Pankhania, I.P., A.M. Spormann, W.A. Hamilton and R.K. Thauer. (1988). Lactate Conversion to Acetate, CO₂ and H₂ in Cell Suspensions of *Desulfovibrio vulgaris* (Marburg): Indications for the Involvement of an Energy Driven Reaction. *Archives of Microbiology* 150: 26–31
- Parkes, R.J., B.A. Cragg, and P. Wellsbury. (2000). Recent Studies on Bacterial Populations and Processes in Subseafloor Sediments: a Review. *Hydrogeology Journal* 8:11-28.

- Parnell, J. and S. McMahon. (2016). Physical and Chemical Controls on Habitats for Life in the Deep Subsurface Beneath Continents and Ice. *Philosophical Transactions of the Royal Society A* 374: 20140293.
- PEBS. (2012). The Early Evolution of the EBS in Safety Assessments: A PEBS WP1 Report. Long-Term Performance of Engineered Barrier Systems. Retrieved from http://www.pebs-eu.de/PEBS/EN/Downloads/D1_1_pdf.pdf?__blob=publicationFile&v=7
- Pecini, E.M. and M.J. Avena. (2013). Measuring the Isoelectric Point of the Edges of Clay Mineral Particles: The Case of Montmorillonite. *Langmuir* 29: 14926-14934.
- Pedersen, K. (1993). The Deep Subterranean Biosphere. *Earth-Science Reviews* 34: 243-260.
- Pedersen, K. (1999). Evidence for a Hydrogen-Driven, Intra-terrestrial Biosphere in Deep Granitic Rock Aquifers. In *Eighth International Symposium on Microbial Ecology*, pp. 335–341. (eds. C. R. Bell, M. Brylinsky and P. Johnson-Green). Halifax, NS: Canadian Society for Microbiology.
- Pedersen, K. (2000). Microbial Processes in Radioactive Waste Disposal. Swedish Nuclear Fuel and Waste Management Company Report, SKB TR 00-04.
- Pedersen, K. (2005). Microorganisms and Their Influence on Radionuclide Migration in Igneous Rock Environments. *Journal of Nuclear and Radiochemical Science* 6(1): 11-15.
- Pedersen, K. (2008). Numbers, Biomass and Cultivable Diversity of Microbial Populations Relate to Depth and Borehole-Specific Conditions in Groundwater from Depths of 4–450 m in Olkiluoto, Finland. *ISME Journal* 2: 760–775.
- Pedersen, K.A. 2010. Analysis of Copper Corrosion in Compacted Bentonite Clay as a Function of Clay Density and Growth Conditions for Sulfate-Reducing Bacteria. *Journal of Applied Microbiology* 108: 1094-1104.
- Pedersen, K. and F. Karlsson. (1995). Investigations of Subterranean Bacteria. Their Influence on Performance Assessment in Radiactive Waste Disposal. SKB Technical Report, 95-10, Swedish Nuclear Fuel and Waste Co., Stockholm.
- Pedersen, K., M. Motamedi and O. Karnland. (1995). Survival of Bacteria in Nuclear Waste Buffer Materials. The Influence of Nutrients, Temperature and Water Activity. SKB TR 95-27, Svensk Kärnbränslehantering AB.
- Pedersen, K., L. Hallbeck, J. Arlinger, A-C Erlandson, and N. Jahromi. (1997). Investigation of the Potential for Microbial Contamination of Deep Granitic Aquifers during Drilling Using 16s rRNA Gene Sequencing and Culturing Methods. *Journal of Microbiological Methods* 30:179-192.
- Pedersen, K., M. Motamedi, O. Karnland and T. Sandén. (2000a). Cultivability of Microorganisms Introduced into a Compacted Bentonite Clay Buffer under High-Level Radioactive Waste Repository Conditions. *Engineering Geology* 58: 149-161.
- Pedersen, K., M. Motamedi, O. Karnland and T. Sandén. (2000b). Mixing and Sulphate-Reducing Activity of Bacteria in Swelling, Compacted Bentonite Clay under High-level Radioactive Waste Repository Conditions. *Journal of Applied Microbiology* 89:1038-1047.

- Perdrial, J.N., L.N. Warr, N. Perdrial, M-C. Lett and F. Elsass. (2009). Interaction Between Smectite and Bacteria: Implications for Bentonite as Backfill Material in the Disposal of Nuclear Waste. *Chemical Geology* 264: 281-294.
- Plackett, R.L. and J.P. Burman. (1946). The Design of Optimum Multifactorial Experiments. *Biometrika* 66: 305-325.
- Posiva. (2009). Olkiluoto Site Description 2008, Part 1. Report POSIVA 2009-01.
- Postgate, J.R. (1984). The Sulfate Reducing Bacteria. Cambridge University Press, London.
- Poulain, S., C. Sergeant and M. Simonoff. (2008). Microbial Investigations in Opalinus Clay, an Argillaceous Formation under Evaluation as a Potential Host Rock for a Radioactive Waste Repository. *Geomicrobiology Journal* 25(5):240-249.
- Priyanto, D.G., J.A. Blatz, G.A. Siemens, R.B. Offman, J.S. Powell and D.A. Dixon. (2008). The Effects of Fluid Composition on the One-Dimensional Consolidation Behaviour of Clay-Based Sealing Materials. NWMO TR-2008-20. Nuclear Waste Management Organization, Canada.
- Priyanto. D.G. (November, 2013). Swelling Pressure of Long-Term Microbial Study (Specimen 2.0 DDW (9)). Unpublished document.
- Pusch, R. and R. Weston. (2003). Microstructural Stability Controls the Hydraulic Conductivity of Smectitic Buffer. *Applied Clay Science* 23:35-41.
- Rappe, M.S. and S.J. Giovannoni. (2003). The Uncultured Microbial Majority. *Annual Review of Microbiology* 57: 369-394.
- Ratto, M. and M. Itavaara. (2012). Microbial Activity in Bentonite Buffers – Literature Study. Espoo 2010. VTT Technology 20.
- Ravi, K. and S.M. Rao. (2013). Influence of Infiltration of Sodium Chloride Solutions on SWCC of Compacted Bentonite–Sand Specimens. *Geotechnical and Geological Engineering* 31:1291–1303.
- Reasoner, D.L., and E.E. Geldreich. (1985). A New Medium for the Enumeration and Subculture of Bacteria from Potable Water. *Applied Environmental Microbiology* 1: 1–7.
- Rickard, D. (2006). The Solubility of FeS. *Geochimica et Cosmochimica acta* 70(23): 5779-5789.
- Rochelle, P.A., J.C. Fry, R.J. Parkes and A.J. Weightman. (1992). DNA Extraction for 16S rRNA Gene Analysis to Determine Genetic Diversity in Deep Sediment Communities. *FEMS Microbiology Letters* 100(1): 59-65.
- Roden, E.E. and Q. Jin. (2011). Thermodynamics of Microbial Growth Coupled to Metabolism of Glucose, Ethanol, Short-Chain Organic Acids, and Hydrogen. *Applied Environmental Microbiology* 77(5): 1907-1909.
- Rong, X., Q. Huang, X. He, H. Chen, P. Cai and W. Liang. (2008). Interaction of *Pseudomonas putida* with Kaolinite and Montmorillonite: A Combination Study by Equilibrium Adsorption, ITC, SEM and FTIR. *Colloids and Surfaces B: Biointerfaces* 64: 49-55.
- Roose-Amsaleg, C.L., E. Garnier-Sillam and M. Harry. (2001). Extraction and Purification of Microbial DNA from Soil and Sediment Samples. *Applied Soil Ecology* 18: 47-60.

- Saitou, N. and M. Nei. (1987). The Neighbour-Joining Method: A Method for Reconstructing Phylogenetic Trees. *Molecular Biology Evolution* 4: 405-425.
- Sauzeat, E., T.F. Villieras, M. François, M. Pelletier, O. Barrés, J. Yvon, D. Guillaume, J. Dubbessy, C. Pfeiffert, R. Ruck and M. Cathelineau. (2001). Caractérisation Minéralogique, Cristalochimique et Texturale de l'argile MX-80, ANDRA Technical Report, France.
- Saylor, G. and A. C. Layton. (1990). Environmental Application of Nucleic Acid Hybridization. *Annual Review of Microbiology* 44:625-648.
- Schiller, N.L. S.R. Monday, C. Boyd, N.T. Keen and D.E. Ohman. (1993). Characterization of the *Pseudomonas* alginate Lyase Gene (algL): Cloning, Sequencing and Expression in *E. coli*. *Bacteriology Journal* 175: 780-789.
- Schlebeck, D., N. Barraud, J. Klenbensberger, J.S. Webb, D. McDougald and S.A. Rice. (2009). *Pseudomonas aeruginosa* PAO-1 Preferentially Grows as Aggregates in Liquid Batch Cultures and Disperses upon Starvation. *PLoS ONE* 4(5): e5513.
- Schneegurt, M.A., S.Y. Dore and C.F. Kulpa, Jr. (2003). Direct Extraction of DNA from Soils for Studies in Microbial Ecology. *Current Issues in Molecular Biology* 5: 1-8.
- Scully, J.R. and M. Edwards. (2013). Review of the NWMO Copper Corrosion Allowance. Nuclear Waste Management Organization, Toronto, Ontario NWMO TR-2013-04.
- Selenska-Pobell, S., G. Kampf, K. Flemming, G. Radeva and G. Satchanska. (2001). Bacterial Diversity in Soil Samples from Two Uranium Waste Piles as Determined by Rep-APD, RISA and 16S rDNA Retrieval. *Antonie van Leeuwenhoek* 79: 149-161.
- Setlow, P. (2003). Spore Germination. *Current Opinion in Microbiology* 6:550–556.
- Sheng, G.-P., H.-Q. Yu and X.-Y. Li. (2010). Extracellular Polymeric Substances (EPS) of Microbial Aggregates in Biological Wastewater Treatment Systems: A Review. *Biotechnology Advances* 28: 882-894.
- Sheppard, M.I., Stroes-Gascoyne, S., Motycka, M., Haveman, S.A. (1997). The Influence of the Presence of Sulphate on Methanogenesis in the Backfill of a Canadian Nuclear Fuel Waste Disposal Vault; A Laboratory Study. Atomic Energy of Canada Limited Report, AECL-11764, COG-97-21-I.
- Sherwood Lollar, B. (2011). Far-field microbiology Considerations relevant to a Deep Geological Repository – State of Science Review. Nuclear Waste Management Organization, Ontario, Canada TR-2011-09.
- Simon C. and R. Daniel. (2011). Metagenomic Analyses: Past and Future Trends. *Applied and Environmental Microbiology* 77(4): 1153-1161.
- SKB. (2006). Buffer and Backfill Process Report for the Safety Assessment. Swedish Nuclear Fuel Waste Management Company, Stockholm, Sweden. SKB TR-06-18.202
- Small, J., M. Nykyri, M. Helin, U. Hovi, T. Sarlin and M. Itävaara. (2008). Experimental and Modelling Investigations of the Biogeochemistry of Gas Production from Low and Intermediate Level Radioactive Waste. *Applied Geochemistry* 23: 1383-1418.

- Smart, N., A. Rance, B. Reddy, S. Lydmark, K. Pendersen and C. Lilja. (2011). Further Studies of *In Situ* Corrosion Testing of Miniature Copper-cast Iron Nuclear Waste Canisters. *Corrosion Engineering, Science and Technology* 46:142-147.
- Sorokin, D.Y., I.I. Rusanov, N.V. Pimenov, T.P. Tourova, B. Abbas and G. Muyzer. (2010). Sulfidogenesis under Extremely Haloalkaline Conditions in Soda Lakes of Kulunda Steppe (Altai, Russia). *FEMS Microbiology and Ecology* 73: 278-290.
- Stahl, D.A. (1997). Molecular Approaches for the Measurement of Density, Diversity and Phylogeny. In *Manual of Environmental Microbiology* (eds. Hurst C.J., G.R. Knudsen, M.J. McInerney, L.D. Stetzenbach, M.V. Walters). Washington ASM Press, pp, 102-114.
- Stevens, T. (1997). Lithoautotrophy in the Subsurface. *FEMS Microbiology Reviews* 20(3-4): 327-337.
- Stevens, T.O. and J.P. McKinley. (1995). Lithoautotrophic Microbial Ecosystems in Deep Basalt Aquifers. *Science* 270: 450-453.
- Stone, W., O Kroukamp, A. Moes, J. McKelvie, D.R. Korber and G.M. Wolfaardt. (2016a). Measuring Microbial Metabolism in Atypical Environments: Bentonite in Used Nuclear Fuel Storage. *Journal of Microbiological Methods* 120: 79-90.
- Stone, W., O Kroukamp, J. McKelvie, D.R. Korber and G.M. Wolfaardt. (2016b). Microbial Metabolism in Bentonite Clay: Saturation, Desiccation and Relative Humidity. *Applied Clay Science* 129: 54-64.
- Stotzky, G. (1985). Mechanisms of Adhesion to Clays, with Reference to Soil Systems. In *Bacterial Adhesion, Mechanisms and Physiological Significance* (eds. Savage, D.C. and M. Fletcher). New York and London: Plenum Press, pp. 195-253.
- Stotzky, G. (1986). Influence of Soil Mineral Colloids on Metabolic Processes, Growth, Adhesion and Ecology of Microbes and Viruses in Interactions of Soil Minerals with Natural Organics and Microbes. pp. 356-364.
- Stotzky, G. and L.T. Rem. (1967). Influence of Clay Minerals on Microorganisms. IV. Montmorillonite and Kaolinites on Fungi. *Canadian Journal of Microbiology* 13(11): 1535-1550.
- Stroes-Gascoyne, S. (1989). The Potential for Microbial Life in a Canadian High-Level Nuclear Fuel Waste Disposal Vault: A Nutrient and Energy Source Analysis. Atomic Energy of Canada Limited Report, AECL-9574.
- Stroes-Gascoyne, S. (2005). A Review of International Experience with Microbial Activity in Bentonite-Based Sealing Materials and Argillaceous Host Rocks. Atomic Energy of Canada Limited. Report No: 06819-REP-01300-10109-R00.
- Stroes-Gascoyne, S. (2010). Microbial Occurrence in Bentonite-Based Buffer, Backfill and Sealing Materials from Large-Scale Experiments at AECL's Underground Research Laboratory. *Applied Clay Science* 47: 36-42.
- Stroes-Gascoyne, S. and J. West. (1997). Microbial Studies in the Canadian Nuclear Fuel Waste Management Program. *FEMS Microbiology Reviews* 20:573-590.

Stroes-Gascoyne, S. and M. Gascoyne. (1998). The Introduction of Microbial Nutrients into a Nuclear Waste Disposal Vault During Excavation and Operation. *Environmental Science and Technology* 32: 317-326.

Stroes-Gascoyne, S. and F. King. (2002). Microbiologically Influenced Corrosion Issues in High-level Nuclear Waste Repositories. In *Proceedings of CORROSION/2002 Research Topical Symposium Microbially Influenced Corrosion* (ed. Little, B.). NACE International, Houston, TX, p. 79.

Stroes-Gascoyne, S. and C.J. Hamon. (2008). The Effect of Intermediate Dry Densities (1.1-1.5 g/cm³) and intermediate porewater salinities (60-90g NaCl/L) on the Culturability of Heterotrophic Aerobic Bacteria in Compacted 100% Bentonite. Nuclear Waste Management Organization, NWMO TR-2008-11, Toronto, ON.

Stroes-Gascoyne, S. and C.J. Hamon. (2010). The Effects of Elevated Temperatures on the Viability and Culturability of Bacteria Indigenous to Wyoming MX-80 bentonite, Nuclear Waste Management Organization, NWMO TR-2010-08, Toronto, ON.

Stroes-Gascoyne, S., L.M. Lucht, J. Borsa, T.L. Delaney, S.A. Haveman and C.J. Hamon. (1995). Radiation Resistance of the Natural Microbial Population in Buffer Materials. *Materials Research Society Symposium Proceedings* 353: 345-352.

Stroes-Gascoyne, S., K. Pedersen, S. Daumas, C.J. Hamon, S.A. Haveman, T.-L. Delaney, S. Ekendahl, N. Jahromi, J. Arlinger, L. Hallbeck, and K. Dekeyser. (1996). Microbial Analysis of the Buffer/Container Experiment at AECL's Underground Research Laboratory. Atomic Energy of Canada Limited Report AECL-11436/COG-95-446.

Stroes-Gascoyne, S., C.J. Hamon, P. Vilks and P. Gierszewski. (2002). Microbial, Redox and Organic Characteristics of Compacted Clay-based Buffer after 6.5 Years of Burial at AECL's Underground Research Laboratory. *Applied Geochemistry* 17: 1287-1303.

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, Nuclear Waste Management Division Report 06819-REP-01200-10016-R00.

Stroes-Gascoyne, S., C.J. Hamon, D.A. Dixon, C.L. Kohle and P. Maak. (2007a). 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, M. Simanoff, C. Le Marrec, S. Altmann, T. Nagaoka, L. Mauclaire, J. McKenzie, S. Daumas, A. Vinsot, C. Beaucaire and S.-M. Matray. (2007b). Microbial Community Analysis of Opalinus Clay Drill Core Samples from the Mont Terri Underground Research Laboratory, Switzerland. *Journal of Geomicrobiology* 24:1-17.

Stroes-Gascoyne, S., C.J. Hamon, D.A. Dixon, D.G. Priyanto. (2010a). The Effect of CaCl₂ Porewater Salinity (50-100g/L) on the Culturability of Heterotrophic Aerobic Bacteria in Compacted 100% Bentonite with Dry Densities of 0.8 and 1.3 g/cm³. Nuclear Waste Management Organization, NWMO TR-2010-06, Toronto, ON.

Stroes-Gascoyne, S., C.J. Hamon, P. Maak and S. Russell. (2010b). The Effects of the Physical Properties of Highly Compacted Smectitic Clay (Bentonite) on the Culturability of Indigenous Microorganisms. *Applied Clay Science* 47:155-162.

Stroes-Gascoyne, S. C. Sergeant, A. Schippers, C.J. Hamon, S. Neble, M.-H. Vesvres, V. Barsotti, S. Poulain and C. Le Marrec. (2011). Biogeochemical Processes in a Clay Formation *In Situ* Experiment: Part D – Microbial analyses – Synthesis of Results. *Applied Geochemistry* 26: 980-989.

Stucki, J.W. and P.J. Getty. (1986). Microbial Reduction of Iron in Nontronite. In *Agronomy Abstracts, 1986 Annual Meetings of the Soil Science Society of America*. P. 249.

Stucki, J.W. and J.E. Kostka. (2006). Microbial Reduction of Iron in Smectite. *Comptes Rendus Geoscience* 338: 468-475.

Studds, P.G., D.I. Stewart, and T.W. Cousens. (1998). The Effects of Salt Solutions on the Properties of Bentonite-Sand Mixtures. *Clay Minerals* 33: 651-660.

Štyriaková, I., K. Jablonovská, A. Mockovčiaková, A. Bekényiová, I. Štyriak, I. Kraus, M. Osacký, and M. Lovás. 2010. Dissolution of Iron from Quartz Sands by Basin Bioleaching Under Static In-Situ Conditions. *Hydrometallurgy* 104 (3–4): 443–47.

Subramanian, S.B., S. Yan, R.D. Tyagi and R.Y. Surampalli. (2010). Extracellular Polymeric Substances (EPS) Producing Bacterial Strains of Municipal Wastewater Sludge: Isolation, Molecular Identification, EPS Characterization and Performance for Sludge Settling and Dewatering. *Water Research* 44(7): 2253-2266.

Tamura, K. G. Stecher, D. Peterson, A. Filipski and S. Kumar. (2013). MEGA6: Molecular Evolution Genetics Analysis version 6.0. *Molecular Biology Evolution* 30: 2725-2729.

Templeton, A.S., E.J. Knowles, D.L. Eldridge, B.W. Arey, A.C. Dohnalkova, S.M. Webb, B.E. Bailey, B.M. Tebo and H. Staudigel. (2009). A Seafloor Microbial Biome Hosted Within Incipient Ferromanganese Crusts. *National Geoscience* 2: 872-876.

Tetre, E., S. Castet, G. Berger, M. Loubet and E. Giffaut. (2006). Surface Chemistry of Kaolinite and Na-Montmorillonite in Aqueous Electrolyte Solutions at 25 and 60 °C: Experimental and Modeling Study. *Geochimica et Cosmochimica Acta* 70: 4579-4599.

Thauer, R.K., K. Jungermann and K. Decker. (1977). Energy Conservation in Chemotrophic Anaerobic Bacteria. *Bacteriology Reviews* 41(1): 100-180.

Thury, M. and P. Bossart. (1999). The Mont Terri Rock Laboratory, A New International Research Project in a Mesozoic Shale Formation, in Switzerland. *Engineering Geology* 52: 347-359.

Torsvik, H. J. Goksøyr and F.L. Daae. (1990). High Diversity in DNA of Soil Bacteria. *Applied and Environmental Microbiology* 56(3): 782-787.

Trüper, H. G., and H. G. Schlegel. (1964). Sulphur Metabolism in *Thiorhodeaceae*. I. Quantitative Measurements on Growing Cells of *Chromatium okenii*. *Antonie van Leeuwenhoek* 30:225-238.

Tyson, G.W., J. Chapman, P. Hugenholtz, E.E. Allen, R.J. Ram, P.M. Richardson, V.V. Solovyev, E.M. Rubin, D.S. Rokhsar and J.F. Banfield. (2004). Community Structure and

Metabolism through Reconstruction of Microbial Genomes from the Environment. *Nature* 428: 37–43.

Urios, L., F. Marsal, D. Pellegrini and M. Magot. (2012). Microbial Diversity of the 180 Million-Year-Old Toarcian Argillite from Tournemire, France. *Applied Geochemistry* 27: 1442-1450.

VanEngelen, M.R., E.K. Field, R. Gerlach, B.D. Lee, W.A. Apel and B.M. Peyton. (2010). UO Speciation Determines Uranium Toxicity and Bioaccumulation in an Environmental *Pseudomonas* sp. isolate. *Environmental Toxicology and Chemistry* 29: 763–769.

Veening, J.W., W.K. Smits, L.W. Hamoen, and O.P. Kuipers. (2006). Single Cell Analysis of gene expression patterns of competence development and initiation of sporulation in *Bacillus subtilis* grown on chemically defined media. *Journal of Applied Microbiology* 101: 531–541.

Venter, J.C., K. Remington, J.F. Heidelberg, A.L. Halpern, D. Rusch, J.A. Eisen, D., I. Paulsen, K.E. Nelson, W. Nelson, D.E. Fouts, S. Levy, A.H. Knap, M.W. Lomas, K. Nealson, O. White, J. Peterson, J. Hoffman, R. Parsons, H. Baden-Tillson, C. Pfannkoch, Y.H. Rogers, and H.O. Smith. (2004). Environmental Genome Shotgun Sequencing of the Sargasso Sea. *Science* 304: 66–74.

Vilks, P. (2009). Sorption in Highly Saline Solutions—State of the Science Review. Nuclear Waste Management Organization, Toronto, Canada. NWMO Report TR-2009-18

Vilks, P., N.H. Miller and K. Felushko. (2011). Sorption Experiments in Brine Solutions with Sedimentary Rock and Bentonite. Nuclear Waste Management Organization, Toronto, Canada NWMO TR-2011-11.

Volckaert G., F. Bernier, X. Sillen and M. Van Geet. (2004). Similarities and Differences in the Behaviour of Plastic and Indurated Clays. In *Radioactive Waste Management - Community Policy and Research Initiatives The Sixth International Conference on the Management and Disposal of Radioactive Waste* (ed. P.F. Ruiz). Euradwaste 2004, France, p. 955. France

Vreeland, R.H., A.F. Piselli, S. McDonnough and S.S. Meyers. (1998). Distribution and Diversity of Halophilic Bacteria In a subsurface salt formation. *Extremophiles* 2(3): 321-331.

Wallace, S.S. (1998). Enzymatic Processing of Radiation-Induced Free Radical Damage in DNA. *Radiation Research (Supplementary)* 150: S60-S79.

Wang, X.-C. and C. Lee. (1993). Adsorption and Desorption of Aliphatic Amines, Amino Acids and Acetate by Clay Minerals and Marine Sediments. *Marine Chemistry* 44(1): 1-23.

Wang, Y. and A.J. Francis. (2005). Evaluation of Microbial Activity for Long-term Performance Assessments of Deep Geologic Nuclear Waste Repositories. *Journal of Nuclear Radiochemical Sciences* 6: 43-50.

Wang, Q., A.M. Tang, Y.-J. Cui, P. Delage and B. Gatmari. (2012). Experimental Study on the Swelling Behaviour of Bentonite/Claystone Mixture. *Engineering Geology* 124(1): 59-66.

Ward, D.M., R. Weller and M.M. Bateson. (1992). 16S rRNA Sequences Reveal Numerous Uncultured Microorganisms in a Natural Community. *Nature* 345: 63-65.

Weber, K.A., L.A. Achenbach and J.D. Coates. (2006). Microorganisms Pumping Iron: Anaerobic Microbial Iron Oxidation and Reduction. *Nature* 4: 752-764.

- Webster, G., C.J. Newberry, J.C. Fry, and A.J. Weightman. (2003). Assessment of Bacterial Community Structure in the Deep Sub-Seafloor Biosphere by 16S rDNA Based Techniques: A Cautionary Tale. *Journal of Microbiological Methods* 55: 155-164.
- Welch, S.A. and P. Vandevivere. (1994). Effect of Microbial and Other Naturally Occurring Polymers on Mineral Dissolution. *Geomicrobiology Journal* 12:227-238.
- West, J.M., M. Cave, P. Coombs, A.E. Milodowski and C.A. Rochelle. (1998). Alteration of Repository Structural Materials within the First Few Years. *Materials Research Society Symposium* pp. 503-510.
- Widdel, F., and F. Bak. (1992). Gram-negative Mesophilic Sulfate-Reducing Bacteria. In *The Prokaryotes vol. IV* (eds. A. Balows, H. G. Trüper, M. Dworkin, W. Harder, and K.-H. Schleifer). New York: Springer-Verlag, pp. 3352-3378.
- Wilkins, M.J., F.R. Livens, D.J. Vaughan, I. Beadle and J.R. Lloyd. (2007). The Influence of Microbial Redox Cycling on Radionuclide Mobility in the Subsurface at a Low-Level Radioactive Waste Storage Site. *Geobiology* 5: 293–301.
- White, D.C. and D.B. Ringelberg. (1998). Utility of the Signature Lipid Biomarker Analysis in Determining *In Situ* Viable Biomass, Community Structure and Nutritional/Physiological Status of Deep Subsurface Microbiota. In *The Microbiology of the terrestrial deep subsurface*, P. S. Amy, D. Haldeman (ed), LCRC Press, New York, pp 119-136.
- Whitman, W.B., D.C. Coleman and W.J. Wiebe. (1998). Prokaryotes: The Unseen Majority. *Proceedings of the National Academy of Sciences U.S.A.* 95: 6578-6583.
- Whitlock, J. (2013). CANDU Nuclear Power Technology. In *Canadian Nuclear FAQ*. Retrieved from http://www.nuclearfaq.ca/cnf_sectionA.htm#a.
- Woese, C.R. (1987). Bacterial Evolution. *Microbiology Reviews* 51:221-271.
- Wolfaardt, G.M. and D.R. Korber. (2012). Near-Field Microbiological Considerations relevant to a Deep Geological Repository for Used Nuclear Fuel – State of Science Review. Nuclear Waste Management Organization, Ontario, Canada. NWMO TR-2012-02.
- Wolfaardt, G.M., J.R. Lawrence and D.R. Korber. (1999). Functions of EPS. In *Microbial Extracellular Polymeric Substances: Characterization, Structure and Function* (eds. Wingender, J., T.R. Neu and H.-C. Flemming). Springer-Verlag Berlin Heidelberg New York, pp. 172-195.
- Wolfaardt, G.M., D.R. Korber, and J.R. Lawrence. (2007). Cultivation of Microbial Consortia. In *Manual of Environmental Microbiology* (eds. Hurst C.J., R.L. Crawford, J.L. Garland, D.A. Lipson, A.L. Mills and L.D. Stetzenbach). American Society for Microbiology, Washington DC. Pp. 101-111.
- Wong, D. J.M. Suflita, J.P. McKinley and L.R. Krumholz. (2004). Impact of Clay Minerals on Sulfate-Reducing Activity in Aquifers. *Microbial Ecology* 47: 80-86.
- Wouters, K., H. Moors, P. Boven and N. Leys. (2013). Evidence and Characteristics of a Diverse and Metabolically Active Microbial Community in Deep Subsurface Clay Borehole Water. *FEMS Microbiology Ecology* 86: 458-473.

- WNA. (December 2012). What is Uranium? How Does it Work? World Nuclear Association. Retrieved from <http://www.world-nuclear.org/info/Nuclear-Fuel-Cycle/Introduction/What-is-Uranium--How-Does-it-Work/>
- WNA. (2013). Storage and Disposal Options: Radioactive Waste Management Appendix 2. Retrieved from <http://www.world-nuclear.org/info/Nuclear-Fuel-Cycle/Nuclear-Wastes/Appendices/Radioactive-Waste-Management-Appendix-2--Storage-and-Disposal-Options/>
- Wu, Q., X.H. Zhao and S.Y. Zhao. (2006). Application of PCR-DGGE in Research of Bacterial Diversity in Drinking Water. *Biomedical and Environmental Sciences* 19(5): 371-374.
- Yohe, G.R. (1967). Industrial Mineral Notes 31: Clay as Binding Materials. Illinois State Geological Survey, Urbana, Illinois, USA, pp. 1-32.
- Zeikus, J.G., A. Ben-Bassat, T.K. Ng, R.J. Lamed. (1981). Thermophilic Ethanol Fermentations. In *Trends in the Biology of Fermentations for Fuels and Chemicals*. (ed. A. Hollaender). New York: Plenum Press Pp. 397-420.
- Zengler, K. M. Walcher, G. Clark, I. Haller, G. Toledo, T. Holland, E.J. Mathur, G. Woodnott, J.M. Short and M. Luller. (2005). High-Throughput Cultivation of Microorganisms using Microcapsules. *Methods in Enzymology* 397: 124-130.
- Zhilina, T. N., G.A. Zavarzin, F.A. Rainey, E.N. Pikuta, G.A. Osipov, and N.A. Kostrikina. (1997). *Desulfonatronovibrio hydrogenovorans* gen. nov., sp. nov., an Alkaliphilic, Sulfate-Reducing Bacterium. *International Journal of Systematic Bacteriology* 47 (1): 144–149.
- Zhou, J., M.A. Bruns and J.M. Tiedje. (1996). DNA Recovery from Soils of Diverse Composition. *Applied Environmental Microbiology* 62: 316-322.
- Zinder, S.H. and A.A. Salyers. (2001). Microbial Ecology—New Directions, New Importance. In *Bergey's Manual of Systematic Bacteriology. Volume 1: The Archaea and the Deeply Branching and Phototrophic Bacteria*. 2nd ed. (eds. D.R. Boone and R.W. Castenholz). New York: Springer-Verlag, 101-109.

10. APPENDICES

Appendix 10.1: Elemental composition (%) of commercial clays used in the study

Elemental composition	Clay Brand		
	MX-80 (%)	Canapril (%)	National Standard
SiO ₂	63.02	63.28	65.19
Al ₂ O ₃	21.08	14.28	20.86
Fe ₂ O ₃	3.25	4.43	1.8
FeO	0.35	ND	ND
MgO	2.67	1.83	2.43
Na ₂ O	2.57	1.99	2.31
CaO	0.65	1.36	0.94
K ₂ O	ND	1.00	0.29
TiO ₂	ND	0.51	0.15
Cr ₂ O ₂	ND	ND	0.02
MnO	ND	ND	0.06
V ₂ O ₅	ND	ND	0.035

ND – no data

Appendix 10.2: Selenska-Pobell Method (Swiss Protocol)

1. 50 g of powdered Opalinus clay sample was added to a 200 mL Erlenmeyer flask containing 50 mL sterile Millipore Q (MQ) water. The flask was shaken overnight at room temperature. The following day,
2. The clay slurry was transferred to two 50 mL round bottom polypropylene Oak Ridge tubes (Thermo Scientific Nalgene, Waltham, MA) and centrifuged at 8000 g using a Sorvall Superspeed RC2-B centrifuge (Ivan Sorvall, Inc., Norwalk, CT) for 20 min at room temperature. The supernatants were stored at 4 °C in case re-analysis of this sample was required. The clay pellets were transferred into new sterile 200 mL Erlenmeyer flasks containing 60 mL of 0.12 M Na₂HPO₄ (pH=8) and 15 mL of 5 % sodium dodecyl sulfate (SDS). The mixture was incubated at 70 °C in a shaking water bath set at 100 rpm for 60 min.
3. After incubation, the mixture was allowed to settle for 15 min. The upper liquid portion of this mixture was gently decanted to two new 50 mL round bottom polypropylene tubes, leaving the “clay slurry portion” in the bottom of the flask.
4. These tubes were then centrifuged (8000 g for 15 min at 10 °C) wherein the supernatants were decanted into a sterile container (labelled “clay extract”). The pellets from these tubes were then discarded. The clay extract was stored at 4 °C until use.
5. The clay slurry portion in the flask, another 40 mL of 0.12 M Na₂HPO₄ was added and then the mixture was incubated again at 70 °C in a shaking water bath set at 110 rpm for 25 min.

6. After settling for 10-15 min, the liquid upper portion was decanted to a new round bottom tube, centrifuged at 8000 g for 15 min at 10 °C, and the solid-free supernatant was combined with the “clay extract” container. The remaining clay slurry portion was added again with 20 mL 0.12 M Na₂HPO₄, incubated at 70 °C shaking water bath set at 110 rpm for 20 min. The clay slurry was centrifuged after which the final supernatant was transferred to the “clay extract” container.
7. The contents (approximately 75-120 ml) of the “clay extract” container were portioned into several sterile round bottom tubes (~1/3 of tube volume) and stored on ice for 2 h to crystallize the SDS. Subsequently,
8. These clay extracts were centrifuged at 4 °C using a pre-cooled rotor at 15000 g for 30 min in order to sediment the precipitated SDS. The pellet-free supernatant was then collected in a new flask and gently mixed with sterile 0.1 volumes of 5 M NaCl and 0.4 volumes of 50 % w/v polyethylene glycol (PEG) 6000. The mixture was stored overnight at 4 °C. The following day,
9. The mixture was centrifuged at 15000 g for 30 min at 4 °C, after which the supernatant was discarded. In preparation for concentrating the DNA, the resulting pellets were dissolved in 2.5 ml of enzymatic digestion mix (10 ml of TE-Buffer, 100 µl of RNase A [10 mg/mL] and 125 µl of Proteinase K [20 mg/mL]), transferred into one 50 ml round bottom tube and then incubated at 37 °C for 45 min.
10. To the dissolved sample above, 3-mL aliquot of Buffer G4 (3 M guanidium hydrochloride (GuHCl) in 20% Tween 20, buffered to pH 5.5 by NaOH) was added to the sample. The mixture was then incubated at 50 °C for another 30 min. Thereafter,
11. One volume of Buffer N2 (containing 100 mM Tris, 15% (vol/vol) ethanol, 900 mM KCl, and 0.15% Triton X-100, buffered to pH 6.3 with H₃PO₄) was added to the mixture and vortexed at maximum speed for 15 s.
12. To collect the DNA, the above mixture was loaded onto a NucleoBond® AXG-100 column (Macherey-Nagel, GmbH & Co. KG, Duren, Germany) placed above a 15 mL Greiner tube (initially equilibrated with 2 mL of Buffer N2), and the sample was allowed to pass through the column via gravity flow.
13. The column was washed three times with 4 mL of Buffer N3 (containing 100 mM Tris, 15% (vol/vol) ethanol, and 1150 mM KCl, buffered to pH 6.3 with H₃PO₄).
14. After the washing step, the DNA was eluted twice with 5 mL of Buffer N5 (containing 100 mM Tris, 15% (vol/vol) ethanol, and 1,000 mM KCl, buffered to pH 8.5 with H₃PO₄) and collected in a sterile Greiner tube. The collected flow-through was mixed with 0.7 volume of sterile isopropanol, incubated at room temperature for 45 min, and centrifuged at 15000 g for 30 min at 4 °C.
15. The supernatant was removed and the pellet was air-dried under laminar flow at room temperature for 15 min. The pellet containing extracted DNA was dissolved in 20 µL of TE-Buffer and stored at -20 °C.

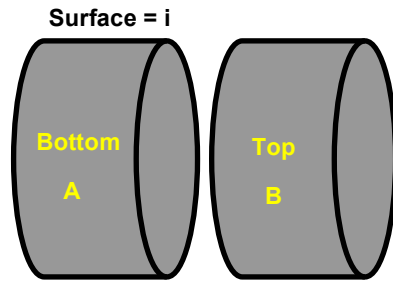
Appendix 10.3: Modified Swiss Protocol

1. 50 g sample of Opalinus clay, MX-80 or Avonlea clay was aseptically mixed with 50 ml sterile deionized water in the pre-autoclaved 500 mL screw-cap shaker flask. The mixture was incubated for 12 h on a rotary shaker set at 110 rpm at room temperature.
2. The slurry was aseptically dispensed into four 50 ml sterile polypropylene round bottom tubes and centrifuged at 4000 g using a Sorvall Superspeed RC2-B centrifuge (Ivan

Sorvall, Inc., Norwalk, CT) for 45 min at room temperature. The supernatant (SP1) was collected in a sterile screw-mouth Erlenmeyer flask and stored at 12 °C while the slurry precipitate was aseptically transferred to a new Erlenmeyer flask.

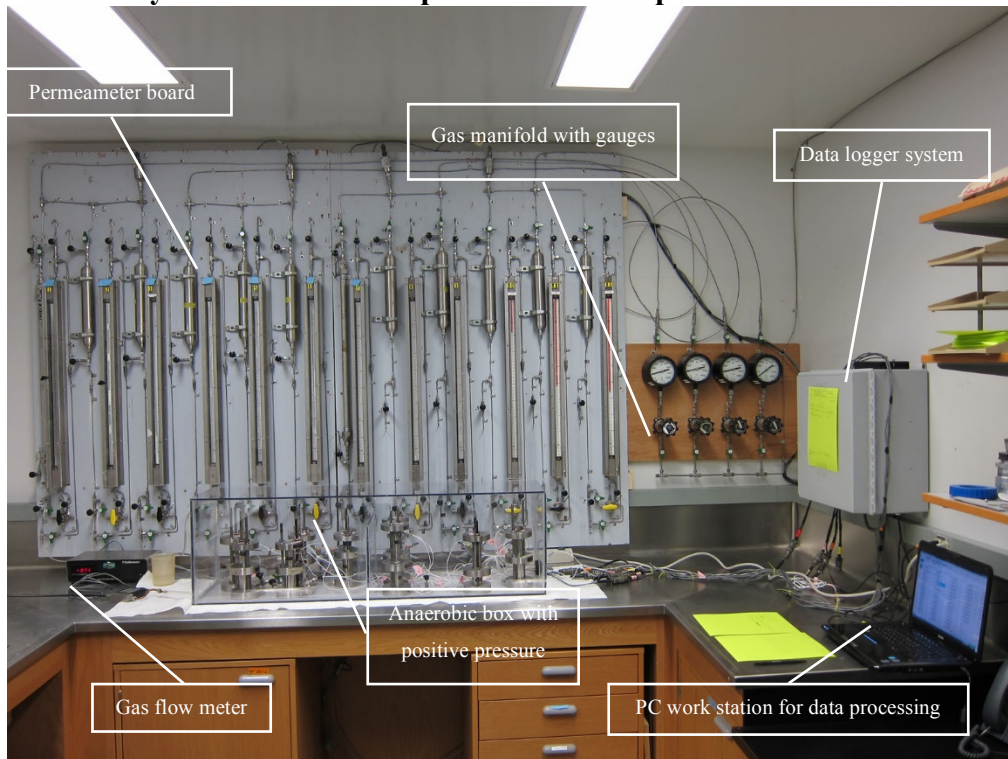
3. To the slurry precipitate or clay pellet, a similar Na₂HPO₄ and SDS treatment and the method from the Swiss method above were applied. The only difference was that the samples were centrifuged at 4000 g for 45 min at room temperature.
4. The supernatants acquired (SP2, SP3 and SP4) were combined with SP1 and stored at 12 °C. Thereafter, the combined supernatants were also treated with 0.1 volumes of 5 M NaCl, but this time, 0.4 volumes of 50% w/v PEG 8000 solution was used. Finally, the mixture was stored at 4 °C overnight. The following day,
5. The mixture was centrifuged at 15000 g for 60 min at 4 °C.
6. The pellets were combined, centrifuged again at 15000 g for 10 min at 4 °C, washed with 1.5 mL NaCl-EDTA (0.15 M NaCl, 0.1 M EDTA, pH 8.0) solution, and then resuspended in 300 µl NaCl-EDTA solution in a pre-sterilized 1.5 ml microcentrifuge tube.
7. A 20 µl aliquot of lysozyme (5 mg/ml) was added to the microcentrifuge tubes, followed by 10 min incubation at 37 °C in a thermo block.
8. To each clay extract, 32 µl of 25% SDS solution was added and the tubes were incubated in a 60 °C water bath to cause cell lysis. A 72 µl aliquot of 5 M NaClO₄ and ~450 µl of chloroform/isoamylalcohol (24: 1) was then added to the suspensions. The tubes were capped tightly and then mixed gently on a rotary platform for 1 h.
9. After mixing, suspensions were centrifuged at 4 °C for 2 min and the supernatants were transferred to new microcentrifuge tubes.
10. A 2.5 times the volume of ice cold 95% ethanol (-20 °C) was added to the tubes and subsequently they were gently inverted.
11. The DNA was collected by spinning at 4 °C for 20 min. DNA was again washed with 500 µl of ice cold 70% ethanol and then dissolved in 200 µl of TE buffer (10 mM Tris-HCl, 0.1 mM EDTA; pH 8.0).
12. A 25 µl aliquot of RNase A (10 mg/ml) was then added and the tubes left for ~15 min at room temperature.
13. A 10 µl portion of Proteinase K (10 mg/ml) was added followed by 1 h incubation.
14. An 80 µl of TE-saturated phenol (pH=8) was added to the tubes and then mixed gently on a rotary platform for ~1 hr.
15. After mixing, the suspensions were centrifuged at 4 °C for 3 min. The top aqueous layers (~220 µl) were transferred to new microcentrifuge tubes.
16. DNA was precipitated with 550-600 µl of DNA precipitation mixture (containing 90% (w/w) ethanol, 10% (w/w) 2.5 M NaOAc; pH 5.5) and centrifuged for 15 min at 4 °C.
17. The DNA pellet was then washed with 70% ethanol. After drying at room temperature, DNA was dissolved in 50 µl of TE buffer and stored at 4 °C.

Appendix 10.4: Sampling schematic for microbiological assessment of clay plugs



The clay plug was sub-sampled at: (i) the outer, or interfacial, regions, (A) the inner portion of the bottom half of the plug, and (B) the inner region of the top half of the plug. The clay plug was removed from the pressure cell prior to microbiological analysis. The plug dry bulk density, water activity and swelling pressure were measured.

Appendix 10.5: May 2014 – Anaerobic pressure cell setup



Permeameter board station with anaerobic box and gas pressure gauges, a nitrogen gas flow meter, data logger and computer with software to process data received by logger from the load cells and pressure transducers. These components were used to deliver and monitor hydrostatic and swelling pressure in steel cells containing HCB.

Appendix 10.6: R2A medium adapted from Reasoner and Geldreich (1985)

Chemical compounds	Amount (g/L)
Peptone	0.5
Yeast extract	0.5
Casamino acids	0.5
Glucose	0.5
Soluble starch	0.5
Sodium pyruvate	0.3
K ₂ HPO ₄	0.3
MgSO ₄ ·7H ₂ O	0.05
pH	7.2 ± 0.2

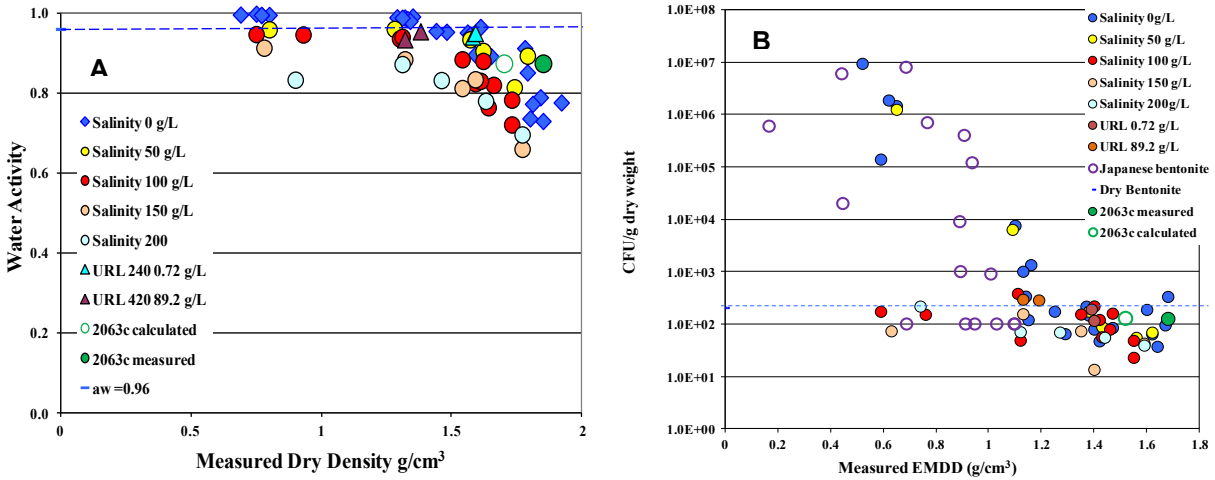
Appendix 10.7: Postgate B medium adapted from Postgate (1984)

Chemical compounds	Amount (g/L)
NaCl	10
KHPO ₄	0.5
NH ₄ Cl	1.0
CaSO ₄	1.0
MgSO ₄ ·7H ₂ O	2.0
60% sodium lactate	3.5
FeSO ₄ ·7H ₂ O	0.5
Na ₂ S·9H ₂ O	0.2 mM
Yeast extract	1.0
pH	7.2 ± 0.2

Appendix 10.8: Zeikus medium adapted from Zeikus *et al.* (1981)

Media	Chemical compounds	Amount (g/L)
Zeikus medium	NH ₄ Cl	0.9
	NaCl	0.9
	MgCl ₂	0.2
	KHPO ₄	0.75
	K ₂ HPO ₄	1.5
	NaHCO ₃	2.5
	FeSO ₄ ·7H ₂ O	0.015
	T-337 trace elements	1 mL/L
	Sodium acetate	2.2
	Na ₂ S·9H ₂ O	0.5 mM
	Yeast extract	1.0
T-337 trace elements	EDTA	4.0
	CaCl ₂	1.5
	FeSO ₄ ·7H ₂ O	1.0
	MnSO ₄ ·2H ₂ O	0.35
	NaMoO ₄ ·2H ₂ O	0.5

Appendix 10.9: Graphs obtained from Stroes-Gascoyne *et al.* (2010b) to compare Section 5 results with water activity, measured dry density, and EMDD.



Appendix 10.10: Composition of trace element solution added to the alkaliphilic bacteria medium (Zhilina medium) – adapted from Pfennig and Lippert (1966)

Chemical compounds	Amount added
EDTA	5.00 g
FeSO ₄ ·7H ₂ O	2.20 g
ZnSO ₄ ·7H ₂ O	0.10 g
MnCl ₂ ·4H ₂ O	0.03 g
H ₃ BO ₄	0.03 g
CoCl ₂ ·6H ₂ O	0.20 g
CuCl ₂ ·2H ₂ O	0.03 g
NiCl ₂ ·6H ₂ O	0.03 g
Na ₂ MoO ₄ ·2H ₂ O	0.03 g
Distilled Water	1.00 L
pH 3.9	using HCl (ion)

Appendix 10.11: Composition of medium for alkaliphilic bacteria (per Liter) – adapted from Zhilina *et al.* (1997)

Basal Medium (Medium Zh-SO ₄ 20)		Amount added			
NH ₄ Cl		1.0 g			
K ₂ HPO ₄		0.2 g			
NaCl		10.0 g (microcosm study A) 50.0 g (microcosm study B)			
KCl		0.2 g			
T-337 Trace element solution (Appendix 10.8)		1 ml			
Resazurin, 1% (wt/vol)		4-5 drops			
<i>Additives</i>					
0.5M Na ₂ S·9H ₂ O		0.5 ml			
5% Yeast Extract		0.5 ml			
5% FeSO ₄ ·7H ₂ O		0.25 ml			
1M Na ₂ SO ₄		20 ml (20 mM*)			
<i>Further supplements</i>					
		Medium Zh7-SO ₄ 20		Medium Zh8-SO ₄ 20	
		Lac	Ac	Lac	Ac
1M NaHCO ₃		30ml	30ml	-	-
0.9M NaHCO ₃ /0.1M Na ₂ CO ₃		-	-	90ml	90ml
1M Sodium Lactate		10ml (10mM)		10ml (10mM)	
1M Sodium Acetate			10ml (10mM)		10ml (10mM)

* Values in parenthesis indicate the final concentration of supplement

Appendix 10.12: Experimental determination of effective methylene blue protocol. Adaptation of modified methylene blue assay for sulfide determination in microcosm containing 10% clay with lactate at pH 7 after 24 days. (Underlined data showed maximum sulfide detected by the assay). Data provided with permission from Dr. Alexander Grigoryan.

ID	Sample	V _{sample} (μl)	Spin ¹	ZnAc ² (μl)	Spin	PDA ³ (μl)	Spin	Fe(III) ⁴ (μl)	H ₂ O (μl)	Spin	A _{670nm}
1	10% clay	39.5	+	632	-	789	-	39.5	0	-	.031; .036; .039
2	10% clay	39.5	+	0	-	789	-	39.5	632	-	.033; .031; .037
3	10% clay	39.5	-	632	-	789	-	39.5	0	-	.279; .381; .440
4	10% clay	39.5	-	0	-	789	-	39.5	632	-	.172; .225; .252
5	10% clay	39.5	-	632	+	789	-	39.5	0	-	.060; .059; .061
6	<u>10% clay</u>	<u>39.5</u>	-	<u>632</u>	-	<u>789</u>	<u>+</u>	<u>39.5</u>	<u>0</u>	-	<u>.831; .877; .998</u>
7	10% clay	39.5	-	632	-	789	-	39.5	0	+	.266; .131; .171
8	10% clay	39.5	-	0	-	789	+	39.5	632	-	.542; .815; .617
9	10% clay	39.5	-	0	-	789	-	39.5	632	+	.149; .132; .134

¹ Centrifugation at 14000 rpm for 90 s

² 2% zinc acetate (in 0.1% w/v glacial acetic acid)

³ 0.2% w/v of [(CH₃)₂NC₆H₄NH₂]₂*H₂SO₄ in 20% w/v H₂SO₄

⁴ 10% FeNH₄(SO₄)₂*10H₂O in 2% w/v H₂SO₄

Appendix 10.13: M-Salt media and T-337 trace element

Chemical compounds	Amount (g/L)
NaCl	2.0
NH ₄ NO ₃	1.0
MgSO ₄ (anhydrous)	0.248
K ₂ HPO ₄	5.2
KH ₂ PO ₄	2.7
T-337 Trace Element (Appendix 10.6)	1.0 mL/L
pH	7.2 ± 0.2