Near-field Microbiological Considerations Relevant to a Deep Geological Repository for Used Nuclear Fuel – State of Science Review

NWMO TR-2012-02 **December 2012**

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ABSTRACT

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Report No.: **NWMO TR-2012-02**
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Abstract

A literature review-based study has been undertaken to evaluate the possible near-field impacts of microorganisms and their activity on a deep geological repository (DGR) for used nuclear fuel. The term “near-field” refers to the Engineered Barrier System (EBS) and those parts of the host rock in contact or near the EBS, whose properties have been affected by the presence of the repository. The EBS includes the buffer and backfill materials, as well as the used fuel containers.

In 2007, the Government of Canada selected *Adaptive Phased Management* (APM) as the approach for the long-term management of used nuclear fuel. The goal of APM is long-term containment and isolation of used nuclear fuel in a DGR, constructed in a suitable rock formation at a depth of approximately 500 metres. Consistent with international designs for nuclear waste repositories, the Canadian concept involves the use of steel or copper/steel containers, surrounded by a low-permeability, swelling clay buffer material. When considering the long-term performance of such an installation, the activity of microorganisms is relevant, along with the related effects of microbial activity on the engineered barriers. The implications of microorganisms in a deep geological repository on geochemical evolution, gas production, radionuclide transformation and transport, and EBS performance are reviewed in this report.

Given their ubiquity and metabolic capabilities, it is assumed that with sufficient time and appropriate conditions, microbes have the potential to impact the EBS, including the lifetime of used nuclear fuel containers through microbially influenced corrosion. Should this occur, containment of radioactive material would be provided by the used fuel itself, the clay-based sealing sytems, and the enclosing host rock.

A challenge inherent to the evaluation of microbiological influences on the near-field repository environment is that long-term behaviour predictions must be based on short-term, and sometimes limited, data. Accordingly, a comprehensive understanding of the key microbiological parameters which could affect repository barrier components is necessary so that these factors can be incorporated, along with margins for error, into the repository safety case. It is also important that these microbiological parameters be described in the physical and chemical context of the EBS. Over the last 20 years, there has been significant advances in subsurface microbiology in the context of a DGR for used nuclear fuel. However, the number of established research groups with extensive experience in this sub-discipline is notably small relative to that of mainstream microbiology, and it needs to be determined how accurately the relatively unknown effects, and particularly the indirect effects, can be predicted based on the finite existing knowledge base. Further understanding of microbiology has the potential to refine long-term predictions regarding repository evolution and safety.

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1. INTRODUCTION

This report presents a state of science review of international literature and knowledge on the role of microorganisms, in particular near-field microbial processes, in relation to the key issues affecting the characterization, design and performance of a deep geological repository (DGR) for used nuclear fuel. Based on the definitions of the OECD NEA (2003), the near-field includes the Engineered Barrier System (EBS) and those parts of the host rock in contact with, or near, the EBS whose properties have been affected by the presence of the repository. The far-field is defined as the geosphere (including the biosphere) beyond the near-field. Sherwood Lollar (2011) provided a summary of the state of science on far-field microbiology considerations for a DGR for used nuclear fuel.

The Canadian DGR concept for used nuclear fuel involves multiple barriers to ensure the long-term safety of humans and the environment (NWMO, 2005). These barriers include: i) the low solubility of the used nuclear fuel and zircaloy cladding; ii) the corrosion-resistant copper/steel or steel metal containers that contain the used nuclear fuel, iii) the high-density clay barrier and sealants that surround the used nuclear fuel containers, and iv) the location of the repository deep within a stable geologic formation. Hence, the multiple protective barriers proposed in Canada's DGR concept include both naturally-occurring (the stable geologic formation, considered the far-field) and engineered (the used nuclear fuel containers and surrounding clay buffer, seal and backfill materials, termed the near-field) components. Overall, the concept entails a network of tunnels and placement rooms located approximately 500 m below ground level in either an appropriate crystalline or sedimentary rock formation, connected by a shaft to the surface. Examples of multibarrier system conceptual designs under consideration for a deep geological repository for Canada's used nuclear fuel are illustrated in Figure 1 and Figure 2.

This report draws on peer-reviewed scientific literature, publically-available reports from nuclear waste management programs, both internationally and in Canada, and from relevant microbiological investigations in the resource sector to evaluate near-field microbiological considerations for repository engineering and long-term safety assessment of a deep geological repository. The specific objectives were to:

1. Review microbiological research (both experimental and modelling-based) on microbial impacts, activity and survivability in a DGR for used nuclear fuel conducted in support of Adaptive Phased Management (APM) and conducted by other nuclear waste management agencies;
2. Review potential Canadian host rock and repository design options and discuss the implications of microbial activity on those designs; and
3. Review findings related to long-term EBS performance in relation to evolving conditions with the goal of identifying any gaps in knowledge and understanding of microbial processes in a deep geological environment that could affect the long-term performance of the EBS.

On the basis of the conducted review, this report outlines a suggested approach for an investigative strategy and for development of a microbiology technical program that could be implemented in support of APM.

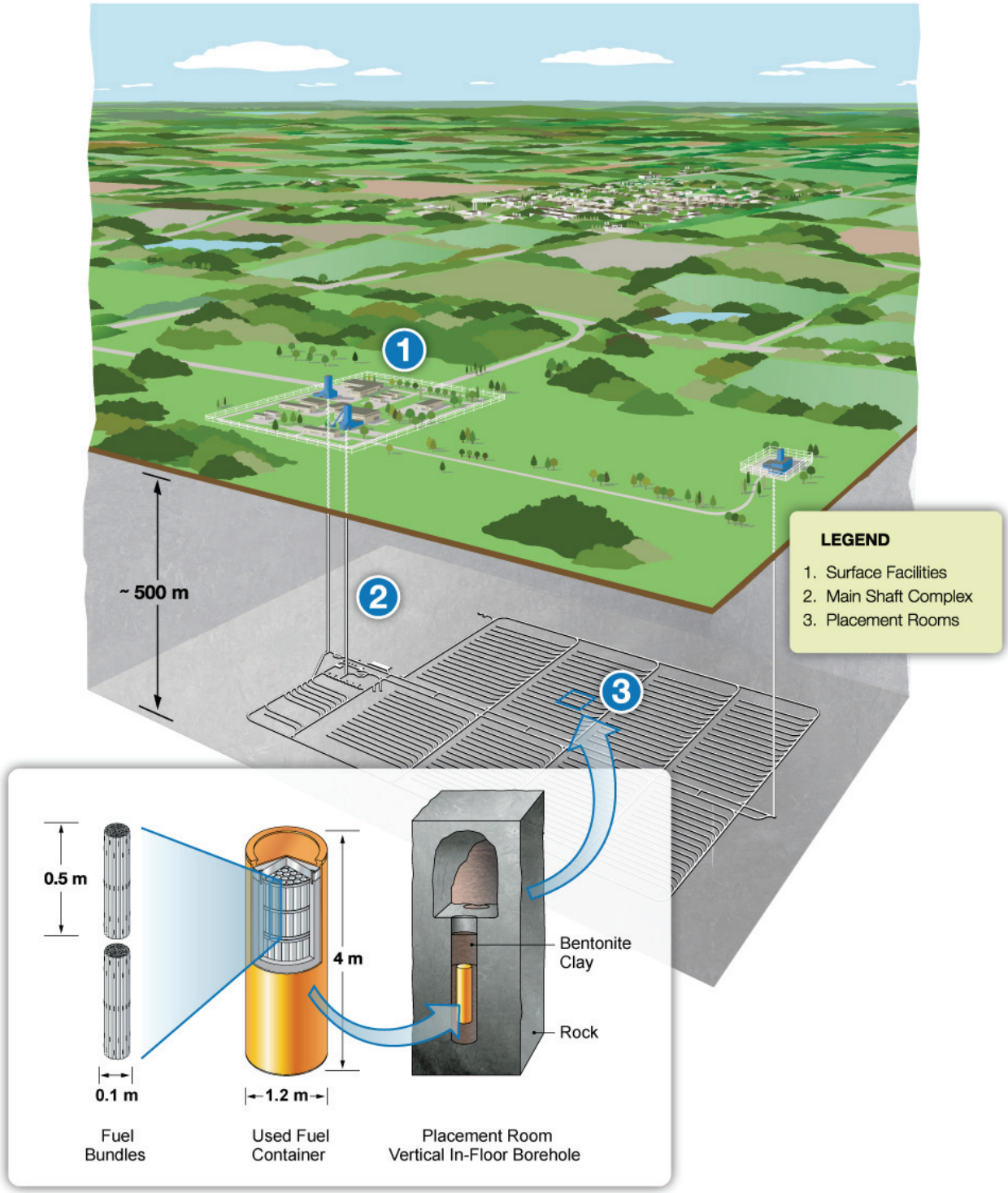


Figure 1: Illustration of a reference deep geological repository (Copper-shell used nuclear fuel container with vertical in-floor borehole placement) (Russell, 2011).

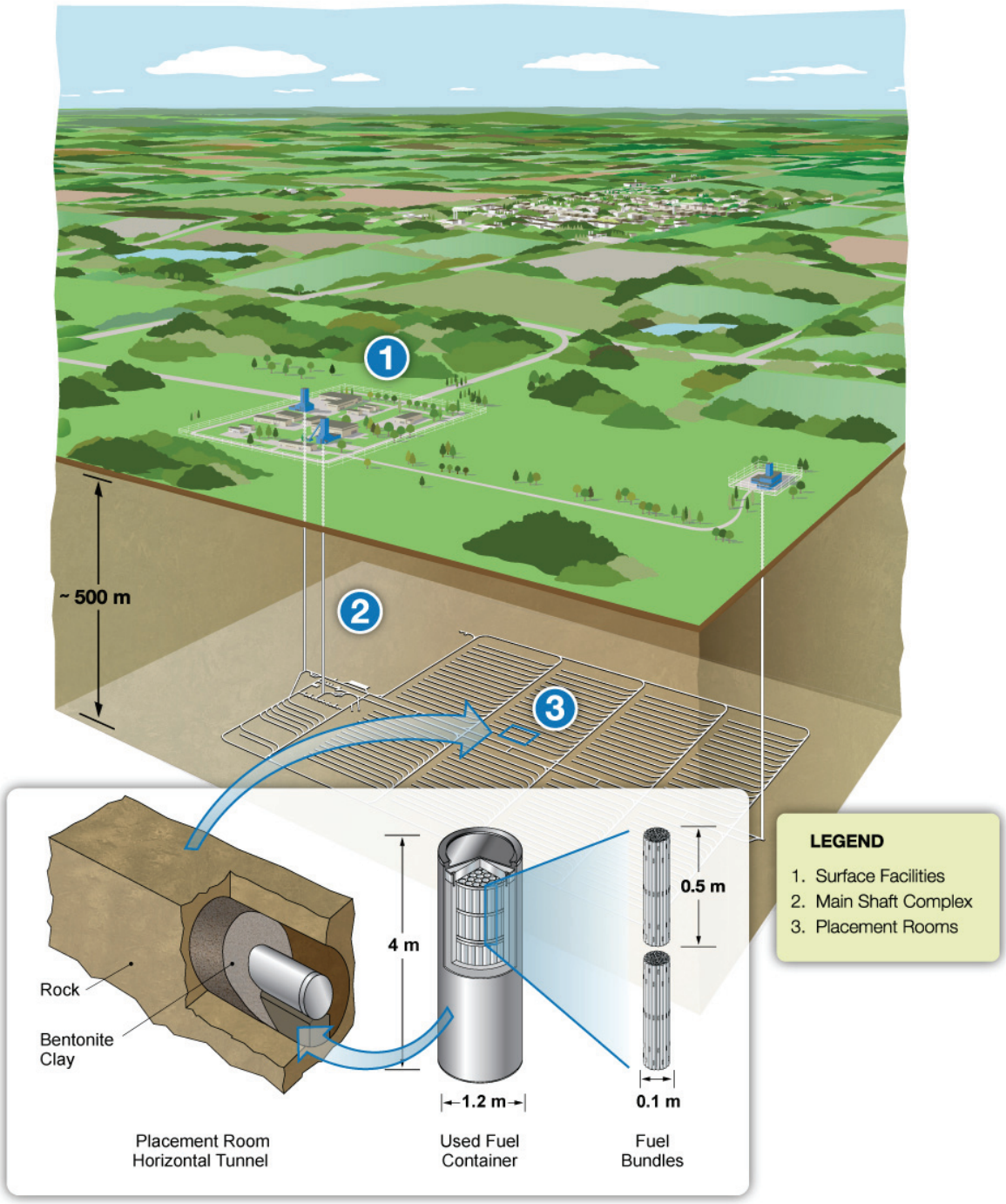


Figure 2: Illustration of a reference deep geological repository (Steel used nuclear fuel container with horizontal tunnel placement) (Russell, 2011).

2. NEAR-FIELD MICROBIOLOGY IN THE CONTEXT OF THE HOST ENVIRONMENT

Used nuclear fuel is highly radioactive, particularly just after removal from the reactor core. The source of radiation includes a number of reaction products (i.e., radioactive isotopes including iodine, molybdenum, cesium, technicium, palladium, etc.) that form upon splitting of the fissionable U^{235} uranium atoms, as well as those elements (known as transuranic actinides - elements with atomic numbers of 93 or greater, such as neptunium and plutonium) that are formed when the uranium atoms that absorb bombarded neutrons do not split. The radioactive material is unstable, and undergoes decay or breakdown over time, during which radiation is emitted. Some of this radiation is gamma radiation, which is highly energetic and penetrating, like x-rays, and thus damaging to biological (living) material due its direct and indirect (formation of damaging radicals) effects. Those fission products with very short half-lives tend to rapidly emit large amounts of radiation energy, and thus are typically very hazardous, but do not require long-term management. Due to the presence of significant amounts of radionuclides with long half-lives in used nuclear fuel, Canadian safety assessments consider a time frame of one million years.

On initial emplacement of used nuclear fuel, the near-field environment would experience a sharp increase in temperature due to the release of energy from fission products with short half-lives. Based on known decay rates and proposed density of used nuclear materials, peak container surface temperatures of 100°C (Maak et al., 2010) or possibly higher in some designs (120°C; Jolley et al., 2003) would occur around 10 years after placement and then begin to drop. Peak temperatures and timeframes will be design- and site-specific, however, the temperatures of the host rock in a DGR would likely range from 55-75°C and these elevated temperatures would persist for hundreds to a few thousands of years.

Canada, along with other countries (e.g., Sweden, Finland, Switzerland, France, UK), has evaluated and is considering a number of container emplacement options in the repository, including vertical and horizontal boreholes, as well as horizontal tunnels (Maak et al., 2010). Used nuclear fuel containers would be placed in the rooms or tunnels constructed from main access tunnel leads, or in boreholes. Surrounding the containers would be a clay-based buffer (100% compacted bentonite rings). The facility would be tailored to the geological matrix in which the DGR is situated, with horizontal and vertical boreholes considered suitable for crystalline rock repositories (as well as hard sedimentary rock), and the horizontal tunnel option preferred for a soft sedimentary rock repository (e.g., analogous to the Opalinus Clay Formation in Switzerland). An additional feature of APM design is long-term retrievability (Villigran, 2012).

A detailed review of microbiology in the far-field was recently completed by Sherwood Lollar (2011), which provides information about microbial diversity and methodology of relevance to the far-field. While the focus of this review is near-field microbiology, a brief overview of the larger environment in which repositories will be placed is included below to set the context in which near-field microbiology is being considered. As part of the APM approach, the selection of the repository site involves the ongoing review of relevant geoscientific data, including geological, hydrogeological, geochemical and geomechanical factors. Information collected during site investigation activities can partially serve as a benchmark environment for microbial distribution and activity, with the primary question being whether or not near-field conditions will evolve towards those in the far-field (discussed in the following sections). Notably, heterogeneities within the far-field may present different microbial functional groups that may have different impacts on repository installations.

2.1 NATURALLY-OCCURRING ANALOGUE SITES, BURIED UO_2 AND STABILITY

Due to the long-lived activity of used nuclear fuel, the stability and performance of the DGR must be predicted far into the future. As such, the existence of analogue sites, where uranium or naturally-occurring fission reactions and products either exist or have existed, permits the critical evaluation of some related considerations over time frames relevant to a DGR (i.e., one million years). In these assessments, considerations of the natural geological processes, which have impacted analogue sites over geologic time scales, are useful. A number of naturally-occurring sites possess parallel characteristics and conditions to those expected in the DGR (Mazurek et al., 2008; Smellie et al., 1997; Pedersen, 2000), and, as such, can aid in predicting aspects of microbiological, chemical and radiological processes.

Key DGR characteristics include the role that the clay barrier plays in mitigating radionuclide transport. For some repositories, the candidate geological repository matrix itself may consist of clay material (i.e., the Opalinus Clay formation in Switzerland; Boom Clay in Belgium; sedimentary deposits in Canada). Cigar Lake in northern Saskatchewan is one of the world's largest uranium deposits (~11% of the world's UO_2 reserves at approximately 209 million pounds) and is almost entirely UO_2, the same form of uranium used in refined CANDU fuel rods. The high-grade deposit (averaging 17% uranium, with greater than 55% found locally) at Cigar Lake is encased by a dome of clay (Cramer and Smellie, 1994). The Cigar Lake deposit has survived approximately 1.3 billion years without significant movement of radioactive elements through the clay, despite the fact that the clay dome contains fractures. The clay has thus effectively immobilized the uranium 430 m below ground, by restricting movement of groundwater into the deposit, as well as by hindering transport of uranium out of the deposit, such that there is no chemical or radiological signature detectable at ground level (Brown and Sherriff, 1999; Smellie et al., 1997). While the Cigar Lake site models the concepts behind the Canadian DGR, the barrier performance characteristics of the 100% bentonite clay buffer can be expected to exceed that of the *in situ* illite clay underground at Cigar Lake. Smellie and Karlsson (1999) indicated that natural analogues have significantly contributed to the demonstration of long-term stability of bentonite as a stable barrier to radionuclide transport, and indeed pointed out that illite clays are considered inferior to bentonite in this regard. Despite the low organic carbon concentrations (<2.0 mg/L) in the clay at the Cigar Lake site, microorganisms have been detected within the deposit's radiation field using direct and indirect methods (e.g., plate counts, fluorescent staining, adenosine triphosphate measurements, most probable number analyses), with anaerobic bacteria out-numbering aerobes by an order of magnitude (Cramer, 1995; Francis et al., 1994; Stroes-Gascoyne et al., 1994). Sulfate-, iron- and nitrate-reducing bacteria were also detectable in most of the samples tested, and are thus thought to play a role in metal reduction reactions using Fe(III) and U(VI) as electron acceptors at the interface between oxygenated groundwater overlying the deposit, and the anaerobic zone beneath. Maintenance of reducing conditions would enable the more stable, insoluble U(IV) species to predominate.

The only known site where the long-term behaviour of nuclear fission-products has been well documented is in the vicinity of a former "natural reactor" located at Oklo, in Gabon, Africa. At this site, isotope signatures confirm that self-sustaining nuclear chain reactions occurred, resulting in the fission of approximately 1000 tonnes (t) of naturally occurring uranium, 6-12 t of which was ^{235}U. The multiple natural fission reactions occurred more than 2 billion years ago, and due to the moderating effects of overflowing groundwater, actually continued to react for approximately 1 million years. These natural reactors operated at extreme temperature (600°C) and pressure (800-1000 bar) (Miller et al., 1994), producing a variety of radioactive nuclides,

many of which have subsequently decayed, but also including ~4 t of plutonium (Bodu et al., 1972). Traces of these elements have remained in place, and studies confirm that the plutonium has not moved away from the source of uranium from which it was produced, despite the influence of groundwater flow for much of the geological record (Brown and Sherriff, 1999).

Work in the region, performed by Swedish Nuclear Fuel and Waste Management Company (SKB) throughout the 1990's (recounted by Kotelnikova and Pedersen, 1998; Pedersen, 2000) focused on the number and distribution of microorganisms associated with the Oklo site, particularly sulfate reducing bacteria (SRB) and iron reducing bacteria. Microbiological samples (focusing on the 16S rRNA gene) obtained from the reactor zone in this site revealed the presence of a diverse population of microorganisms (Crozier et al., 1999; Pedersen et al., 1996). Comparisons between the diversity and microbial numbers from two sampling boreholes at different depths showed a relatively homogeneous microbial distribution, with microbial numbers in the same range as other groundwater environments: namely between 10^3 and 10^7 bacteria per mL. It was established that there is a strong possibility of a hydraulic connection between the sampling points, based on the similarities in bacterial numbers and diversity, as well as other data such as pH and TOC values. It was further pointed out that parameters, such as redox changes, due to microbial activity, production of complexing agents, uptake of radionuclides, and bacterial mobility should be examined. It is important to note that the test boreholes were drilled in the geological surroundings of the natural reactor. As such, it is the equivalent of the far field, and thus provided a test site for evaluating 'communication' between near- and far-field environments. This is especially interesting in view of the observations made at Cigar Lake where movement of radioactive elements through the clay buffer was limited, despite the presence of fractures in the surrounding clay dome.

Fukunaga et al. (2005) conducted microbiological investigations on four analogue bentonite deposits (two underlying wetlands and two adjacent to galleries in a mine) in Japan that had been undisturbed for between 2 to 30 years. Using fluid-free drilling techniques, the authors found that both microbial cell numbers (determined using culture-based methods) and cell activity (determined using carboxyfluorescein diacetate acetoxymethyl-ester direct staining) declined with distance from the drill core mouth. The authors concluded that bentonite represented an unfavourable microbiological matrix, likely due to its low porosity (high dry density) and bacterial filtration properties. A filtration effect was supported by the failure of higher numbers of microorganisms found at the drill core mouth to migrate deeper into the bentonite matrix, and these results overall correlated well with findings suggesting that clay matrices are not highly conducive to either microbial growth or advective (i.e., active) transport.

The thermal properties of clay can also be evaluated separately from potential interactions with radionuclides through examination of geologic analogues. Clay thermal properties require consideration because the clay buffer in the near-field zone will become hot due to internal heat (up to ~100 degrees C) generated from the used nuclear fuel. Clays underlying volcanic rock in Sardinia, and buried a kilometer below the island of Gotland, provide evidence that thermally-altered clay retains the swelling and sealing properties, for function in a DGR, even after millions of years (Pusch and Kärnland, 1988). The thermal and geochemical influences on the Morrón de Mateo bentonite deposit are also considered analogous to those that would occur in a DGR; however, an investigation by Pelayo et al. (2011) reached the opposite conclusion. In this case, the authors suggested that the thermal transformation of Spanish repository candidate Al-rich smectite clays into Fe-Mg-rich smectites and corrensites could lead to failure of physiochemical and mechanical properties of the EBS. However, SKB review of high temperature effects on smectite to illite conversion and the time scales indicated by their results suggest that while an effect may exist, this would not occur over timescale relevant to a DGR, and that the

temperature would not be sustained for the levels necessary (i.e., the temperatures would fall to 60ºC within the first 200 years). While the occurrence of a narrow altered buffer zone close to the used fuel containers cannot be excluded, illitisation and the transformation of montmorillonite in the buffer is expected to be slow and is not expected to have any significant effect on the important buffer properties, since it is unlikely that a majority of the buffer would be transformed resulting in a drop in buffer swelling pressure below design criteria (OECD, 2012). Thus, while this phenomenon has been shown to occur in the laboratory under strictly defined conditions, it is not thought to reflect what would occur within the *in situ* DGR condition. However. these studies underscore the need for consideration of thermal and geochemical influences on clay host rock properties, along with variable temperature affects on microbial activity that may influence radionuclide transport during safety assessment activities.

2.2 IMPLICATIONS OF FAR-FIELD CONDITIONS ON NEAR-FIELD MICROBIOLOGY

Different DGR concepts place different emphases on engineered and natural barriers in order to attain overall performance targets. The host rock dictates the effectiveness of natural containment, and in turn, influences whether in-floor boreholes, horizontal boreholes, or tunnels are most suitable as a waste container emplacement method (Maak et al., 2010).

The Canadian DGR concept currently encompasses options for emplacement of waste in either crystalline or sedimentary rocks at a depth of approximately 500 m. The far-field host rock presents a barrier component of the repository. The host rock also determines factors for microbial growth and represents a possible source of microorganisms. Thus, the potential for microorganisms to affect the performance of the EBS located in either host rock type warrants discussion.

In design concepts where the used nuclear fuel would be deposited within fractured igneous/crystalline rock, more stringent performance criteria of the EBS (particularly with respect to container life expectancy) would be required vs. emplacements in intact rock. Groundwater movement through the fractured rock matrix could transport viable microbes and nutrients into and out of the proximity of the EBS where they could exert effects, including: i) affecting waste container integrity (e.g., via microbially-influenced corrosion), and ii) altering radionuclide mobility (in the event of a container failure) by complexation with various microbial components, such as biofilms and their extracellular products (Pedersen and Albinsson, 1992c), or iii) alteration of the redox state, thereby influencing radionuclide solubility (Pedersen, 1996; Anderson and Lovley, 2002). These effects could occur in the DGR near-field (e.g., between the buffer material or backfill and the host rock), or occur within the cracks/fissures in the far-field matrix.

Repository concepts where the host rock will be sedimentary rock or clay typically place greater emphasis on the host rock as a low permeability barrier against radionuclide movement, and thus the required container lifetimes are relatively shorter. Such sedimentary rock typically does not support extensive microbial growth; evidence to support the existence of bacteria, which would have been emplaced millions of years before the present time is still a topic of conjecture. A preliminary study of microbial communities in potential Canadian sedimentary host rock types with low water activity suggested low to negligible microbial biomass (Stroes-Gascoyne and Hamon, 2008a), which in turn suggests that a large contribution of microorganisms from sedimentary host rock types to the EBS is not likely. Others predicted that the small pore space (e.g., <0.02 µm diameter), and low water availability within clay matrices, would significantly restrict the number and activity of microorganisms (Stroes-Gascoyne et al., 2007a, 2010b;

Poulain et al., 2008). In such an environment, the movement of nutrients to, and metabolic wastes from, microbial cells would similarly be hindered, causing nutrient limitation and end-product inhibition, resulting in low-frequency cell division. Small pore throats would further prevent any significant bacterial translocation. The putative mechanisms by which bacteria would survive under the above conditions are as of yet unknown. It follows that the potential impact of these scarcely-distributed microorganisms present within a clay formation would likely be negligible relative to the potential effects of introduced organisms at time of DGR construction.

As summarized by Maak et al. (2010) the two potential types of host rock media under consideration in the Canadian used nuclear fuel DGR program are subject to differing geologic properties that might influence their performance, including the potential for rock “creep” (the slow movement of rock due to gravity and gradients in pressure), as well as occurrence or presence of fractures. The presence of fractures in crystalline rock could provide conduits for the transport of water as well as bacterial cells to the EBS; however, crystalline rock is not expected to undergo significant rock creep over the lifetime of the repository. Although rock creep would be greater in soft sedimentary rock, the outcome would similarly not be expected to affect the barrier properties.

The hydrogeochemistry associated with the deep subsurface is site-specific; however, there are identifiable trends that exist, and as indicated by Maak et al. (2010), many of the technologies developed for repository concepts in crystalline rock are transferable to self-supporting (i.e., not subject to rock creep) hard sedimentary rock. This is potentially helpful, because the availability of instruments developed for long-term monitoring in sedimentary rock formations, as well as relevant experience, is more limited in Canada than it is for monitoring in hard crystalline rock media. Maak et al. (2010) performed an extensive review on used fuel container placement methods in crystalline rock, hard sedimentary rock and soft sedimentary rock. Overall, the potential influence of the chemical and physical conditions, and related microbial activity, in the far-field (as discussed by Sherwood Lollar, 2011) on the effect of biotic reactions in the near-field appear to be minimal, which indeed is the objective of EBS: namely, to isolate these two zones. Nevertheless, the numbers, distribution and activity of microorganisms in the far-field provide a realistic scenario of the endpoint towards which these characteristics may evolve in the near-field, with the probable exception of the area directly adjacent to the containers, which is described in Section 5 of this review.

2.3 ENGINEERED BARRIER SYSTEMS

In most repository concepts, the EBS refers to the metal container, bentonite clay buffer, backfill and sealing materials, and the surrounding host rock within the influence of the near-field. Each of these EBS components performs a function in the isolation and protection of the used nuclear fuel, and the potential microbial impact on each component should be considered.

A number of concepts for the disposal of used nuclear fuel have been proposed or are under consideration by the various nuclear waste management organizations that employ a system of engineered barriers (clay, seals, and metal container). The KBS-3 design concept of SKB and POSIVA, the Swedish and Finnish nuclear waste management agencies, respectively, use copper-iron containers, consisting of an outer copper shell and a cast iron insert, for encapsulation of used nuclear fuel prior to emplacement in their deep geological repository. In contrast, the engineered barrier system design of NAGRA, the Swiss nuclear waste

management agency, uses a steel container for their sedimentary host rock design. Despite different container designs, both concepts surround the used nuclear fuel containers with bentonite clay to protect the containers with used nuclear fuel and to limit groundwater access and the potential transport of microbial metabolites, such as sulfide, via groundwater, to the container surfaces (Bennett and Gens, 2008; Smart et al., 2011; Pedersen, 1996, 1999b). Bennett and Gens (2008) provided a concise overview of, as well as relevant references to, the current repository concepts and EBS designs for high-level waste and used nuclear fuel disposal in European countries. The microbial implications of the different Canadian design concepts on the engineered barrier system are discussed below.

2.3.1 Metal containers

It is highly unlikely that biological processes will have an impact on the used nuclear fuel itself, or the inside of the containers holding the used fuel, due to the combination of high temperatures, high radiation, the absence of water and lack of nutrients (McMurry et al., 2003; Meike and Stroes-Gascoyne, 2000). The zircaloy fuel cladding tubes used in nuclear reactors are heat and corrosion resistant. However, because the cladding is thin, the Canadian postclosure safety assessment uses conservative assumptions about the long-term performance of the zircaloy as a barrier. The main anticipated microbial effects are expected outside the containers.

Nuclear waste management agencies and governments around the world have considered a variety of container designs and components as part of their engineered waste containment systems. NWMO has two reference used fuel container designs, both approximately 4 metres in length, under consideration. Similar to the Swedish and Finnish designs, one design consists of a 10.25 cm thick carbon-steel inner vessel within an outer copper shell approximately 2.5cm thick (Figure 1). The second design, involves a carbon-steel container similar to the Swiss and French designs (Figure 2). Potential microbial effects on used fuel containers are discussed in Section 5.2.2.

2.3.2 Seal and backfill options

The Canadian APM approach to used nuclear fuel waste repository development considers a number of options for the isolation of used nuclear fuel containers from the host rock. Many of these options have been adopted, or are being considered, by other nuclear agencies around the world. Most concepts include: highly-compacted clay buffer, which would encase the container; clay and crushed rock backfill which surrounds the clay buffer; and repository seals which isolate the disposal room from the repository access shaft and prevent the mass of clay/clay-rock backfill from expanding into the access tunnels. The repository seal enables the swelling clay surrounding the used fuel containers to meet density/water activity specification targets.

Overall, bentonite-based sealants play a number of important functional roles (Stroes-Gascoyne, 2005), including: 1) limiting the rate of liquid movement by diffusion, 2) providing mechanical support to the container, thereby protecting it from movement or shifting of the surrounding host rock, 3) retention of the radionuclides, 4) thermally-conducting heat to the surrounding host rock, and 5) limiting the numbers, activities and transport of microorganisms near the container. Options for seals also include bulkheads consisting of cement or compacted

clay plugs placed at the entrance to the waste repository rooms. Clay-based sealants have been the focus of intense research as related to EBS performance, including comprehensive research programs by Canada and Sweden over more than 2 decades. Clay-based seal and backfill materials have been shown to contain indigenous aerobic and anaerobic microflora, including SRB (e.g., *Desulfovibrio africanus* in MX-80 bentonite (Masurat et al. 2010)). It has been the focus of many studies to demonstrate how buffer and backfill parameters are likely to affect the survival and activity of these and other organisms under relevant EBS conditions (see e.g., Stroes-Gascoyne et al., 1996a, 1997b, 2007a,b, 2010b; Stroes-Gascoyne, 1997, 2005, 2010; Pedersen, 1993a, 2000; Pedersen et al., 2000a,b). Potential microbial effects on backfill and buffer are discussed in Section 5.2.1.

2.3.2.1 Bentonite

Bentonite is widely included in various DGR concepts as a key component of the EBS design. For instance, Stroes-Gascoyne et al. (2007c, 2011b) indicated that countries such as Switzerland, France and Belgium consider clay deposits as a potential host rock for deep geological repositories because of various desired physical and hydrogeochemical properties. Due to the very low hydraulic conductivities, typically in the range of 10^{-13} to 10^{-14} m s^{-1}, clay formations, such as the Opalinus Clay Formation in Switzerland, are characterized by the absence of significant advective groundwater flow and are therefore expected to have excellent isolation properties (Stroes-Gascoyne et al., 2007c). In the absence of advective flow, clay matrices are typically diffusion-dominated, with water residence times estimated on the order of several millions of years (Stroes-Gascoyne et al. 2007c, 2011b). The clays being considered as potential host rocks are therefore expected to have excellent isolation properties, and it is thought that since the bentonite used in repository designs has similar properties, it is consequently a highly suitable material for barriers and seals.

Since the activity of microorganisms within the near-field has the potential to cause microbially influenced corrosion, and may also contribute to the generation of gases, much attention has been focused on the ability of the clay buffer to inhibit microbes and their activity. Microorganisms can grow over a large range of water activities (a_w of 0.75–0.999), but most favour a_w values of 0.98 or above (corresponding to a salinity of water of ~3.6% or less) (Jay et al., 2005). Studies have shown that pure bentonite that has been compacted to 2 Mgm^{-3} (or as dry density of about 1.6 Mg/m^3) and is water-saturated (approximately 26% v/w) has an a_w of 0.96, which is sufficient to inhibit the activity of the large majority of bacteria likely to be problematic in a repository (Stroes-Gascoyne et al., 2011b). Furthermore, water-expanded bentonite clay physically restricts the movement of water and whether emplaced or naturally-occurring, hydrated clay matrices are known to result in a low permeability environment, with hydraulic conductivities in the range of 10^{-12} to 10^{-14} m s^{-1} (Pusch and Weston, 2003). This would serve to directly influence the diffusion of radionuclides, as well as that of nutrients needed by microorganisms for metabolic activity and growth. Given that the average pore size of the clay matrix is on the order of hundreds of times smaller than a bacterial cell, cell growth and movement would also be physically restricted.

In the current Canadian EBS reference design, highly-compacted 100% bentonite buffer material surrounding the waste containers is proposed to prevent or minimize potential negative consequences of microbial activity, such as damage to the container or barrier integrity. Early Canadian EBS design options were based on a blended mix of sand and clay; however, findings reported by Stroes-Gascoyne (2010) related to microbial behavior led to stipulation of use of 100% bentonite. In order for the highly-compacted 100% bentonite to inhibit the activity of

bacteria and germination of bacterial spores, it has been established that the bentonite would need to meet one or both the following criteria (Stroes-Gascoyne et al., 2006, 2007a,b): i) have a water activity of less than or equal to 0.96, resulting from either a bentonite dry density of at least 1.6 Mg m^{-3} or a pore-water salinity of greater than 60 g NaCl/L; or ii) yield a swelling pressure of at least 2 MPa.

2.3.2.2 Cement and related materials

Waste repository concepts considered by NWMO and other waste management agencies around the world include options for the use of high-performance cement (Portland cement) as seals, grouts, tunnel liners, bulkheads and floors. Used nuclear fuel repository applications rely on cement primarily for its mechanical support and sealant properties in the repository, rather than the direct containment of used nuclear fuel containers (although this approach is an option for containment of low and intermediate level nuclear wastes (L/ILW)). Concrete forms the backbone of the building industry and its use goes back for centuries. Thus, there is a relatively large body of data available on the susceptibility of concrete to microbial attack. One of the seven microbiological concerns posed with regard to systems for safe storage of radioactive materials by Petersen (1999) was the question related to the impact of alkaliphilic microbes on concrete: “Do relevant microorganisms survive at pH equivalent to that of repository concrete and can they possibly influence repository performance by concrete degrading activities such as acid production”. Evidence from surface (e.g., roads, bridges) and underground cement structures (e.g., sewer pipes) has shown that the integrity of concrete over extended timescales can indeed be influenced by microorganisms, particularly the well-known sulfur-oxidizing bacteria such as *Acidithiobacillus thiooxidans* (previously *Thiobacillus thiooxidans*), which produces sulphuric acid under aerobic conditions through the oxidation of reduced sulfur, sulfide, and thiosulfate compounds. The oxidation product, sulfuric acid, contributes to the degradation of concrete by dissolving the calcium silicate hydrate and calcium hydroxide cement matrix constituents. Nitric acid, produced by the combined action of nitrosifying and nitrifying bacteria that use inorganic nitrogen compounds (i.e., ammonium, nitrite), may similarly lead to acid-mediated concrete deterioration. It is noteworthy that within a DGR, oxygen would be in finite supply in sharp contrast to the situation of roads and bridges; thus, once utilized during corrosion, mineral dissolution and microbial redox reactions, oxygen would no longer be able to contribute to the formation of sulphuric acid.

Further evidence for the detrimental effects of microbial activity on concrete has been described in a number of reviews. For instance, Sanchez-Silva and Rosowsky (2008) pointed out that the action of live organisms has been shown to be a major contributor to the deterioration of infrastructure systems such as underground structures, sewage systems and at-sea structures. It is further noted that this phenomenon is usually overlooked because microbial activity typically accelerates other processes that may eventually lead to unacceptable structural performance, or cause failure such as corrosion and cracking. In the repository near-field, either situation could lead to the creation of conduits for moisture movement. Production of extracellular enzymes and organic acids that solubilize concrete (e.g., Gutarowska, 2010; Jayakumar et al., 2011) and the negative impact of biofilms on infrastructure materials (e.g., Camper et al., 2003) have been described; however, it should be pointed out that the emphasis in most literature on the biodeterioration of concrete and other cement-based products relate to conditions that are conducive to microbial proliferation. In contrast, as described in Section 5, the repository environment poses a number of challenges to microbial activity, including the extremely low porosity that will exclude all but those with an ultra-small cell size. It is recognized though, that the study of microbes in the deep subsurface and in highly-consolidated materials is at a

relatively early stage, and there are likely microbes to be discovered in addition to ultra-small bacteria that are adapted for persistence in these extreme environments. Also, cement has a high pH (~13) and contains relatively low concentrations of carbon-containing nutrients to support microbial growth. Cement compounds for repository applications typically utilize 0.5 to 1% of a superplasticizer (SP) (i.e., sulphonated naphthalene condensates), which functions to reduce the amount of water required for cement mixing and to improve the workability and strength of the cement for repository applications. These plasticizers are carbon-containing compounds and thus they may be subject to microbial breakdown as a possible carbon source. The study of plasticizer leaching from concrete still remains incomplete, and despite the small quantities of SP added, there is evidence to suggest that some microorganisms use these compounds as a carbon source (Haveman et al., 1996; Stroes-Gascoyne, 1997). As indicated above, microbial activity can accelerate other processes in the cement components and potentially affect other repository component requirements (e.g., the ability to maintain swelling pressure of the buffer, and limiting water activity in the clay matrix). It is also known that disturbance of an environment (e.g., introduction of air and nutrients) results in a temporary increase in microbial activity; therefore, the period during emplacement and soon after may be critical to the potential impact of microbial activity and may warrant consideration (Wersin et al., 2011). As such, the Canadian postclosure safety assessment uses conservative assumptions about the long-term performance of cement in the repository and does not give it credit as a component of the multibarrier system.

2.3.3 Host rock within the possible influence of the near-field

As indicated above, a number of counties have adopted, or are considering adopting, clay-based or argillaceous materials as either the host rock for placement of a DGR (Switzerland, France and Canada), or as essential components (bentonite seals or grouts) of various EBS designs in both sedimentary and crystalline rock (Canada, Sweden, Finland). In either of these potential applications, bentonite is expected to serve as buffer between the used nuclear fuel containers and host rock, where it will influence hydraulic, mechanical, thermal, and chemical processes, as well as radionuclide diffusion, similar to the host rock (Stroes-Gascoyne, 2005).

In the Canadian design, the used nuclear fuel containers will be placed within a hole or a tunnel within the host rock and encased in 100% high-density compacted bentonite. Subsequent to this, the clay will be expanded following saturation by groundwater. Microorganisms indigenous to the water and bentonite will be present in the zones spanning from the container surface to the surrounding host rock. The redox conditions of the infiltrating groundwater may be oxic initially, with oxygen being introduced in the repository atmosphere during installation, but will return to anoxic conditions due to minor container corrosion, mineral oxidation or microbial respiration. While repository environments are generally not conducive to large amounts of microbial activity, there are potentially significant microbially-mediated outcomes that may subsequently occur given changing conditions and sufficient time. These include further microbial reduction processes (e.g., iron and sulfate reducing bacteria) that could affect the *in situ* redox conditions.

Overall, the current knowledge of microbial distribution and activity in previously undisturbed deep-subsurface environments, which were subsequently disturbed by, for example, mining activities, provides clues as to what may happen at the outer extremes of the EBS. It is noteworthy that gradients of water activity, pressure, pore space and temperature will occur locally within the EBS as well as over the larger repository (e.g., it is expected that temperatures in the center of the repository will be higher than those attained at the periphery of the

installation). A realistic assumption is that there will be: i) indigenous microorganisms, which may or may not be stimulated by the disturbance, and ii) microorganisms introduced during EBS installation. As such, the impact of microbes on repository performance requires evaluation. Specifically, what are the potential outcomes with regards to microbial behavior and how much of an effect could microorganisms have considering the *in situ* physical-chemical properties and geochemical evolution? Over what time scale are microorganisms active before transforming from active metabolism – to survival – to preservation, or the reverse scenario? Establishing the boundaries of these behaviors and their effects continues to remain a goal of research and reviews of potential microbial outcomes on the DGR near-field.

3. MICROBIAL ECOLOGY OF SUBSURFACE ENVIRONMENTS

It has long been recognized that an active and diverse community of microorganisms exist in a variety of subsurface habitats, including fractured crystalline/granitic rocks, saturated and unsaturated sedimentary formations, subsurface aquifers, metal mines, and marine sediments (West et al., 1985, 1986; Ghiorse and Wilson, 1988; Balkwill, 1989; Pedersen and Ekendahl, 1992a,b; Baker et al., 2003; Newby et al., 2004). In fact, the abundance of organisms (~6.1×10^{30} cells) in the subsurface, or geosphere, is approximately the same as the photosynthetically-derived green plant biomass present on the earth's surface (Whitman et al., 1998; Amend and Teske, 2005; Harvey et al., 2007). Hazen et al. (2012) pointed out that deep microbial ecosystems rival the total surface biomass by some estimates, but are mostly unknown and ignored. Furthermore, the diversity of microorganisms residing within the geosphere is known to be considerably greater than previously appreciated, thereby providing a possible explanation for the range of potential activities observed within subsurface environments.

Factors influencing the numbers, diversity and activity of microbes will primarily be reflective of the subsurface physical and chemical environment (e.g., pore size, water activity, temperature, pH, available carbon sources, and electron acceptors), as well as the nature of any previous anthropogenic activities (e.g., drilling, drilling muds, blasting, and introduction of backfill materials) (Stroes-Gascoyne and West, 1997; Wang and Francis, 2005). The geochemistry will thus largely dictate the physiological nature of the resident microbes, as well as the biogeochemical reactions that they catalyze (Fredrickson and Balkwill, 2006). In other words, microbial response to their physical-chemical environment also determines how they affect the surrounding macro- and micro-environmental conditions. In the case of disturbed subsurface environments, the introduction of allochthonous (foreign) microorganisms would be of potential importance because these organisms would be emplaced along with other materials. It is therefore anticipated that a variety of microorganisms with a range of metabolic capabilities will inhabit any constructed underground facility. To evaluate the potential for these microorganisms to influence the integrity of a DGR facility over relevant time frames, an understanding of the ecology of both native and introduced microorganisms, in the context of the subsurface geochemistry, is necessary.

3.1 SUBSURFACE MICROBIAL DIVERSITY

Based on decades of subsurface research using culture-dependent approaches, and more recent work using culture-independent analyses, the diversity and range of metabolic activities of subsurface life has become much better known (Ghiorse and Wilson, 1988; Balkwill, 1989; Fredrickson and Balkwill, 2006; Colwell and Leadbetter, 2007; Biddle et al., 2008). A variety of

factors dictate which organisms will be present in a particular subsurface region. These factors will determine if the organisms present will be generalists or specialists in terms of their metabolism. Under conditions of increasing isolation, such as within sedimentary clay deposits, the physical and chemical properties of the geologic matrix become more important. Because of the low permeability of the repository system, limitations of space, water, and nutrients would increasingly influence microbial growth and survival (Stroes-Gascoyne et al., 2002, 2007a,b). In particular, the supply of carbon and energy sources have the potential to be growth-limiting, given that primary photosynthetic production in the deep subsurface would not be operative. Oxygen is increasingly unavailable as a function of depth in subsurface environments; thus alternative (oxygen-independent) metabolic strategies (i.e., anaerobic respiration, fermentation, chemoautotrophy) are necessary for organisms in these locations.

The physical constraints of most subsurface environments mean that only microorganisms will be of importance, with the exception of karst landscapes formed in limestone deposits where caves often provide habitats for a variety of surface animals. Phylogenetic analyses (the evolutionary relatedness of organisms based primarily on their gene sequences; Olsen et al., 1986; Olsen and Woese, 1993; Liu and Stahl, 2007) have established the evolutionary positions of the organisms that make up the three main groups of life on Earth; the Bacteria, the Archaea, and the Eukarya. The Bacteria and Archaea are exclusively microorganisms, the smallest of which are approximately 0.2 μm in diameter, whereas unicellular eukaryotes (i.e., protozoa, fungi, primitive animals) may be as large as 1 mm in size.

In nature, microorganisms may exist as free-living individuals or cell aggregates in liquid suspension, or as surface-attached colonies, in what has been termed biofilms (Costerton et al., 1978, 1995). All microorganisms replicate by doubling or binary fission, where each individual cell receives a complete copy of the genetic blueprint (the genome, or chromosome), as well as the necessary biomolecules for carrying out the genome's instructions. Due to their small size and simple, often specialized, metabolism, many microbial representatives have the potential to survive within the small matrices of subsurface environments, as evidenced by numerous reports of various members of Bacteria (Gram positive and Gram negative bacteria, DeFlaun et al., 2007; Rastogi et al., 2009) and Archaea having been recovered from subsurface samples. Reports of yeast and fungi being detected in subsurface environments have also been made, and, although much more rarely, the existence of a food chain which includes higher-level bacterivorous predators is not beyond the realm of possibility. Gene sequences from flagellated and ciliated protozoa have been recovered from the deep subsurface (Pedersen, 1999a.b). Furthermore, Borgonie et al. (2011) reported the recent discovery of a species of the phylum Nematoda from fracture water 0.9-3.6 km deep in South African mines. Interestingly, the nematodes were not detected in the mines' service waters but in palaeometeoric fracture water with an age of 3,000–12,000 years based on carbon-14 data. The authors suggested that these subsurface nematodes might control microbial population density by grazing on biofilms on fracture surfaces. They also noted that they are able to enter anabiosis for extended periods, and metabolize aerobically when oxygen partial pressures are as low as 0.4 kPa. Even though these larger organisms will be confined to fractures and fissures, their discovery demonstrates that the metazoan biosphere reaches further than previously recognized, and more generally, that deep ecosystems are more complex than previously accepted. Although many of the reports suggest that the complexity and representatives of subsurface communities rival that of surface environments, this clearly is not the case for deep subsurface consolidated habitats, especially those where oxygen is not readily available. Under the latter conditions, the diversity of organisms would be much lower and their mode of survival more specialized (i.e., involving (chemolitho-) autotrophy and anaerobic respiration, which tends to revolve around hydrogen, bicarbonate, methane and acetate). Furthermore, the existence of bacteriophages, or bacterial

viruses, within deep granitic groundwater Äspö, Sweden, has also been recognized and reported (Kyle et al., 2008). Up to 10^7 virus-like particles were detected per mL and it was suggested by the authors that these agents could act to control microbial populations in the deep subsurface.

It has been noted that the concentrations of microorganisms found in different deep subsurface environments vary with location, ranging from none detected (750 m salt deposits at Asse, Germany) to 10^7 bacteria/mL of water in deep granitic environments in Japan and Sweden (West and McKinley, 2002). In the context of deep subsurface installations, the characterization of the existing microbial community may be more useful for analysis of the hydrogeochemistry of the system (and potential long-term influence) than in predicting the performance of the installation itself, because the latter would likely introduce organisms associated with the various construction materials as well as microorganisms associated with worker activities (e.g., species from workers, surface dust and airborne/aerosolized microorganisms, machinery). However, there would most certainly be long-term equilibration of the installation with its surrounding environment. Thus, the ability of indigenous organisms to be metabolically-active over extended periods of time remains a possibility.

3.2 MICROBIAL ENERGETICS IN THE DEEP SUBSURFACE GEOSPHERE

A recurring theme in subsurface microbiology is the source of energy available for sustained microbial growth. It has been demonstrated that a linkage exists between the characteristics of a subsurface environment and the presence of specific microorganisms (Fredrickson and Balkwill, 2006; Lovley, 2006). Where subsurface microbial activity is high, an active or recent linkage of the subsurface environment with surface terrestrial processes can usually be demonstrated. For example, subsurface microbial communities may demonstrably be linked with the aerobic or anaerobic breakdown of dissolved or particulate photosynthetically-derived organic matter, that either: i) percolates via surface water into subsurface habitats, or ii) is deposited via sedimentary processes (e.g., kerogen, petroliferous deposits, lignin, etc.).

Deeper in the geosphere, chemoautotrophic systems may also become established independent of photosynthesis (Stevens, 1997). For such a chemoautotrophic microbial community to develop and be sustained, a sufficient source of energy would need to be available. Relatively new evidence supports the existence of subsurface ecosystems based on the geochemical production of hydrogen via radiolysis, where primary production supporting this system would be provided by chemoautotrophic homoacetogens, as well as acetoclastic and hydrogenotrophic methanogens, and subsequent heterotrophic organisms based on the autotrophic production of organic carbon (Pedersen, 1993b, 1997, 1999a,b; Stevens and McKinley, 1995; Fredrickson and Balkwill, 2006). This premise is not improbable because the conditions in the deep subsurface (high temperatures, reducing gases, etc.) show much resemblance to conditions that persisted when life first formed on earth. Still, the energy requirements of microbial cells may be considerable in comparison to the small energy yields and fluxes that dominate in much of the deep subsurface environment. This difference in energy requirement, and the maximum potential rates of energy supply, imposes a significant constraint on the habitability of consolidated materials, and, as indicated by Hoehler (2004), this important determinant of the presence, distribution, and productivity of life in photosynthesis-independent subsurface environments is seldom tested. Thus, in the absence of the comparatively high-energy light- and oxygen-based metabolic potential energy that are the primary drivers of the Earth's surface biosphere, life in the deep subsurface is limited to low-energy anaerobic processes dependent primarily on the mineral geochemistry of the host

environment. Jones and Lineweaver (2010) described terrestrial liquid waters that are uninhabited because of limiting nutrients and energy, and amongst others, restrictions on pore space. Whether certain habitats are indeed uninhabited continues to remain a topic for discussion, and may be a combined result of our inability to detect very low numbers of organisms with stringent growth requirements.

Hoehler (2004) described the magnitude of the energy required to sustain basic biochemical integrity and function in terms of two concepts: biological energy quantum (BEQ) and maintenance energy (ME). With respect to BEQ, for free energy to be usefully harnessed it must be available at levels equal to or larger than the specific required finite minimum energy needed to drive the synthesis of adenosine triphosphate (ATP) from adenosine diphosphate (ADP) (i.e., the minimum free energy that must be available in a given environment to sustain life). Importantly, this free energy change ($\Delta G_{ADP \rightarrow ATP}$) is sensitive to the prevailing physical and chemical conditions, and the parameters with specific relevance to the environment of interest in this review are: i) magnesium concentration; ii) ionic strength; as well as iii) pH. Most bentonite types have high magnesium content, which has a strong affinity for ATP, ADP and phosphate, and the $\Delta G_{ADP \rightarrow ATP}$ increases with ionic strength. Similarly, $\Delta G_{ADP \rightarrow ATP}$ becomes more positive with increases in pH. It should be noted that, in contrast to BEQ requirements described above, Jackson and McInerney (2002) indicated that some fermentative bacteria do have the ability to couple substrate metabolism directly to ATP synthesis to obtain energy for growth via reactions in which the change in free energy is less than what is needed for ATP synthesis. However, with reference to earlier observations, and based on a combination of theoretical and experimental observations, Hoehler (2004) suggested that the BEQ required for actively growing populations would be twice as much as that required for static populations in maintenance mode. The author further pointed out that the emergence and proliferation of a population is a prerequisite to its survival in maintenance mode. However, given the stability of the deep subsurface geosphere, it is not clear how such proliferation could occur in stages other than deposition and early consolidation.

To preserve life, organisms require a minimum rate of energy intake to maintain molecular and cellular function and / or integrity. As dictated by the environment, energy is used by microorganisms in one of three broad categories: growth, maintenance of basic metabolism without growth, or survival that involves little or no metabolic activity and energy is primarily used to preserve the integrity of amino acids and nucleic acids (such as may be operative in consolidated materials). *Overall, it is clear that ignoring the important constraints on energy flux in the deep subsurface geosphere, and failure to carefully consider maintenance requirements of microbial cells in context of this important control of energy availability, will result in a significant overestimation of related microbial activity.* Indeed, Morita (1999) suggested that when the presence of active bacteria in ancient material is reported it could be possible that the suspended animation state has been broken by giving the cells a substrate or electron acceptor, different conditions from their *in situ* environment, or other forms of perturbing the sample. This concern has also been raised in a number of seminal publications (e.g., Krumholz, 1998; Chapelle and Lovley, 1990; Phelps et al., 1994a; Kieft and Phelps, 1997). In brief, these authors made a few important observations, namely: i) that the use of inappropriate methodology can lead to a significant difference between potential activity and actual *in situ* activity of microbes; ii) that laboratory estimates of microbial activity are often orders of magnitude higher than actual *in situ* rates, potentially by factors up to 10^6 over what geochemical models and knowledge of groundwater flows would substantiate; iii) that perturbation, container effects, and typical addition of small amounts of energy to activate the cells all have an effect on microbial activity; and, iv) that calculated rates of microbial activity are typically averaged over time and distance, while the actual rates of activity are temporally and

spatially heterogeneous. Considering the extremely slow rates of energy flux and the levels required for growth, it is evident that the slow rates of metabolism in the deep subsurface will be directed primarily toward survival rather than growth. The adaptive ability of microorganisms, when stressed by one set of conditions (e.g., starvation) to initiate cross-protection against other stresses, such as osmotic stress, heat stress, or temperature extremes, in general, contribute to survival success to the degree that microorganisms in lithotrophic ecosystems have seemingly developed mechanisms for survival and extended periods of anabiosis (Krumholz, 1998). Similarly emergent is the theory that individual microorganisms can survive in subsurface environments for millennia while carrying out cellular metabolism at an extremely slow rate (Morita, 1999).

It is evident that the deep subsurface imposes significant physical (e.g., extremely small void space) and chemical (e.g., low available energy and energy flux) constraints on microorganisms, and as discussed by McCollom and Amend (2005), a rigorous accounting of controls, such as energy flow, is needed to get an improved understanding of the potential biological productivity of chemolithoautotrophic communities and to better describe limits to habitability in subsurface environments. Such information is needed to derive more accurate estimates of microbial contribution to geochemical evolution. Interfaces and transition zones often support increases in microbial diversity and metabolic activity. In the immediate vicinity of the containers containing used nuclear fuel, the availability of complex carbon sources would be in limited supply (Loewen and Flett, 1984; Stroes-Gascoyne and West, 1996); however, it is also likely that the construction of the EBS will introduce some sources of carbon. Thus, there may be some enhanced capacity for metabolic activity of heterotrophic organisms vs. within the unperturbed rock. As mentioned previously, the near-field environment is expected to gradually become more reducing as the organisms present in this zone consume O_2 as a terminal electron acceptor and produce CO_2, or minor container corrosion occurs to consume O_2. Development of reducing conditions could enable anaerobic organisms like SRB to become active, providing that sufficient sulfate is present, along with the presence of appropriate electron donors like hydrogen. Within the appropriate pH conditions, the outcome of this metabolic process would be the production of bisulphide/HS^-, which could possibly diffuse through the clay buffer and contribute to corrosion of the metal container.

In addition to the constraints imposed on life by the subsurface environment described above, along with its predominant physical and chemical conditions, the selective pressure on organisms or groups of organisms, and how they will survive and/or proliferate, should also be considered. The following section focuses on a rather general discussion of microbial metabolic diversity, using as a point of departure Baas-Becking and Lourens' statement that "Everything is everywhere, but the environment selects" (1934). For example, organisms that require complex organic materials for their carbon and energy requirements (heterotrophic organisms) cannot grow in the absence of such compounds. However, heterotrophy may occur aerobically (using oxygen as terminal electron acceptor) or anaerobically (by facultative anaerobes using a range of alternate electron acceptors of varying oxidative states). Examples of this would be the initial predominant use of O_2 as electron acceptor for breakdown of organic material (such as would exist within a newly-constructed EBS). However, once all of the oxygen has been consumed, the next most-oxidized, relatively-abundant alternate electron acceptor (based on the chemistry of the geologic system) would then be utilized (e.g., NO_3^-). After all of the NO_3^- has been consumed, the electron acceptor would switch to NO_2^- followed by species such as manganese(IV), ferric iron, sulfate, and lastly, CO_2. Organisms generally use electron acceptors that yield the greatest amount of energy (Amend and Teske, 2005) providing that these species are sufficiently abundant.

Under saturated conditions, the deep subsurface environment is typically reducing, thus anaerobic processes predominate in these zones (Lovley, 2006). Anaerobic chemotrophic organisms can utilize various chemicals as sources of energy (generally redox reactions where electrons are shuttled between donor molecules to microbial electron acceptors, with liberation of potential energy), and therefore, depending on the chemistry of the system, have the potential for being highly active. The type of chemotrophy that occurs is dependent on the microbes, with some organisms requiring pre-formed organic materials as a source of energy (chemoorganotrophs or chemoheterotrophs), and others requiring or being able to use inorganic materials (chemolithotrophs). In the case of chemolithoautotrophic organisms (e.g., *Acidithiobacillus ferroxidans,* and *Nitrosonomas spp*.), the source of reducing energy comes from the geosphere and includes reduced inorganic electron donors such as H_2, NH_3, NO_2^-, Fe^{2+}, S^o and H_2S and the utilization of relatively oxidized compounds as electron acceptors (e.g., O_2, NO_3^-, SO_4^{2-}). Thus, when groundwater with high concentrations of reduced species of, for example, iron, manganese or sulfur, contacts the oxygenated environment of a newly commissioned DGR, the chemical gradients necessary for the early proliferation of chemolithoautotrophic bacteria will develop; of course, as oxygen is consumed, this proliferation will diminish substantially.

In order for a photosynthesis-independent ecosystem to be sustained, a primary reductant is necessary; in the subsurface, this reductant could be geologically-evolved hydrogen. In a paper published in Science, Stevens and McKinley (1995) offered proof for the existence of such a system, and presented evidence where autotrophic organisms outnumbered heterotrophs, and where stable carbon isotope analysis indicated that autotrophic methanogenesis was linked to the disappearance of inorganic carbon. They further demonstrated that the production of H_2 from the reaction of basaltic rock with anaerobic water supported microbial growth in laboratory experiments. Pedersen (1999a,b) offered support for a hydrogen-driven microbial ecosystem in that hydrogen (termed by Pedersen as “geogas”) can be generated from basaltic rocks by hydrolytic reactions of naturally-occurring radioisotopes with water. For example, the decay of alpha-emitting particles, such as radon and radium, causes water hydrolysis, yielding hydrogen. Pedersen’s model explains how the produced hydrogen would support the growth of methanogens and acetogens, as described in equations 1 and 2, respectively:

$$4H_2 + CO_2 \rightarrow CH_4 + 2H_2O$$ equation 1

$$4H_2 + 2CO_2 \rightarrow CH_3COOH + 2H_2O$$ equation 2

The products of these reactions would then provide the reduced carbon necessary to support other microorganisms in the H_2-driven ecosystem (for example: the production of methane and CO_2 from acetate; the production of H_2S, CO_2 and water from acetate and sulphate; and the production of water, ferrous iron and CO_2 from acetate and ferric iron (and acid)), as shown in equations 3, 4 and 5, respectively:

$$CH_3COOH \rightarrow CH_4 + CO_2$$ equation 3

$$CH_3COOH + SO_4^{2-} + 2H^+ \rightarrow 2CO_2 + 2H_2S + 2H_20$$ equation 4

$$CH_3COOH + 8\mathrm{FeOOH} + 16H^+ \rightarrow 2CO_2 + 8Fe^{2+} + 14H_20$$ equation 5

Fermentation reactions are also common under anaerobic conditions, but rely on the presence of organic compounds that function as either electron donors or electron acceptors. Typical fermentative end products include CO_2, H_2, acetate, propionate, butyrate, ethanol, lactate, and formate, with typical substrates including cellulosic and other carbon-rich materials (e.g., carbohydrates). Fermentation often occurs in syntrophic association with organisms that use H_2 and CO_2 for methane generation (it is well-known, for example, in the rumen), as well as those acetoclastic organisms that strictly use acetate for methanogenesis. Thus, where sufficient organic material exists, these organisms will play a functional metabolic role. Notably, within sedimentary rock environments, organic material is often not present in quantities that are sufficient to sustain significant fermentation reactions. In the EBS environment, any labile carbon source present in the bentonite buffer or backfill material, or transported along with the groundwater, would be expected to be consumed during either aerobic or anaerobic respiration (Wang and Francis, 2005). The largest contribution of organic matter in the EBS is expected to be from the buffer and backfill. Once materials for buffer and backfill have been chosen, their organic matter content and potential bioavailability for microbial processes should therefore be assessed further (OECD, 2012).

3.3 MICROBIAL GROWTH, ACTIVITY AND SURVIVAL

Microbial cells reproduce by binary fission, and thus grow exponentially (i.e., by doubling). A typical first order exponential growth equation (equation 6) can accordingly predict the change in the number of microorganisms over a given period of time (t), providing that the initial number (N_o) of bacteria is known as well as the organism's specific growth rate (μ).

$$N = N_o e^{\mu t} \qquad \text{equation 6}$$

Microbial growth in any habitat is dependent on the availability of nutrients, and is usually controlled by the concentration of the most limiting factor (Kieft and Phelps, 1997). Limiting factors could be major macronutrients (e.g., carbon, nitrogen or phosphorus), but could also be physical/chemical conditions such as osmotic pressure, temperature, or pH. In general, microbial growth rates in most deep subsurface environments have been estimated to be quite low, more than 1000's of times lower than rates observed for surface environments (Thorn and Ventullo, 1988), with doubling times on the order of centuries or longer (Chapelle and Lovley, 1990; Phelps et al., 1994a; Fredrickson and Onstott, 1996). As discussed above, it has been speculated that in some deep subsurface environments, microbial metabolism would primarily be directed towards cell maintenance rather than growth (Stevens, 1997). Considering the *in situ* growth rate potential for microbes in low-nutrient, subsurface habitats and the antimicrobial features of engineered barrier systems (low water activity, small pore size, high temperature, and radiation), the activity and reproductive potential for microbes is expected to be low in the near-field.

3.3.1 Spores, cell dormancy and death

The capacity for microorganisms to survive under extreme conditions is well known, and includes the cells entering either an inactive state (synonymously known as moribund, latency, dormancy, and cryptostatic) or resting state (sporulation) involving chemical-morphological

adaptations (Davey, 2011). The possibility that bacteria may survive via these inactive states requires consideration if such cells were present in the surrounding matrix of a DGR. Repositories positioned in argillaceous clay deposits create conditions likely conducive for cell entry into dormancy due to the low permeability environment and associated low-nutrient concentrations, accumulation of endogenous wastes, and low availability of water. The best known "resting-state" mechanism is that of the formation of an endospore (spore), these are heat- and desiccation-resistant, non-vegetative structures formed by members of, for example, *Bacillus* and *Clostridia* genera, that have undergone stress or changes in their growth conditions. Spores have a complex, layered cell wall containing high amounts of calcium, dipicolinic acid, and peptidoglycan, as well as a full copy of the cell's genome, and are much more capable of surviving stress conditions than their vegetative counterparts. Accordingly, spores undergo extended periods of dormancy until conditions are once again appropriate for vegetative growth and reportedly have been recovered from archeological samples as old as 15,000 to 40,000 years (Grant et al., 2000); reports of million year-old spores revived from insects entrapped in amber (Cano and Borucki, 1995; Greenblatt et al., 1999), though highly-controversial, have also been published. A variety of corrosion-causing bacteria, such as *Desulfosporosinus* spp. can form spores, and some of them have been isolated from bentonite clays such as those that would be used in EBS buffer materials. Consequently, in view of the longevity of bacterial spores that could be present within the EBS, repository safety assessments should take into account the development of corrosion-inducing environments, and the production of corrosive metabolites, that may result if hospitable conditions develop during the repository lifetime (i.e., sufficient nutrients and water, diminished radiation, decreased bulk density of buffer, etc).

3.3.2 Biofilms and Extracellular Polymeric Substances (EPS)

The role of microbial activity as a key control on the functioning of ecosystems is widely recognized. Typically, microbial aggregates and complex biofilm communities living on, or in association with, surfaces are characterized by increased activity (Rothemund et al., 1996; Kargi and Eker, 2003). This recognition of the importance of biofilms resulted in a remarkable increase in the number of studies in this field over the past two to three decades, which demonstrated that surface-associated growth has a distinct effect on microbial physiology and genetic regulation. In addition, attached cells can: i) utilize different carbon sources than planktonic (free-floating) cells; ii) produce different enzymes and extracellular polymeric substances (EPS); and iii) are more resistant to antimicrobial compounds. A generic description of a biofilm is the aggregation of microbial cells and their EPS on a surface. Numerous research articles and reviews describe biofilm formation as a sequence of events, and most of these descriptions have the common features as described by Busscher and Van der Mei (2006). These authors indicated that microbial adhesion to surfaces is the onset of biofilm development, which is typically preceded by the formation of a conditioning film of macromolecular components, which enables initial microbial adhesion. The initial stages of biofilm formation are typically described as reversible, but the formation becomes irreversible once the cells anchor themselves through EPS matrix production. Within this matrix, the cells start to grow and form microcolonies that ultimately lead to a mature biofilm from which viable cells are released back to the environment. Microbial transport to the substratum is typically enabled by microbial motility as well as mass transfer processes such as convection, diffusion, or sedimentation.

This generalized biofilm model, sometimes described as a community of cells, is not likely relevant to consolidated materials such as clays, bentonite, etc., and in the case of the near-

field repository would only be possible in: i) fractures; ii) areas of incomplete sealing at interfaces between different zones; or iii) fissures that form as a result of gas formation or uneven drying. In these cases, biofilm formation may enable increased microbial activity through synergistic interactions widely attributed to the biofilm mode of growth. However, even in the presence of larger void spaces that would allow multi-layered structures, low nutrient concentrations will probably inhibit extensive biofilm formation. Nevertheless, like all porous matrices, the microenvironment will be dominated by surfaces, and therefore it can be expected that microbes present will have a typical surface-associated existence. An interesting question is whether the advantages frequently attributed to the biofilm mode of growth will be of relevance in view of the very scarce nutrient supply and limited liquid flow. For example, increased EPS production by biofilm cells has been described as a mechanism to expand microbial habitat range by trapping nutrients and preventing desiccation; however, the energy requirements for EPS production is poorly described, especially in environments with limited water supply. Amongst the few articles on energy expenditure related to EPS production, Harder and Dijkhuizen (1983) calculated that in aqueous environments bacteria may invest more than 70% of their carbon and energy for the production of EPS. It is indeed possible that delineating the survival mechanisms of the surface-associated cells in oligotrophic, dense matrices may reveal information relevant to biofilms as a survival mechanism, which may provide more plausible information toward the understanding of survival in the deep subsurface environment than is currently known. Overall, introducing information gained through biofilm research may open up a new model or system for studying microbial metabolism in such environments.

Reference has been made to the potential relevance of biofilms during the storage of used nuclear fuel. It is possible that biofilms existing at the interfacial regions between the host rock and the buffer matrix, or the container and buffer, could function to effectively plug these regions. However, relevant biofilm research to date has focused primarily on those DGR's in groundwater systems in crystalline rock. Anderson et al. (2007) pointed out that the dominant transport medium for radionuclides in non-porous crystalline rock is groundwater flow through fractures, and that these fractures also support biological growth on fracture walls. These authors further indicate that the resulting biofilms can significantly affect subsurface geochemical interactions, and notably, that they decrease the rocks' adsorption capacity for a range of migrating radionuclides. A number of earlier studies examining the potential for biofilm formation in granitic systems have employed recirculating flow cells and biofilm samplers connected to boreholes with outlet valves set to maintain pressure close to that in the boreholes (Vandergraaf et al., 1997; Stroes-Gascoyne et al., 2000; Anderson et al., 2006). In the Anderson et al. study (2006), the authors described their observations on biofilms cultivated in flow cells that were connected in a closed loop with a borehole that intersected a hydraulically-conductive fracture zone, where they showed significant biofilm formation (up to 1.86×10^4 cells / mm^2) and the production of copious amounts of EPS. It was found that the EPS also had functional groups involved in adsorption, which led the authors to suggest that biosorption was mediated by functional groups on the microbial cell wall and the EPS matrix, without the need for energy from metabolically-active cells. In a subsequent manuscript from the research group (Jagevall et al., 2011), the presence of significant numbers of microorganisms on fracture surfaces of natural hard rock aquifer was also reported, as measured by quantitative polymerase chain reaction analyses and clone libraries. In general, the results examining biofilms in DGR systems indicate that they are most likely to develop in the groundwater-conducting aquifers in deep granitic rock. Overall, from these studies it is clear that the effect of biofilms, including the EPS matrix, should be considered when the fate of radionuclides that may escape from DGR's is assessed. Attachment-detachment dynamics, as well as facilitated transport of radionuclides by motile cells that can move much faster than groundwater flow,

should also be considered because biofilm formation can have a significant impact on the porosity and permeability of fractures and porous media (Coombs et al., 2010 and references therein). Biofilms can reduce fluid flow by constricting pore throats and increasing tortuosity of pore flow paths and in addition, can alter pH, redox, as well as groundwater and rock chemistry (Coombs et al., 2010). Porosity and permeability can be reduced by microbially-mediated precipitation (i.e., biomineralization), which can result in plugging or cementation of pore spaces (Coombs et al., 2010). However, biofilm growth that covers mineral surfaces can also potentially block access to sorption sites and decrease sorption (Bass et al., 2002). The relationship between planktonic (which could sorb and then transport radionuclides) and attached microbial communities in the subsurface is complex and poorly understood (Onstott et al., 2010) and will require site-specific investigation.

As reviewed by Sherwood Lollar (2011), the effect of microorganisms on geochemical conditions has implications for radionuclide fate since both pH and Eh can have significant impacts on solubility, sorption and transport of radionuclides. According to Bass et al. (2002) radionuclide solubilities are likely to be higher in the neutral to low pH far-field relative to the more alkaline near-field. The significance of microbial activity, and specifically biofilms, in this regard has yet to be established; there has been some *in situ* demonstration at Palmottu, Finland, at the Äspö Hard Rock Laboratory (Haveman et al., 1999), although the authors indicated that it was not possible to determine if the observed redox potentials of the sampled groundwaters were due to the reducing capability of the microbial populations identified.

3.4 MICROBES OF PARTICULAR RELEVANCE

A relatively large variety of microbial functional groups that may affect the overall performance of deep geologic storage facilities for used nuclear fuel has been described, including: i) those with the potential for directly damaging storage containers; ii) those organisms with potential for creating corrosion-aggressive environments where the diffusion of metabolic end products to the container could result in indirect container damage; iii) those that produce metabolites that could lead to the deterioration of EBS components such as concrete and seals; and iv) those that may impact the mobility of leaked radionuclides. Specific bacterial groups have received much attention in the literature for their known involvement in metal deterioration, such as the SRB and metal reducing bacteria. The potential for microbially influenced corrosion of used nuclear fuel containers is a consideration in safety assessments for DGR performance. However, to obtain realistic data, it is important that these metabolic reactions be considered in the context of the EBS environment (and also from a microbial ecology perspective). For example, as pointed out by Chen et al. (2011), the most likely corrosive agent in the groundwater to which containers will be exposed will be sulfide/bisulfide (formed by either mineral dissolution or produced by SRB). In their experiments performed in solution, they showed that once HS^- became depleted in solution, its diffusion in the bulk solution became rate-determining. Considering the extremely slow rate of microbial metabolism that prevails in low permeability environments, it is highly improbable that the source values for microbially-produced sulfide would be sufficient to create a notable concentration gradient (Chen et al., 2011). While the SRB may derive some benefit from the presence of other microorganisms (e.g., specifically those that lower the redox potential to levels conducive for SRB growth), they would also compete with these cells for nutrients in this oligotrophic environment, thus further limiting the source rates.

In addition to physical and chemical controls on microbial activity, microbial metabolic activity could also create conditions conducive to the growth of other specialized functional microbial

groups, or even organisms that might compete with them for scare resources. Typically, these microorganisms would be adapted for purposes other than just survival in oligotrophic environments and will span a variety of functional groups. One example is halophilic / halotolerant microorganisms. The potential for halophiles to be present within subsurface microbial communities is not surprising, as halophiles have long been isolated from most environments (particularly within soils where salts periodically become concentrated during dry periods) (Stewart, 1938). Extremely halophilic organisms have also have been detected, albeit in low numbers, in subsurface environments associated with highly-saline environments from the Waste Isolation Pilot Plant located 650 m below ground surface in a bedded salt formation located in Calsbad, New Mexico (Vreeland et al., 1998). In the long-term test experiment of buffer performance, the moderately-halophilic bacterium, *Desulfovibrio salexigens,* isolated from the deep groundwater of the Äspö hard rock laboratory (HRL), was used to evaluate bacterial survival under different clay swelling pressures (Pedersen, 2000). This organism did not survive as well, or penetrate as deeply into the clay (~6 mm), as did a *Bacillus subtilus* test strain; although, it should be pointed out that it is almost impossible to realistically simulate *in situ* conditions, including the role of microbial species interaction, when performing pure culture studies. In the context of a Canadian DGR in crystalline rock, salt concentration would not likely be sufficient so as to, by itself, inhibit microbial growth or survival. A guide for future considerations in this respect is the comprehensive study by Stroes-Gascoyne et al. (2007b) that examined the effect of salinity on the fate of microorganisms existing within bentonite buffers of differing dry densities relevant to the EBS environment.

While there is little evidence to suggest a strong presence of eukaryotes in the deep subsurface, some indication does exist that eukaryotes are present at depth (see also Section 3.1). For example, investigations of groundwater from the Äspö HRL have suggested that small fungi may inhabit Fennoscandian Shield igneous rock aquifers, although this would not likely have any direct relevance to a DGR (Pedersen, 2000). Given the unique metabolic processes that eukaryotes participate in, fungal chelating agents could offer possible mechanisms for radionuclide transport, and more generally, would affect the activity of those functional microbial groups that are of interest in the EBS environment.

3.5 CANADIAN MICROBIOLOGY RESEARCH CONDUCTED WITH RELEVANCE TO ADAPTIVE PHASED MANAGEMENT

The NWMO has agreements to freely exchange information resulting from research with agencies with similar used nuclear fuel repository concepts, including SKB (Sweden), POSIVA (Finland), NAGRA (Switzerland) and ANDRA (France) (Bennett and Gens, 2008). The following section is not an exhaustive review of Canadian research in this field, but rather a summary of findings from selected studies with significant involvement of Canadian scientists, along with relevance to future Canadian research. There have been three main activities that the Canadian research program has undertaken in support of APM: i) collaborative research at the Mont Terri Underground Rock Laboratory (URL) in Switzerland; ii) underground research at Atomic Energy of Canada Limited's URL in Pinawa, Manitoba; and iii) laboratory experiments assessing microbial growth, distribution and activity in bentonite buffer.

In terms of the microbiology in candidate host rock environments, the general description by Stroes-Gascoyne et al. (2011) of microbial presence/distribution and potential activity in Opalinus Clay cores from the Mont Terri URL, Switzerland, provides a realistic baseline against which other findings can be compared. The Mont Terri results suggest the presence of a small viable microbial community, based on: i) growth in 25% of inoculated cultures; ii) the presence

of phospholipid fatty acids (PLFAs) corresponding to 10^5 to 10^6 viable cells/gram dry weight that is indicative of anaerobic Gram negative bacteria and SRB; and iii) nutrients in quantities sufficient to support growth of indigenous and non-indigenous microorganisms for two months under suitable conditions. Conversely, the inability to detect active cells in 75% of core samples analyzed by microscopy-based methods, such as catalyzed reported deposition-fluorescence *in situ* hybridization, failure to extract polymerase chain reaction (PCR)-amplifiable DNA from cores, and up to 14 times more PLFA markers indicative of cell debris, rather than those indicative of viable cells, all point to a relatively low-abundance, low-activity microbial community. Considering the very small pore sizes and low water content in undisturbed clay, this is not an entirely unexpected result, and in fact, a body of evidence obtained from other systems have yielded similar results. However, the somewhat conflicting indicators (i.e., PLFA vs. PCR and culture data) do suggest that the available methods may not be adequate for quantifying microorganisms in high clay-content environments.

As pointed out by Stroes-Gascoyne et al. in their earlier studies, any disturbances that would provide space, water and nutrients (e.g., during excavation and construction) will probably revive dormant microorganisms. This possibility was subsequently evaluated when the authors (Stroes-Gascoyne et al., 2011b) performed a series of microbial analyses during an experiment on porewater chemistry, where a diverse microbial community consisting of aerobic and anaerobic species was found. In that porewater experiment, a test borehole was drilled (in the Opalinus Clay formation) and filled immediately after drilling with synthetic porewater using a chemical composition that could be expected *in situ*. The experimental setup allowed the introduced synthetic porewater to equilibrate with the porewater in the surrounding host rock through polyethylene filter screens with a mesh large enough so that microbes could freely pass through. A thriving microbial community was also found in the adjacent clay. Plausible explanations for this contrast with the low activity in the undisturbed clay were presented, and included: the possible introduction of microorganisms during drilling and installation of the experimental apparatus; as well as the introduction of nutrients and atmospheric air that revived dormant clay-based cells that were in numbers too low in the undisturbed environment to be detected with conventional and PCR-based methods. The likelihood that organic matter present in clay could have leached into the synthetic water through the filter screen with subsequent utilization by the introduced microorganisms, was also acknowledged. Of particular interest is that the increased microbial activity contributed significantly to the geochemical evolution of the synthetic porewater, which is an indication of the potential impact that microbial processes may have in the near-field following disturbance associated with drilling and installation of facilities. In a related study, Tournassat et al. (2011) applied reactive transport modelling to demonstrate that microbial processes were amongst the most important processes controlling the fluid composition. However, based on their simulations, it was concluded that factors such as the carbonate system [the redox state of the system is controlled by the S^{2-}/S^{6+} couple, whereby the S^{2-} and S^{6+} activities are buffered by pyrite (FeS_2) and a Fe-carbonate phase ($FeCO_3$) (Tournassat and Gaucher, 2004)], as well as the surface reactivity of the Opalinus Clay rock formation, resulted in a high buffering capacity against chemical perturbations due to microbial activity, the result of which being that the impact of microbial activity was predicted to extend only a few centimeters from the borehole. An important point raised by the authors was the reminder that biological activities are highly non-linear; therefore, processes occurring in the experimental borehole could not be modeled using a purely mechanistic approach without fitting parameters, which still leaves the challenge and need for physical model experiments to generate relevant data.

Microbial investigations at the AECL URL have found that heterotrophic aerobes, anaerobes and sulphate-reducing bacteria were present in the buffer, backfill and sealing materials studied.

Stroes-Gascoyne et al. (1997b) performed a microbiological investigation that used an electric heating system to simulate the heat that would be expected to emanate from a used nuclear fuel container in a Canadian repository as well as causing moisture content gradients in the surrounding buffer zone ranging from 13% closest to the heater, to 23% at the rock wall of the deposition hole. The temperature was 85°C at the heater, with a 20°C drop over each of the sand and the bentonite mix layers. The buffer consisted of an inner 5-cm layer of pure sand and an outer layer of a compacted mixture consisting of 50% sand and 50% sodium bentonite. The major finding after the 2.5-year emplacement period was that moisture content was the key determinant of microbial culturability. A range of temperatures were included in the incubations and it was found that many bacteria could be cultured from zones in the system with temperatures as high as 54°C, provided the moisture content was above 15%. Under the prevailing conditions, this translated to a water activity of 0.96. The authors cited an earlier study showing that SRB were viable after 60 days at a_w of 1, but that all cells died within one day when kept in bentonite at a_w of 0.96. Based on these observations, the authors suggested that in the case of used nuclear fuel storage, the initial high radiation emitted from the containers might act in concert with the low a_w in the area near the containers, along with temperature, to effectively sterilize the material, while the extremely small pore size of the buffer material would prevent microbial repopulation. Of note is that a 50% sand / 50% sodium bentonite buffer was used. Substituting the sand with 100% bentonite would result in lower free water available to bacteria, although the low moisture content (15%) in the 50/50% mix was sufficient to prevent microbial activity.

Laboratory experiments were also conducted with 100% bentonite to assess the effect of elevated temperature (60 to 130°C) and dry density on microbial viability and culturability (Stroes-Gascoyne and Hamon, 2010). Cells were not particularly sensitive to a temperature of 60°C and some culturability remained after exposure to 80°C at all dry densities. At temperatures ≥ 121°C, culturability was reduced in both low- and high-density bentonite samples. However, the effect of temperature on culturability in low dry density bentonite was reversible once the heat source was removed and saturation was allowed to occur, highlighting the importance of maintaining high dry density to keep microbial activity to a minimum (Stroes-Gascoyne and Hamon 2010).

Additional experiments were conducted with 100% highly compacted bentonite to identify repository conditions and possible design provisions that might be required to suppress microbial activity and prevent MIC of copper containers in a repository. Stroes-Gascoyne and Hamon (2010) assessed the culturability of microbes indigenous to Wyoming MX-80 bentonite when compacted and infused with $CaCl_2$ porewater, and compared the results with those of earlier experimentation (Stroes-Gascoyne et al., 2006) in which NaCl porewaters were used. In the 2006 study, the authors showed that microbial culturability remained at, or below, background levels (i.e., ≤ 2×10^2 Colony-Forming Units (CFU)/g) in highly-compacted bentonite buffer at a dry density of 1.6 g/cm^3 or using a porewater salinity of > 100 g NaCl/L at dry densities of 0.8–2.0 g/cm^3. In a more recent study, the authors (Stroes-Gascoyne and Hamon, 2008b) repeated the work over a range of NaCl porewater concentrations varying from 0 to 100 g/L at target dry densities ranging between 1.1 and 1.8 g/cm^3, and verified that a dry density of ≥ 1.6 g/cm^3, or a porewater salinity of > 50 g/L, would be needed to keep microbial culturability at or below background levels in the compacted 100% bentonite buffer. In these experiments, which lasted up to 92 days, R2A medium was used to cultivate aerobic and anaerobic heterotrophic bacteria, and modified Postgate B medium was used to cultivate SRB. The numbers of SRB were mostly below 10 MPN/g in the experiments with porewater salinities of 50 – 100 g/L. Similarly, the highest anaerobic heterotrophic counts were 7.5×10^1/g. It is possible that these numbers represented the bacterial cells that were indigenous to the bentonite and

survived the different saline levels without any growth at all. It is also possible that the activity of the aerobic microorganisms was not sufficient to create anaerobic microenvironments to initiate the growth of anaerobes over the 92-day incubation period.

4. APPROACHES AND METHODS FOR SUBSURFACE MICROBIOLOGY

As indicated by Davey (2011), repeated division of a cell on an agar surface, until a colony is visible, is the standard requirement in microbiology to affirm whether a cell is viable. However, what can be determined from the absence of colony formation is not so clear-cut. Contrary to the usual interpretation that a sample containing no visible signs of growth contains no viable cells, it may be possible that: i) the medium and/or incubation conditions were not conducive to support growth, ii) the cells were damaged/stressed and therefore unable to grow on solid medium, iii) the population density was too low for required cell-cell communication or cometabolic reactions to occur, resulting in no observable growth, and iv) insufficient time was allowed for visible colony development by slow growing cells (Davey, 2011). The author further pointed out that microscopy and flow cytometry offer an alternative approach to assess the viability of individual cells, when combined with fluorescent stains that discriminate subpopulations of cells according to characteristics such as metabolic activity, membrane energization or permeability, and RNA and/or DNA content. However, lack of agreement of different methods under *in situ* conditions can complicate interpretations of microbial activity (Stroes-Gascoyne et al. 2011c).

Figure 3 is a generalized representation of the continuum that exists between cells that are alive and dead, as described by Davey (2011), where the exact point of no return between alive and dead is difficult to determine. In most laboratory studies, or in clinical or industrial settings where microbial presence and / or numbers are determined, the emphasis is on obtaining the data in a relatively short time, seldom longer than a few weeks. Because the microorganisms evaluated in these instances typically have short generation times, such time scales are realistic; in fact, there are sustained efforts to develop more rapid analyses. However, relatively little mention has been made about the applicability of commonly-used microbiology techniques to account for the remarkably slow rates of microbial growth in the subsurface environment due to factors such as low concentrations of nutrients. Under these circumstances, the relative position of the perceived 'point of no return' will likely be different from what is generally applied in every-day (high nutrient) microbiology. For example, cells in consolidated subsurface environments go into a state of preservation in which the calculated doubling time, based on predicted rates of flux of growth requirements, can be on the order of centuries (see Section 3.3). Three forms of microbial existence can be proposed based on estimated CO_2 production rates and community doubling times in the literature: active growth, survival and preservation (Figure 4). The increasing number of successful resuscitations, described in the literature for ancient microbial cells sampled from the deep subsurface, is a clear indication that a time frame for the point of no return from alive to dead cannot definitively be assigned for microbial cells; rather, that environmental conditions have a strong influence on microbial persistence. There is a wide range of conditions under which microorganisms are uncultivable. There is, thus, a need to expand on the potential offered by genomic and proteomic techniques to delineate the viability of cells from these extreme environments. Similar to efforts aimed at deriving actual *in situ* metabolic rates in laboratory columns, a justified concern is that these methods may overestimate rates. This further emphasizes the importance of applying appropriate methods when performing subsurface microbiology, and, importantly, interacting with specialists from other disciplines.

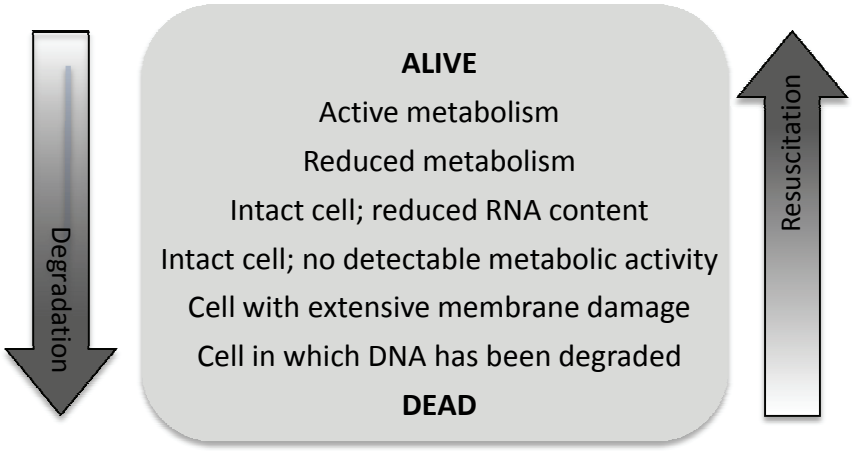
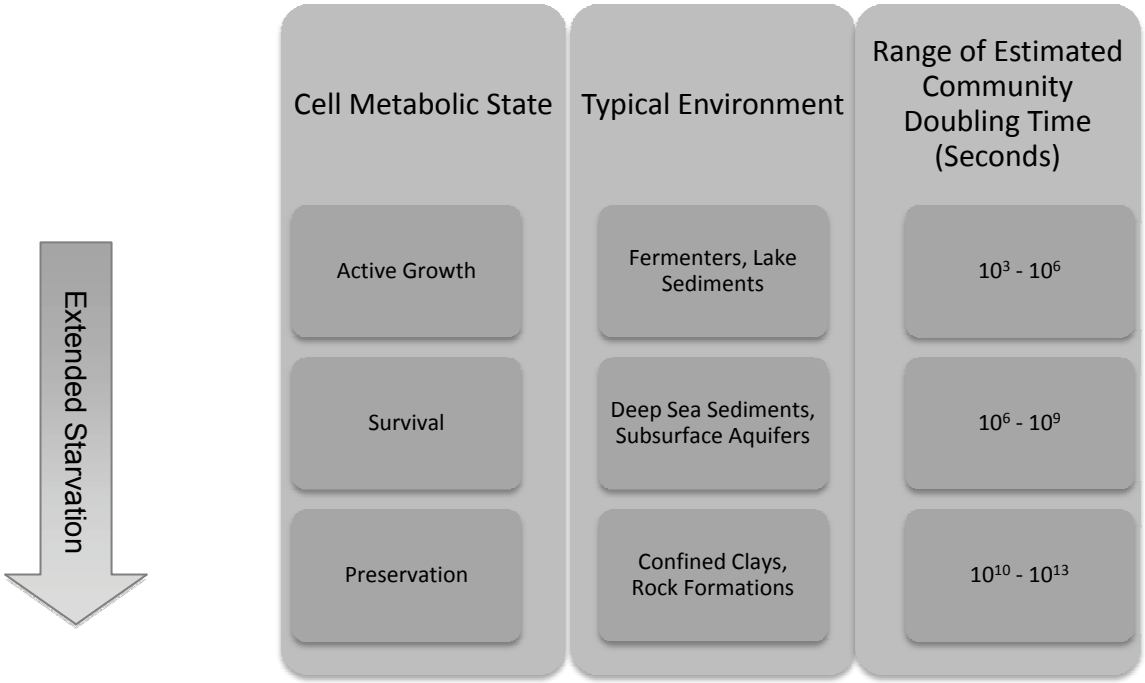


Figure 3: The route from live to dead for a microbial cell, adapted from Davey (2011); light end of the arrow is where the process is less likely to occur.



Note: Knowledge of rates in confined clays, dry vadoze zones and consolidated rock formations gives an indication of target rates to achieve minimal biotic impact, and information to estimate best scenario outcomes for DGR's.

Figure 4: Metabolic state and estimated community doubling times for cells in various environments as reported in the literature (see, for instance, manuscripts authored over the last 10 – 15 years by T.C. Onstott, T.J. Phelps, J.K. Fredrickson, T. Kieft, and others).

4.1 CULTURE-DEPENDENT AND CULTURE-INDEPENDENT APPROACHES

The discipline of microbial ecology has yielded a growing array of methods appropriate for the analysis of complex microbial communities. These developments have stemmed from: i) the increasing realization that only a small fraction of the total microbial diversity has been discovered (<1-5%), with an even smaller proportion of this diversity actually being culturable *in vitro* (Bass et al., 2002; Colwell and Leadbetter, 2007), and ii) rapid advances in molecular techniques. One result of limitations associated with culture-based methods is that our current view of subsurface microbiology remains limited in terms of total diversity and metabolic potential. More recent efforts have incorporated aspects of both culture-dependent and culture-independent analyses to improve sensitivity to the various organisms that exist in these systems. A growing body of ecological work has focused on the microbiological assessment of DGR concepts. Such studies have typically focused on two areas: i) characterization of the geological site, including the diversity of microbes that inhabit the environment (Mauclaire et al., 2007; Basso et al., 2009; Rastogi et al., 2009); and ii) evaluation of the potential effects that microbial metabolic activity might have on the stability of the used nuclear fuel containers and the near-field environment (Herrera and Videla, 2009; El Hajj et al., 2010). An overview of culture-dependent and culture-independent methodologies suitable for the analysis of microbial phenomena associated with nuclear waste repositories is provided below.

4.1.1 Culture-dependent methods

Subsurface microbial communities include a diverse mixture of microorganisms whose presence and relative abundance reflect the predominant environmental and chemical conditions. Culture-based approaches have been used to “reconstruct” culturable isolates into operative communities employing the use of selective (e.g., for SRB, iron- and manganese-reducing bacteria, methanogens, acetogens, methanotrophs; Basso et al., 2009; Kaster et al., 2009; Stroes-Gascoyne et al., 2007c) and non-selective (e.g., trypticase soy broth, plate count, m-HPC, R2A and NWRI agar; Lawrence et al., 2000; Stroes-Gascoyne et al., 2007c; Pedersen et al., 2008) sets of media and culture conditions (APHA, 2005). Due to the slow growth tendencies of the organisms present, and the broad range of environmental conditions, incubation times may range from days to weeks; whereas, incubation temperatures can span the full range of temperatures where microbial growth is known to occur. The use of different medium composition or growth conditions can be expected to influence both the numbers and types of microorganisms detected.

Culture-based methods have been applied to characterize microorganisms from environments and matrices associated with DGR’s (Stroes-Gascoyne et al., 2007c; Fru and Athar, 2008; Hallbeck and Pedersen, 2008; Poulain et al., 2008). It is also appreciated that the sub-surface environment chemistry will impact the type of organisms that are likely to be found (i.e., the environment selects the microbial community). The corollary is that culture-based approaches typically target physiologic groups that match the types of electron acceptors and donors that dominate the environment (Reed et al., 2002; Miller et al., 2010; Wilkins et al., 2010). Indeed, improved culture-based results have been obtained by simulating the original habitat of the microorganisms (Zinder and Salyers, 2001). However, while refining a medium for an particular environment (e.g., containing relevant concentrations of nutrients and micronutrients, appropriate pH, solutes, etc.) can improve recovery and growth of specific organisms, the actual functional role of those organisms may not be reflected by the conditions under which they were isolated and enumerated. For example, isolates may use oxygen as a terminal electron

acceptor in the laboratory even though a different terminal electron acceptor was used *in situ*. Lastly, integration of liquid culture media with the most probable number (MPN) technique (APHA, 2005) has been shown to improve estimations of the numbers of the targeted organisms (Phelps et al., 1994a,b; Lawrence et al., 2004; Basso et al., 2009; Wilkins et al., 2010); however, the method is biased to those organisms which grow in solution.

While culture-based methods are not sensitive to all organisms of potential interest, they have enabled the isolation and subsequent detailed investigation of numerous microorganisms, and proven instrumental to a variety of studies of relevance to subsurface DGR's. These include: i) quantifying the survival of key microorganisms under controlled (e.g., atmosphere, pH, porosity, radiation, temperature) experimental conditions (Bagwell et al., 2008); ii) defining multi-species trophic interactions, electron transfer events, and metal reduction potential (Miller et al., 2010); estimating microbial involvement in radionuclide sorption and transport phenomena (Anderson et al., 2007); iii) describing microbe-bentonite barrier interactions (Perdrial et al., 2009); and iv) assessing microbially influenced metal corrosion (Alfaro-Cuevas-Villanueva et al., 2006). It has also become standard practice to employ 16s rRNA gene polymerase chain reaction (PCR)-sequencing (see below) to identify organisms isolated using culture-based methods. Accordingly, culture-based techniques have isolated and enumerated Bacteria and Archaea representative of most phyla (e.g., firmicutes, α, β, δ, γ, ε groups of proteobacteria) for subsequent study and archival storage from a diverse range of environments.

Determining microbial activity

Radiolabeled substrates have been used to estimate microbial metabolic potential in various environments, including deep subsurface sedimentary matrices, river sediments and biofilms, as well as estuarine and freshwater sediments (Phelps et al., 1994b; Wellsbury et al., 1996; Lawrence et al., 2008a,b). Accordingly, radiolabeled (^{14}C, ^{3}H, ^{35}S) amino acids, sulfate, acetate, bicarbonate, carbohydrates and nucleotides have been used to demonstrate involvement of microorganisms in various key environmental processes such as methanogenesis and sulfur reduction (Wellsbury et al., 1996; Masurat et al., 2010b).

Useful variations on culture-dependent approaches include quantifying community metabolic potential (e.g., the Biolog system) to characterize the ability of isolates or microbial communities to utilize various sole carbon sources. This approach has been adapted for the study of various environmental samples, including soil, water and biofilms (Lehman et al., 1995; Lawrence et al., 2005; Massol-Deya, 2005). Tiquia et al. (2008) screened Rouge River bacterial communities from shallow groundwater and river water samples using Biolog Eco-Plates, revealing significant differences between river water and groundwater samples. While carbohydrates, polymers and amino acids were highly-utilized by the microbial communities in the river and groundwater samples, river samples were found to utilize carboxylic acids, and groundwater samples utilized phenolic compounds. However, it is noteworthy that the Biolog sole carbon source utilization approach has not seen extensive use in subsurface environments relevant to DGR's.

The concept of organisms being viable but non-culturable (VBNC) underlies a key issue associated with obtaining representative data using culture-based techniques from various ecological systems. There are basically two situations under which living microbes might be considered "unculturable": i) when formerly-culturable organisms undergo some physiological shift as a consequence of a change in their environment, including, for example, a decline in nutrient availability, an increase in the concentration of inhibitory compounds, or cellular damage and/or stress; and ii) when organisms have highly-stringent growth requirements involving nutritive or biological factors. Either situation could occur in subsurface environments,

particularly within the near-field of a DGR, where interplay between temperature, water availability and radiation could result in varying degrees of stress. The *in situ* roles that some of these formerly under-represented organisms play in the geosphere is only recently coming to light through the development and use of culture-independent methods (Smith et al., 2000; Liu and Stahl, 2007; Poulain et al., 2008; Masurat et al., 2010b).

4.1.2 Culture-independent methods

Molecular approaches for profiling microbial community diversity and assigning evolutionary relationships (phylogeny) to community members continue to rapidly evolve (Amann et al., 1995; Hugenholtz and Pace, 1996; Hugenholtz et al., 1998; Liu and Stahl, 2007). Most molecular techniques used in ecological studies (including subsurface and groundwater systems) are DNA-based (although RNA may also be converted to a cDNA copy, and used for gene expression analysis), where total nucleic acids are extracted from an environmental sample, are PCR-amplified, and then analyzed through techniques such as “shotgun cloning” (e.g., creation of a clone library) (Basso et al., 2009; Rastogi et al., 2009), or DNA/cDNA probe hybridization (i.e., the Phylochip; Yergeau et al., 2009, 2010). Direct sequencing (e.g., 454 pyrosequencing) (Biddle et al., 2008) of total extracted DNA is gaining rapid acceptance for the study of community diversity and is seemingly paralleling the exponential growth in DNA sequencing speed and capacity. Analysis of PCR amplicons may also be used to provide a fingerprint of the genetic diversity of the community; PCR is used to amplify specific genetic markers from community DNA, followed by amplicon separation and/or melting-point behaviour determined using electrophoresis, or amplicon fragment length polymorphisms (Liu and Stahl, 2007; Muyzer et al., 1993; Muyzer and Smalla, 1998). The above approaches have seen growing use in the study of environmental systems, which are known to consist largely of uncultivated microorganisms. The following section reviews the application of molecular techniques for the analysis of microbiological community diversity in subsurface systems, particularly as employed for the study of deep geologic repositories; a schematic overview of culture-independent analyses is provided in Figure 5.

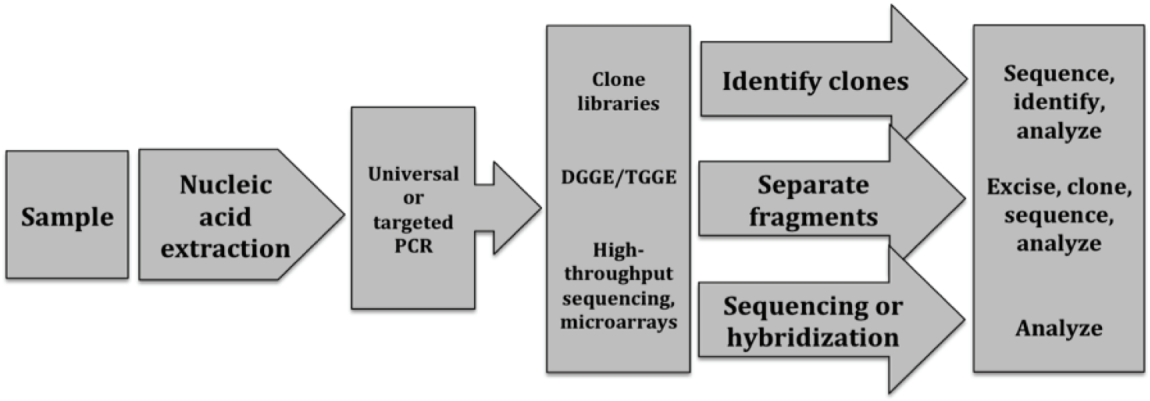


Figure 5: Overview of culture-independent options for the examination of complex microbial communities (adapted from Humphreys et al., 2010).

Extraction of nucleic acids

A variety of methods have evolved which are suitable for extraction of nucleic acids from subsurface sediments, including a number that have the advantage of being commercially-available (e.g., the FastDNA SPIN Kit for Soil [MP Biomedicals, Inc., ON, Canada] or the UltraClean Soil DNA Isolation and RNA PowerSoil® Total RNA Isolation kits from Mo Bio Laboratories, Inc. [Carlsbad, CA, USA]). However, the success of extraction of viable DNA using kits or otherwise, is often environment-specific. Extraction of DNA from subsurface microbial communities can, for example, be confounded by the presence of organic matter and clay materials, which complex and co-extract with the nucleic acids and interfere with nucleic acid purification due to their strong binding tendencies. To extract DNA from cells, it is necessary to break the cells' membranes; certain bacteria require more aggressive cell lysis techniques (i.e., bead-beating) to disrupt the cells and release nucleic acids (i.e., spores versus vegetative cells). The requirement for longer exposures to these techniques correlate with clay and humic/fulvic acid content of the matrix, and thus shearing of DNA increasingly becomes an issue to the point where biased results may result (Hirsch et al., 2010) (e.g., metagenomic libraries require larger genome fragments than 16s rRNA-based analyses). DNA-binding interference by clays was proposed following microbiological studies of Opalinus clay samples, where culture-independent detection methods failed although PLFA and enrichment cultures demonstrated the presence of low numbers of viable cells (Mauclaire et al., 2007; Poulain et al., 2008). The inhibition of RNA-degrading enzymes (RNases), and elimination of contaminating DNA, is also critical in studies where RNA is to be extracted. Sample handling for gene array work involves: i) the initial extraction of the DNA/RNA from the environmental sample (microbial community) (Kieft et al., 2007); and ii) amplification of the genomic DNA using universal bacterial and archaeal primers targeting the 16s rRNA gene, or the preparation of cDNA from mRNA, with each step presenting the potential to material loss. Sample integrity is especially critical for work with mRNA, which is easily contaminated and rapidly-degraded at room temperature.

Target genes for microbial community analysis

Microbial community DNA analysis typically initiates with PCR amplification, which uses specially-designed and custom-synthesized oligonucleotide primers in conjunction with DNA-polymerase, to amplify target DNA specific for: target organisms, domains of organisms (e.g., Archaea or Bacteria, providing estimates of community biodiversity or taxa richness), metabolic processes (various functional genes), or whole genome amplification (WGA). The amplified DNA is characterized in a number of different ways, including cloning and sequencing (clone libraries) as well as DNA fingerprinting (i.e., DGGE, T-RFLP). Either approach typically involves the sequencing of the isolated and purified fragments of the PCR-amplified DNA, followed by comparison with public databases for identification. Despite an on-going lack of consensus on what defines a prokaryotic "species", the PCR target generally considered as the gold standard for biodiversity work is the sequence of the small ribosomal subunit 16s rRNA, or more commonly, its gene (Ward et al., 1990). The 16s rRNA gene is universal in bacteria and is highly-conserved (Pace et al., 1986; Speigelman et al., 2005; Liu and Stahl, 2007; Sanz and Kochling 2007; Malik et al., 2008), permitting sequence alignments, and possesses highly-specific regions of variability, thereby enabling discrimination of different organisms or groups of organisms. For example, primers specific for the amplification of 16s rRNA genes of the domain Bacteria (Lane, 1991; Nakatsu et al., 2000), domain Archaea (Ovreas et al., 1997), methanogenic Bacteria (Torsvik et al., 1993; Ovreas et al., 1997), sulphate reducing bacteria (Amann et al., 1992; Devereux et al., 1992), Legionella (Calvo-Bado et al., 2003), α-proteobacteria, β-proteobacteria, Bacilli, and Actinobacteria (Blackwood et al., 2005) from a

variety of habitats, have been successfully developed. The 16s rRNA molecule also functions as what is known as a molecular chronometer, and accordingly sequence comparisons enable the phylogenetic analysis (study of genetic relatedness) of sequences from different organisms.

Despite the primary advantage of very large and highly-evolved databases (EMBL; http://www.ebi.ac.uk/, RDP10; Cole et al., 2009), the use of 16s rRNA as a PCR target has potential drawbacks. Multiple, heterogeneous copies are common in bacteria isolated from the environment, and may lead to the detection of multiple ribotypes within a single species. Other organisms may simply not reliably produce 16s rRNA PCR amplicons. Therefore, 16s rRNA gene-based community analysis may result in an overestimation of microbial diversity (Case et al., 2007).

Community-level fingerprinting methods

PCR-derived methods have evolved for the culture-independent fingerprinting, or characterization, of complex microbial communities. Temperature gradient gel electrophoresis (TGGE) and denaturing gradient gel electrophoresis (DGGE) both separate amplified 16s rDNA fragments of the same length using electrophoresis, but with different base pair (bp) combinations and melting-point behaviour under denaturing conditions. In DGGE, the denaturants used a mixture of urea and formamide (Muyzer and Smalla 1998; Muyzer et al., 1993, 2004). In TGGE, the denaturant is heat. A high melting domain, known as a GC clamp, is added to one of the primers to prevent complete DNA strand separation. Both DGGE and TGGE enable the detection of almost all possible sequence variations (Sheffield et al., 1989; Muyzer et al., 1993), including separation of single nucleotide variants. During the analysis of microbial communities, the number of bands produced on the gel is proportional to the number of dominant species in the system (Muyzer et al., 2004; Malik et al., 2008).

The PCR-DGGE method has provided microbiologists a means of characterizing the microbial community diversity associated with soils (Nakatsu, 2007; Nakatsu et al., 2000), water (Casamayor et al., 2000; Araya et al., 2003; Jin and Kelly 2007; Lawrence et al., 2008a,b), microbial mats and bacterial biofilms (Muyzer et al., 1993; Ferris et al., 1996). Functional-target DGGE is a relatively simple procedure which involves amplification of catabolic genes (instead of the 16s rRNA gene) linked to various metabolic or other functional processes in a community (Spiegelman et al., 2005), and has also seen growing use for the analysis of subsurface systems relevant to DGR's. Fru and Athar (2008) used 16s rRNA and dissimilatory sulfate reductase-targeted DGGE to aid in the comparison of the microbial community in compacted bentonite with the surrounding aquifer microbes *in situ* at the Äspö HRL in Sweden. Functional gene-DGGE has been performed on a soil microbial community using particulate methane monooxygenase (*pmoA)* gene (Pacheo-Oliver et al., 2002). These studies also provide the basis for targeted studies focusing on a specific process or processes. Dar et al. (2005, 2007) examined the functional gene for dissimilatory sulfite reductase (*dsrB*) in SRB populations; the detection of the presence of the *dsrB* mRNA presumably corresponded to the metabolic activity of SRB at the time of sampling.

It has been estimated that PCR-DGGE is capable of only detecting those community members that represent at least 2% of the overall community, and thus characterization of extremely complex communities is complicated due to the production of numerous weak bands (Spiegelman et al., 2005). Despite this limitation, the DGGE approach, when combined with other microbiological methods, has contributed significantly to our understanding of the genetic diversity of uncharacterized microbial populations in natural environments. Other community-level gene fingerprinting methods include terminal restriction fragment length polymorphism (T-

RFLP) – an enhanced fingerprinting method based on restriction fragment length polymorphism (RFLP). The method involves the PCR amplification of a conserved genetic target (i.e., the gene for small subunit rRNA molecules like 16s rRNA), followed by restriction enzyme digestion and gel fractionation of the digest, where one of the two primers is tagged with a fluorescent probe at its terminal end. These fluorescent products are then detected using laser-induced fluorescence; such well-established PCR-based molecular techniques have become important tools for microbial community analyses. These types of fingerprinting methods are useful for rapid comparative analyses of bacterial populations. However, the most preferable methods to identify individual members of microbial populations generally involve PCR, cloning and sequencing of specific DNA-sequence targets.

The direct cloning and sequencing approach is not considered a fingerprinting method, and although more labour-intensive, it provides a higher-resolution phylogenetic picture of the microbial community. PCR-amplified fragments of target genes (e.g., full length *16s rRNA* or *cpn60a* genes) are ligated directly into appropriate vectors (e.g., pGEM-T from Promega, Madison WI; TOPO from Invitrogen Canada, Burlington, ON) that are then used to transform host *E. coli* cells. Following overnight incubation, individually cloned colonies yield plasmids containing a single gene insertion. Sequence comparison of individual clones with the Ribosomal Database Project II (RDPII), European Molecular Biology Laboratory (EMBL), and cpn60 (Hill et al., 2004; http://cpndb.cbr.nrc.ca) databases permits identification of the individual clones and construction of a phylogenetic tree of the microbial community, as well as an assessment of the most predominant clones in the system (Pronk et al., 2008).

Metagenomic methods

Options for more intensive coverage of diversity, and better estimates of the relative abundance of different cell types, can be obtained from sequence analysis of PCR amplified community DNA (typically using 16s rRNA targets) from an environment, used in conjunction with high-throughput, large-scale parallel DNA sequencing platforms such as 454 Pyro-sequencers or Illumina platforms. The “454 sequencing” system, using the GS FLX Titanium Series, offers sequencing read lengths of up to 1,000 bp along with greater than 600 million bp per run. Other systems provide shorter read lengths, but can sequence a greater number of DNA strands; Applied Biosystems SOLiD™3 system claims 10-15 Gbp (gigabase pairs) total sequence data per run with reads of 75 bp. Ion Torrent sequencers (Glenn, 2011), as of 2012, offered read lengths of ~200 bp, and up to 1 billion bases of sequencing information in a chip-based sequencing platform, with notably lower instrument cost. In general, metagenomic high-throughput systems offer “deeper” analyses than alternative community fingerprinting or clone library approaches, and are not limited by low sensitivity to rare sequences, a problem associated with gel-base fingerprint methods like DGGE.

High-density phylogenetic arrays, such as the Affymetrix G3 PhyloChip, were designed based on Bacterial and Archaeal 16s rRNA sequences available in the 2007 public databases (Yergeau et al., 2009), offering the detection of up to 32,000 unique 16s RNA gene sequences as a high-throughput method for characterizing the diversity of DNA extracted from different environments. Transcript expression arrays can also be used. For example, anonymous DNA microarrays have been used for meta-transcriptomic studies of organisms whose genomes have not been sequenced (Cannon et al., 2006; Hegarty et al., 2005; Parro and Moreno-Paz, 2003; Yergeau et al., 2010). Using this approach, cDNA is hybridized to unsequenced DNA or cDNA fragments from a microbial community that are printed on a microarray, and only the probes that display significant differential responses (relative to the control) to the treatment of interest (e.g., a pollutant, carbon source electron acceptor) are then identified via sequencing

(Yergeau et al., 2009). This provides a large array of functional information, which in the case of samples from unique and extreme environments could yield valuable insight into the dominant active metabolic processes.

Despite the apparent advantage of having increased sensitivity to a large proportion of the microbial community rather than culturable organisms alone, molecular techniques are subject to various limitations and biases. For example, initial harsh extraction methods shear DNA; various extraction methods produce different yields; co-extraction of inhibitory molecules may interfere with subsequent techniques; purification steps may lead to loss of DNA or RNA (von Wintzingerode et al., 1997; Kirk et al., 2004). Also, the detection of genes representative of a particular organism, or group of organisms, does not reflect either the viability or activity of those organisms *in situ*. Differences also exist in terms of DNA extraction efficiency, PCR sequence bias, as well as sequencing accuracy and comprehensiveness. Therefore, careful interpretation, as well as appropriate use of controls, is an essential prerequisite for community-level genetic characterization work, just as it is for culture-dependent analyses.

4.2 MICROBIAL BIOMARKERS

The sparse distribution of microorganisms in many subsurface environments, together with difficulties associated with culture-based analysis, complicate the quantitative determination of microbial abundance and activity in these environments. Phospholipid biomarker techniques are based on the ubiquitous presence of phospholipid fatty acids (PLFA), which are found in cell membranes. Cell membrane PLFA chemistry in living cells is highly responsive to changes in cell metabolic status, and can also be used to discriminate between different bacterial groups (Guckert et al., 1986; Vestal and White, 1989; Hedrick et al., 2005, 2007). PLFA profile analysis provides culture-independent information on viable biomass, microbial community structure, as well as the nutritionally-related physiological status of the resident cells (Ringelberg et al., 1997). Ester-linked PLFA undergo rapid hydrolysis via phospholipid esterase (phospholipase) activity after cell death; thus, the bacterial membrane is rapidly degraded with the subsequent release of the polar (hydrophilic) head groups (White et al., 1979). The activity of the phospholipase on membrane phospholipid fatty acids releases diglyceride fatty acids (DGFA), and the relative abundance of which (PLFA:DGFA) can be used to estimate viable and non-viable microbial biomass. This is particularly relevant to potential DGR sites in clastic sedimentary host rock (e.g., Opalinus clays from the Mont Terri Site, Switzerland) where the culturability and even existence of active microbial populations remains controversial (Mauclaire et al., 2007; Stroes-Gascoyne et al., 2007c). PLFA results from these studies suggest that low numbers of primarily Gram-negative bacteria and SRB exist within the Opalinus clays. The approach has been used by Stroes-Gascoyne et al. (2002) during an *in situ* study at the AECL URL, as well as by Kieft et al. (1997) and Wang and Francis (2005) as part of the microbial characterization of Yucca Mountain tuff samples.

Bacterial lipids are known to undergo significant changes under conditions of gradual starvation (Guckert et al., 1986). The possibility of cells gradually entering an extended starvation state, rather than simply dying, may complicate use of PLFA as a reliable biomarker. Stroes-Gascoyne and Hamon (2010) attempted to evaluate this in their work by deliberately killing bacterial cells in a bentonite matrix, and found the PLFA content corresponded with the cell condition. However, the effect of an extended, gradual cell death – and distinction between cell death and preservation (see Figure 3) – requires more study. Cells fully-entrained from deposition within a water-limited (low a_w) matrix, such as clays with a bulk density approaching 2.0 g/cm^3, may further interfere with the normal degradation of PLFAs in cells, as well as PLFA

extraction, possibly straining the interpretation of PLFA data. Stroes-Gascoyne and Hamon (2010) further suggested that PLFA hydrolysis (i.e., degradation) may be slow, and that the PLFA's in viable cells (with active lipolytic activity) may form complexes with the high density bentonite thereby preserving the PLFA's in a non-representative condition. Accordingly, PLFA results revealing the presence of biomarkers indicative of eubacteria (15:0, 15:0 iso, 15:0 anteiso, and 18:1x7 c) and SRB (C17 monounsaturated fatty acids) in the 170 million year-old Opalinus Clays requires additional study. Due to the potential for misleading results, PLFA analyses have typically been paired with an alternate method of analysis. Extraction and analysis of community DNA (see above) and culture-based methods have commonly been used; however, the different methods have not always been in agreement.

4.3 IN SITU STUDIES AND LABORATORY MICROCOSMS

Countries with nuclear energy programs (e.g., Sweden, USA, Japan, UK, and Canada) have conducted both laboratory and *in situ* studies to evaluate possible effects of microbial activity on EBS components (i.e., container, buffer and backfill materials) and repository environments. Laboratory experiments often evaluate microbial processes in repository materials under relevant environmental conditions using microcosms (an experimental system of reduced size encapsulating the characteristic features of a larger system). Microcosm experiments have generated considerable information about the survival of microorganisms in bentonite under different environmental conditions such as salinity, temperature and radiation, as described in Section 3.5. Microbiology research in underground research laboratories (URLs) has served two general purposes: i) to evaluate the microbiology of the host rock; and ii) to assess the impact of microorganisms on EBS components using demonstration experiments.

In Canada, many studies have taken place at the (now closed) AECL Underground Rock Laboratory (URL) (Stroes-Gascoyne, 2010; Jain et al., 1997; Stroes-Gascoyne et al., 1996a, 1997b, 2002), yielding data on microbiological occurrence, diversity and survival in buffer and backfill materials, as well as from the surrounding granitic rock groundwaters. The Äspö Hard Rock Laboratory (HRL) in Sweden has similarly been integral to the research programs of the Finnish and Swedish nuclear waste agencies. Results from studies conducted at these laboratories have demonstrated that diverse communities exist and thus have potential to be present, or intrude upon, an EBS during or soon after construction in crystalline environments. A diverse range of microbial groups have been identified in crystalline URLs, including autotrophic acetogens, heterotophic acetogens, sulphate reducing, and iron reducing bacteria.

Perhaps the best-studied sedimentary rock underground laboratory is located at Mont Terri, Switzerland (Mauclaire et al., 2007; Stroes-Gascoyne et al., 2007c, 2011b; Poulain et al., 2008), yielding valuable *in situ* data on the occurrence and potential effects of microorganisms within low-permeability clay matrices. Investigations at Mont Terri have been carried out to determine whether low permeability clay environments can prevent movement and growth of bacteria under DGR conditions. Transport of bacteria through the buffer matrices has also been investigated using compacted bentonite model systems, and it has been demonstrated that surrogate bacteria (*Pseudomonas stutzeri*) fail to repopulate a sterilized zone in the presence of liquid, due to the restrictive pore spaces in the matrix. While others (Pusch, 1999; Pedersen et al., 2000a,b) have similarly concluded that buffer clays would prevent the transport of cells in clays, Stroes-Gascoyne and West (1997) observed that the interface zones represented a "special case" where the organisms may persist without penetrating the matrix phase warranting further investigation.

Microcosm studies (e.g., as discussed by Humphreys et al., 2010) have examined the potential for direct microbe-radionuclide interactions, particularly examining the potential for the radionuclides to be involved in redox reactions with prospective changes in radionuclide speciation. Studies focusing on the potential of microbes to be involved in metal container corrosion have similarly been conducted. Most of these studies have been performed using a batch-culture or pressure-cell system approach. Examples of these designs include those described by Fernandez et al. (2006), Huertas et al. (2009) and Stroes-Gascoyne et al. (2011b). It should be emphasized though, that natural subsurface systems are open systems, while microcosms are closed systems with definite boundary layers. The extremely low rates of nutrient and metabolite flux in the consolidated materials, and typical low groundwater flow rates in fissures, necessitate extended time frames of experimentation and make it difficult to design representative model systems. For instance, Pedersen (2010) concluded that microbial sulphate reduction activity in a representative bentonite matrix would be too slow to be resolved by current experimental designs. Nevertheless, access to *in situ* environments is limited and related experimentation is costly. Therefore, there continues to be a need for improved and relevant microcosm studies; efforts should be made to develop techniques that are suitable for these model systems.

Proper sample handling with due attention to aseptic techniques is a critical component of any microbiological investigation. Therefore, in the case of research focused on the isolation and characterization of microorganisms from sites where the presence of a living and active population of microorganisms is subject to question, or any environment where the cells are known to be present at a low abundance, “clean” techniques are especially important. In addition for the need to prevent contamination, the low population densities in materials relevant to the near-field also require extreme caution to preserve the material.

4.4 INTERACTION AND EXCHANGE OF INFORMATION BETWEEN EXPERIMENTAL RESEARCHERS AND MODELLERS

Humphreys et al. (2010) provided an extensive overview of, and a number of useful references on, the development of numerical models for examining the effects of microbiological processes on used nuclear materials. The authors emphasized both the complexity involved in defining these processes and the importance of these effects being quantified in order to provide information to better predict the long-term function of facilities. The authors also pointed out that although the development of these modelling studies and associated codes typically follow a general pattern, including iterative steps from concept to application, the application is diverse and dependent upon factors such as the type of material, type of storage system, the geological environment and exposure pathways of interest. There is thus a significant degree of user interpretation, and as shown in the following two examples, assumptions being made.

Jolley et al. (2003) applied the computer code Microbial Impacts to the Near-Field Environment Geochemistry (MING), which offers a forward-predictive capability to evaluate the effects of microbial communities. Using environmental thresholds for limiting microbial growth to temperatures below 120^{o}C and relative humidity to 90%, this code uses kinetic rates to supply elements from design materials and constituent fluxes to perform separate mass balance calculations as a function of time to estimate biofilm production, CO_2 production, radionuclide sorption, microbial colloid formation and siderophore production. Interestingly, parameters such as salinity, pH, size, and radiation are excluded from the model because, as the authors explained, the effects of these parameters do not impact the results of the calculations, provided that specific assumptions are made with regard to the types of organisms that will be involved

(e.g., it is assumed that halophiles, acidophiles, or radio-resistant organisms will dominate at high salt concentrations, low pH, or elevated radiation doses, respectively).

Askarieh et al. (2000) described use of the GAMMON model to estimate cellulose degradation and gas generation in a repository for low and intermediate level waste. Evaluation of the three possible scenarios considered in their exercise emphasized the need to critically assess the questions being asked, assumptions made, and linkages with experimental data. For instance, in the first scenario considered, in physical experiments the majority of cellulose was degraded within a timescale that was sufficiently short, so that degradation would be expected to be complete by the time of repository closure. This is much faster than estimated from the GAMMON calculations, which estimated that complete degradation of cellulose would take a few hundred years. In the second scenario, degradation of only the amorphous form of cellulose was assumed to occur, and the extent of degradation measured in laboratory experiments was believed to be similar to degradation of the amorphous form, which is typically assumed to be approximately 10% of the total cellulose present. Because the degradation of amorphous cellulose occurs rather fast, it was assumed that all amorphous cellulose had been degraded by the time of repository closure for the performance assessment calculations. In the third scenario, it was assumed that microbial activity removed all significant organic complexants on a timescale much shorter than the period of interest, implying that organic complexants did not have an effect on the solubility and sorption of radionuclides. It was stated that, because of microbial degradation, it was not possible to describe changes in the levels of complexants in the repository (with degradation impacting the release of dissolved radionuclides). It is not clear whether or not the GAMMON model can be adapted for the degradation and utilization of low abundance sources of carbon that would exist in the cellulose-free used nuclear fuel repository scenario.

More relevant to Canadian host rock formations, a model-based evaluation of the microbial evolution of gas (the Gas Generation Model (GGM); Humphreys, 2012) was conducted in support of the Ontario Power Generation (OPG) DGR for low and intermediate level radioactive waste**.** The GGM is primarily based on thermodynamic predictions of energy yield, with more electro-positive terminal electron acceptors being used preferentially over those with lower Eh values. The authors indicated that thermodynamic considerations are not the sole drivers of microbiological processes in a DGR. A variety of other considerations, including physiologic and ecological factors (e.g., cometabolic or mutalistic reactions, interspecies hydrogen or formate transfer) may influence the ultimate process being driven forward. The GGM conservatively assumes that all carbon is bioavailable, and thus would ultimately be converted, in a rate-limiting fashion, by microbial electron transfer redox reactions to various end-products such as reduced forms of iron and manganese, acetate and methane, H_2 and CO_2, with no accumulation of intermediate compounds. The GGM also specifies that methanogenesis is the terminal reaction, and thus all fermentative reactions stemming from the microbial breakdown of polymeric carbon materials (i.e., cellulose, plastics, ion-exchange resins) will yield H_2 and acetate, thereby feeding into the final formation of methane via acetoclastic methanogens, or hydrogen-utilizing methanogens. All other longer chain volatile fatty acids, such propionate and butyrate, are assumed to not be significant. The activity of acetogenic bacteria, that commonly use these substrates for the generation of acetate, is similarly assumed to be negligible. The thermodynamic approach used in the GGM offers potential for the prediction of the geochemical evolution of a DGR. The GGM is a relatively simple model, with primary foci on DGR pressurization under unsaturated conditions, with fewer input parameters, which seemingly does not impact on its ability to reproduce data modeled using more complex algoriths (SMOGG and GRM (Quintessa and Geofirma, 2011). Another model with relevance to Canadian host rock formations is the one derived by King and coworkers (King et al., 2003; 2004), which

predicts microbial corrosion, based on redox conditions, of the containers in a HLW repository. This model includes a number of microbial reactions, described in an earlier document by the same authors (King et al., 2002), with the capability to turn reactions on or off during simulations.

In view of the challenges related to measuring the slow microbial metabolic rates, *in situ* as well as in physical experiments that simulate DGR conditions, numerical models are invaluable in the development of hypotheses as drivers for experimentation, the results of which can then be fed back into the model to develop a more realistic picture of the key processes.

4.5 EXTENT OF INTERDISCIPLINARY RESEARCH

Research enabling the development of DGR concepts and installations by the various nuclear organizations around the world has necessarily been based on highly interdisciplinary research. As evidenced by decades of documentation and reports, the scientific base for establishment of DGR's has involved the geological, hydrological, physical, and material sciences, as well as engineering. During the early phases in the development of the DGR concept, it was determined that the biological/microbiological sciences would also have impacts on the geochemical evolution and integrity of the EBS and repository. Thus, there have been efforts to merge the findings of microbiology with those of other related and involved disciplines. From their respective reports, it is evident that the majority of studies from the various nuclear organizations (e.g., NWMO, SKB, POSIVA, NAGRA) around the world have engaged in significant interdisciplinary research. As discussed in Section 3.2, the physical and chemical characteristics of the near-field and surrounding environment cannot support high levels of microbial activity and it is reasonable to expect that microbial cells in the bulk matrix will be largely restricted to a single-cell existence where cell-cell interaction will be dependent on diffusion through a dense material. Concomitantly, current approaches in microbiology typically rely on cells to be present in relatively high numbers to enumerate them or to measure their metabolic activity. PCR based gene amplification is an exception, but even though related molecular techniques present a notably improved potential to describe microbial diversity, explaining the diversity in terms of metabolic rates and output is still largely indirect. Studies on the microbiology in the near-field necessitate inputs from various disciplines for microcosm design (geochemical and geophysical characteristics), as well as for laboratory experimentation and extrapolation of data (mathematical / computer modelling). Similarly, the role of environmental microbiology in the design of engineered systems, especially where the potential of microbial activity has been identified, should not be underestimated. As indicated by McMurry et al. (2003) and summarized in Section 5, investigation of microbiology is now evaluated in geological disposal programmes for used nuclear fuel throughout the world.

4.6 TIME-SCALABILITY OF LABORATORY STUDIES; HOW REALISTIC ARE THE EXTRAPOLATIONS?

With the exception of a few studies, such as the one described by Hallbeck and Pederson (2008), which determined sulphide and acetate production rates under *in situ* conditions at the Åspö HRL, there is very little reported information on direct measurement of microbial activity in the deep subsurface. A large body of work to date relied on incorporation rates of radiotracers or mineralization of labile nutrients, often in laboratory microcosms, which typically showed turnover times of hours, days and weeks. However, as early as 1990, Chapelle and Lovley reported that estimates of microbial rates, based upon geochemical modelling, more accurately

reflected *in situ* microbial activities than did estimates based upon radiotracer mineralization experiments. While Chappelle and Lovley (1990) were referring to active groundwater flow systems, it can be expected that in the case of consolidated, low permeability systems such discrepancies will be even more notable. Early work by Phelps et al. (1994a) indicated that subsurface microorganisms must be active to affect groundwater chemistry and that, because the findings from time-course experiments may not accurately reflect carbon and energy flow in subsurface environments, these experimental values may be incompatible with geochemical data, as well as *in situ* nutrient resources and oxygen availability.

5. EVOLUTION OF THE ENGINEERED BARRIER SYSTEM (EBS) NEAR-FIELD ENVIRONMENT: MICROBIAL IMPLICATIONS

There are currently two reference designs for the used fuel container being considered for use in Canada's deep geologic repository: steel and copper/steel. The final selection of the material for use in repositories will be made on the basis of the host rock environment, engineering demonstrations of integrity and the repository safety case. Corrosion of these materials has undergone considerable study (King, 1996, 2007; King et al., 2001, 2003). These studies indicate that the potential for corrosion to occur changes as conditions evolve in the repository from relatively warm, dry and aerobic (in the initial phase) to cooler, wet and anaerobic (over the longer term). Regardless of the particular specifications for different repository concepts, the base situation includes the use of compacted bentonite buffer surrounding the fuel containers with backfill material consisting of crushed rock and bentonite clay filling the remaining excavation space. The Canadian DGR reference design uses pure compacted bentonite with a dry density of ≥ 1.6 g/cm^3 to surround the used fuel containers (Stroes-Gascoyne, 2010). Dense backfill that consists of crushed rock, non-swelling clay and bentonite (dry bulk density of 2.1 g/cm^3) would be used to fill remaining excavation spaces. Pellets consisting of either 50:50 bentonite:crushed rock or 100% bentonite would then be blown into remaining areas where the dense backfill cannot be emplaced.

The functions of the backfill material are to: i) support the containers and the 100% compacted bentonite buffer; ii) aid in creation of anaerobic conditions; and iii) slow saturation of the repository. Upon saturation, the hydraulic conductivities of these buffer/backfill materials would be low (10^{-12} to 10^{-14} m/s for 100% bentonite buffer and 10^{-10} to 10^{-11} m/s for backfill (Dixon et al., 2002; Pusch and Weston, 2003)). There would also be seals or bulkheads constructed out of concrete or expandable clays (or both) that would be used to seal emplacement rooms and repository openings.

5.1 MICROORGANISM PREVALENCE AND SURVIVAL IN THE NEAR-FIELD REPOSITORY ENVIRONMENT

A growing body of research (Stroes-Gascoyne et al., 2002; Stroes-Gascoyne, 2010) suggests that conditions within the EBS will result in the significant reduction in the number of surviving, viable microorganisms, or cause cells to enter a dormant, relatively-inactive state. The range of conditions expected to exist in the near-field environment after emplacement of used nuclear fuel (e.g., after saturation of the excavated zone) would include: i) extreme antimicrobial conditions at or near the surface of used nuclear fuel containers, due to the combined effects of radiation (especially in the case of thin-walled containers), heat, and desiccation; ii) low water activity, heat, and restricted pore space within the compacted bentonite clay material; and iii)

higher water activity, pore space and some nutrients (carbon, nitrogen and oxygen) within the backfill; as well as the EBS-host rock interface regions (Stroes-Gascoyne, 2010; McMurry et al., 2003; Pedersen, 2010). For microbes to have any impact on the emplaced barriers or fuel, they will first need to survive long-term exposure to these harsh conditions. The survival and potential activity of bacterial cells will be dependent upon their distance from the waste container, and local conditions of moisture, temperature, nutrients, pore space and time. From Stroes-Gascoyne (2010), it can be concluded that heterogeneities existing at boundaries in the EBS will likely be critical in determining microbial survival; results from this *in situ* emplacement repository study revealed that interfaces between the bentonite-based buffer, backfill and sealing materials contained higher numbers of culturable heterotrophic aerobic bacteria and anaerobic bacteria – including SRB – than did the bulk materials themselves. Fractures and incomplete seals between adjacent blocks of expanded bentonite, the expanded bentonite and the host-rock walls of the repository, and the expanded bentonite and the surface of the waste container, could therefore offer refuges to microbial cells.

Tolerance of microorganisms to nearly every example of extreme conditions is well documented, as indicated in Section 3. West and McKinley (2002) summarized the specialized microbes, which have evolved within various extreme habitats of relevance to deep geological repositories (Table 1), revealing that there is hardly any foreseeable condition under which microorganisms might not be expected to survive. Thus, the potential for resistant microbes to become established within certain regions of a nuclear fuel repository is not only possible, but likely. However, the potential for microorganisms to develop a poly-extremophilic phenotype (e.g., to become multiply-resistant to heat, pressure, low water activity and radiation) so as to survive and proliferate in the immediate proximity of the used nuclear fuel containers is considered most improbable given that these extreme conditions would occur almost instantaneously on placement of the used nuclear fuel, thereby not providing organisms the time necessary for evolutionary or adaptive change.

Table 1: Range of Tolerances of Bacteria to a Variety of Subsurface Conditions. Adapted from West and McKinley (2002)

Condition	Range of tolerance
Temperature	-20 to 113°C
pH	0-12
Salinity/a_w	up to 50% w/w; minimum a_w of 0.62
Radiation	Dosages of 17-30 kGy
Pressure	180 MPa

5.1.1 Effect of radiation

Used nuclear fuel is both radiologically- and thermally-active (i.e., hot), to the extent that within hours or days of emplacement, it is anticipated that the used nuclear fuel container surfaces, along with the first few centimeters of the compacted bentonite buffer, would essentially have become biologically inactive (McMurry et al., 2003).

Radiation emitted from used nuclear fuel includes gamma- and beta-emitting fission products, alpha-emitting actinides, and neutron-emitters like californium (Cf). These forms of radiation are ionizing, meaning they are sufficiently energetic so as to cause electrons to become ejected from the affected parent atom or molecule. In biological tissue, ionizing radiation (IR) causes lesions in cellular DNA and RNA in a genome size-dependent manner; the larger the genome target, the less radiation required to cause lethal damage (Bacq and Alexander, 1961). Bacteria are more resistant to IR than humans and less resistant than viruses. Ionizing radiation may also damage cellular membranes (Suzuki et al. 1982), and in the presence of water, leads to radiolysis yielding highly chemically-reactive free radicals and ions (equation 7 below). Of the radical species formed, the hydroxyl radical is, by far, the most biologically-reactive, causing 60-70% of the radiation-related DNA damage (i.e., phosphodiester DNA strand breakage) that a living cell may experience (Wallace, 1998).

$$H_2O \rightarrow H^+ + OH^- + H_2O_2 + H_2 + {}^{\bullet}OH + e_{aq}^- \qquad \text{equation 7}$$

The potential for the antimicrobial efficacy of IR to interact with a variety of physical and chemical conditions is well-known, due to numerous studies on its application for food preservation and destruction of microbial and insect pathogens (Grecz et al., 1971; Anellis et al., 1972; Jay et al., 2005). For example, conditions of high moisture, high temperature, and presence of oxygen are all known to enhance IR antimicrobial effectiveness.

As indicated in Stroes-Gascoyne (1997), a series of experiments were conducted by AECL from 1991 to 1997 to elucidate the extent to which radiation emitted from used nuclear fuel would impact microorganisms under *in situ* conditions. During these studies, a number of scenarios were explored with respect to microbial survival in the container-buffer zone, including: i) the effects of radiation under near-saturated conditions; ii) the effects of different radiation dosages; iii) the effect of radiation at three temperature regimes (30, 60 and 90°C); and iv) the effect of radiation under various moisture conditions (0, 23 and 47% saturation).

Hanna and Arguner (2001) estimated that the gamma radiation dose rate at the surface of Canada's copper reference container concept (McMurry et al., 2003) would be approximately 0.05 kGy/hr. Using a panel of naturally-occurring and radiation-resistant microorganisms within a clay matrix (i.e., containing either Avonlea or Wyoming bentonite), it was determined that the D_{10} values (dose required for a 1 log reduction in viable cell numbers) for cells exposed to a ^{60}Co radiation source ranged from 0.34 to 1.68 kGy (Stroes-Gascoyne et al., 1995). Relative to Deinococci IR resistance, these values are ~20 times less. Pitonzo et al. (1999) found that indigenous organisms in microcosms remained viable, but non-culturable, after receiving a cumulative radiation dose of 2.33 kGy, at a dose rate of 0.0036 kGy/hr. Previously, King and Stroes-Gascoyne (1995) calculated that the time required for the complete eradication of a population of the most radiation-resistant microorganisms (using a D_{10} value of 1.67 kGy for a filamentous bacterial isolate from the Wyoming buffer) in the zone nearest to an emplaced used

nuclear fuel container would occur within 8 to 96 days. This is essentially instantaneous relative to a DGR (safety assessment time frame considered for a DGR of a million years). It should be pointed out that these calculations were based on thin-walled Ti containers, and it will thus be useful if repeated with dose rates appropriate for thick-walled Cu or Cu/Fe containers. Further experimentation examining other aspects of EBS conditions, as part of an extensive microbial sampling program carried out during the decommissioning of the buffer/container experiment (BCE) at the AECL URL, including dose rate and moisture content, were also conducted (Stroes-Gascoyne et al. 1996a; Stroes-Gascoyne, 1997; Stroes-Gascoyne and West, 1997). The effect of IR under elevated temperature conditions increased resistance of some microorganisms, likely due to a desiccation effect that decreased the formation of hydroxyl radicals. Work specifically examining water content (0, 23 and 47% saturation) on radiation resistance substantiated these observations (Stroes-Gascoyne et al., 1997b).

Studies by Stroes-Gascoyne and West (1997) revealed that the radiation and desiccation effects within the bentonite buffer surrounding nuclear fuel waste containers would essentially create a sterilized zone extending a few centimetres, and a microbe-depleted zone extending tens of centimetres (after ~40 cm, the radiation levels would decrease by several orders of magnitude, no longer inhibiting organisms solely-based on radiation effects) (Stroes-Gascoyne et al., 1995). However, there is also evidence suggesting that microbes could survive at elevated cumulative dosages of radiation. For example, radiation-tolerant bacteria have been found in the reactor core at 3 Mile Island, where 10 Gy/hr radiation was received by the organisms, which were also regularly exposed to biocides and hydrogen peroxide in the system (Booth, 1987). Other reports have shown that specialized organisms have the capacity to survive normally-lethal conditions of radiation exposure. For example, *Deinococcus* species, isolated from Sonoran Desert soils, were demonstrated to survive up to 30 kGy radiation (Rainey et al., 2005) and survival of chronic IR of up to 60 Gy/hr has also been reported (Daly, 2000). Work by Fredrickson et al. (2008), and further reviewed by Meike and Stroes-Gascoyne (2000), described a link between protein oxidation (desiccation) resistance and IR resistance, posing interesting questions about the evolution of IR resistance in bacteria beyond that of genome copy number and DNA repair. As indicted above, those regions closest to the used nuclear fuel container will be lethal given combined temperature and radiation effects, microbes located further from the containers will be subjected to less extreme conditions. Due to shielding provided by the used nuclear fuel container and surrounding buffer material, the effects of radiation will not, in fact, extend very far from the actual container surface. Thus, other controls in the EBS (i.e., clay swelling pressure, water availability, temperature) will primarily be responsible for limiting the activity and resulting effects of microorganisms as the DGR evolves, as discussed in Sections 5.1.2 to 5.1.4.

5.1.2 Effect of temperature and water activity

Conditions within a respository after emplacement of used nuclear fuel containers, would include temperatures of 70-90°C and clay swelling pressures of <5 MPa, combined with a hydrostatic pressure range of 5-10 MPa, and have low amounts of available microbial activity (Stroes-Gascoyne and West, 1997; Stroes-Gascoyne, 2010; McMurry et al., 2003; Pedersen, 2010). The pressures within the highly compacted bentonite buffer would aid in limiting microbial growth by restricting pore space for growth (e.g., average ~0.01 µm pore diameter in compacted bentonite, which is slightly smaller than the thickness of bentonite particles) and restricting available water. Cells would thus be immobilized within the low permeability environment (which will limit the flux of nutrients and movement of sulfide generated by SRB). As part of a large-scale *in situ* experiment (discussed in Section 3.5), an electric heater was

installed in 50% sand and 50% bentonite buffer material at 240 m depth for 2.5 years as part of the Buffer Container Experiment (BCE) at the AECL URL to simulate container emplacement (Stroes-Gascoyne et al., 1997b). Heat surrounding the experimental container resulted in the creation of a gradient of moisture content in the buffer matrix, ranging from 24% at the host rock wall to 13% at the container surface, where temperatures of 50 to 60°C were maintained. Following decommissioning of the experiment, it was revealed that viable microbes, including heterotrophs and specialized organisms, could only be recovered from heated buffer matrix materials where moisture was >15% (an $a_w \geq 0.96$), suggesting that the buffer and backfill materials could be populated or repopulated when higher moisture levels prevailed. A limiting water activity value of 0.96 on SRB survival in bentonite was also reported by Motemedi et al. (1996) using *Desulfomicrobium baculatum* and *Desulfovibrio* sp. over a 60 day incubation at 30°C. However, the presence of an indigenous SRB (e.g., *Desulfovibrio africanus,* a common corrosion-causing microorganism) isolated from dry Wyoming bentonite MX-80 powder suggests that strategies employed by SRB include the formation of a "dormant" state (Masurat et al., 2010a). In this case, the organisms became active upon addition of growth medium containing 4% salt, and temperatures of 40°C. Notably, viable *D. africanus* cells could still be recovered from dry bentonite powder even after heat treatment of 100°C for 20 h (but not after treatment of 120°C for 20 h), providing evidence of considerable tolerance to heat, suggesting that viable SRB are likely to be present in the emplaced buffer material and can be expected to be active if other controls on their activity are not imposed. The enhanced survival of desiccated cells is a well-known phenomenon and is likely linked, in part, to the increased efficacy and penetration of moist heat on various cell components (e.g., protein denaturation effects versus oxidation and dehydration) (Jay et al., 2005; Fine and Gervais, 2005). The potential for spores to survive under conditions of elevated temperature and low moisture must also be considered, on reflection of evidence of survival of organisms in Yucca Mountain tuff at temperatures of 120°C (Horn et al., 1998). Given that the surface temperatures of a used nuclear fuel container would not drop to <60°C for ~10,000 years, in combination with increasing swelling pressure and low water activity within the compacted bentonite, the potential for microbial activity within the most temperature-affected zone is considered extremely remote (King et al., 2003, 2010).

5.1.3 Effect of nutrient availability

The sources of nutrients within a repository will vary depending on whether the repository is located within a granitic or sedimentary rock formation. The nutrient status within Canadian granitic rock has been categorized as "nutrient poor" (Stroes-Gascoyne, 1997); deep groundwaters in the Canadian Shield are similarly limited by availability of organic carbon, which is typically <2 mg/L (Loewen and Flett, 1984, Stroes-Gascoyne, 1989). Inorganic carbon, in the form of carbonates, would range from 0.01 to 0.1 g/L and concentrations of sulfate could be as high as 3 g/L (McMurry et al., 2003) in these groundwaters. Bentonite clay deposits, as in the case for clays used as buffer and backfill, do contain quantities (up to 1.5%; Sheppard et al., 1997) of carbon-containing compounds (e.g., humic and fulvic acids), which overall would not be readily available to microorganisms, but which may be extractable (Lucht et al., 1997). It has been proposed that heat, along with effects of radiation, may cause the degradation of clay-bound organic compounds rendering them more bioavailable (Stroes-Gascoyne et al., 1997a), while altering their potential to react and transport actinides (Vilks et al., 1998). In an experiment designed to elucidate this, heating (60 and 90°C) and irradiation (25 and 50 kGy) of a 50:50 Avonlea bentonite:silica sand buffer preparation, followed by extraction with a 3:1 water:buffer ratio, resulted in an approximate 2 log greater enhancement of cell growth (relative to control extracts not exposed to heat and irradiation) when supplemented with granitic

groundwater. While this suggests that there exists the potential for stimulation of microbes within a repository, any microbial utilization of extracted carbon within these low hydraulic conductivity (~10^{-12} to 10^{-14} m/s) environments would occur far more quickly than the substrate could be replenished by diffusion, precluding any sustained effect on microbial growth.

It is inevitable that repository construction will introduce a variety of materials that could be used by microorganisms for growth, including those associated with intentionally-placed repository materials (e.g., the used nuclear fuel and container, buffer and backfill), as well as those materials which are inadvertently-placed during construction activities, including fuels, detergents, lubricants, wastes from human activities, etc. (Hallbeck, 2011). Stroes-Gascoyne and West (1996) performed an analysis of the nutrients that would be available to heterotrophic and chemolithotrophic microorganisms in intentionally-emplaced EBS materials, and determined that the major nutrients, N and P, would be growth-limiting, even using the unlikely scenario of all of the carbon being available for growth.

Subsequent work has examined other possible nutrient additions to the EBS, with particular focus on the blasting components as potential sources of N and C (e.g., ammonia nitrate fuel oil; ANFO) (Stroes-Gascoyne et al., 1996b; Stroes-Gascoyne, 1997). Within blast rubble, a significant amount of nitrogen and carbon could remain as a result of incomplete combustion or detonation failure of explosive materials, ranging from 4-5% of the total ANFO (Stroes-Gascoyne et al., 1996b) to as much as 10-20% (Forsyth et al., 1995). Excavated rock from repository construction may therefore constitute a significant potential nutrient source for microorganisms if incorporated into EBS backfill (Stroes-Gascoyne et al., 1996b; Stroes-Gascoyne, 1997; Stroes-Gascoyne and Gascoyne, 1998). Factors impacting nutrient concentration in blast-rubble rock include the surface area of rock exposed to the blast and the amount of explosives used, as well as the amount of blasting materials and gases entering the rock via fissures. Direct measurements on leachates from freshly-broken rock at the AECL URL indicated that the amount of N in freshly excavated rock varied from 6.5 to 47% of the total N present in the initial blasting materials (Stroes-Gascoyne et al., 1996b; Stroes-Gascoyne and Gascoyne, 1998), and could potentially stimulate a 2 log increase in microbial numbers.

Concentrations of organic carbon, as high as 120 µg/g, in old and fresh broken rocks could originate from a variety of anthropogenic sources, including leaked oil, greases, and paints. Water used during drilling operations similarly represents a potential source of carbon (Stroes-Gascoyne and Gascoyne, 1998). Because the net effect of stimulation of microbes within the EBS is unknown, it has been suggested that measures (e.g., washing, leaching) should be taken to reduce the potential for nutrient addition via excavated rock or surface processed waters (Stroes-Gascoyne and Gascoyne, 1998).

Lastly, superplasticizer (SP) ingredients in high-strength cement used in bulkheads, or as grouts to seal groundwater leaks within the repository, are not thought to likely play a significant role as a source of carbon due to the low relative abundance compared to the major components of the installation, along with the low concentrations and leachability (10^{-16} kg/m^3) of the SP (Onofrei et al., 1991).

5.1.4 Effect of swelling pressure, pore size and salinity

Montmorillonite clay crystal units are characterized by an alumina sheet sandwiched between two silicate (tetrahedral) layers. The layered crystals of this mineral are loosely held by very weak inter-layer oxygen attractions (Brady and Well, 2007). Water molecules, as well as

cations (as reflected by typically high cation exchange capacity of 80-100 meq/100 g), associate more strongly to regions between the crystal layers, resulting in expansion of the mineral. Due to water adsorption by these expandable clays, there is less available water for microbial growth. There is also a positive relationship between clay swelling pressure and buffer compaction density; the more compacted the clay (i.e., the more expandable clay per unit volume), the greater the resultant swelling pressure (Craig, 1987). When compacted to a density of 2 g/cm^3 (dry density of 1.6 g/cm^3) the swelling pressure exerted by water-saturated 100% bentonite is approximately 5 MPa and has a water content of 26% (Pedersen et al., 2000). In the same study, the SRB isolates *Desulfovibrio aespoeensis* and *Desulfomicrobium bacalatum* from the Äspö Hard Rock Laboratory in Sweden became non-cultivatable at clay densities higher than 1.8 g/cm^3. Similarly, results from Hedin (2006) indicated that swelling pressures of 2 MPa or greater were sufficient to eliminate microbial activity within compacted bentonite. Increasing salinity further decreases water availability due to interaction of water molecules with solute ions. While microorganisms have been documented to survive a_w ranges of 0.74 to 0.99, most bacteria prefer an a_w of 0.98 or higher (Jay et al., 2005). Several studies have assessed the effect of salinity (NaCl and CaCl) on microbial survival in commercial Wyoming MX-80 bentonite clay (Stroes-Gascoyne et al., 2007b, 2010a,b, Stroes-Gascoyne and Hamon, 2008b). Stroes-Gascoyne et al. (2010b) examined the effect of salinity (0, 50, 100, 150 or 200 g/L NaCl) on microbial survival over a 40 to 90 day period in commercial Wyoming MX-80 bentonite clay (75% montmorillonite) over a range of dry densities (0.8, 1.3, 1.6, 1.8 or 2.0 g/cm^3). A relatively high bentonite dry density of at least 1.6 g/cm^3 was required to achieve an a_w of 0.96 and 2 MPa swelling pressure, which together resulted in the number of culturable cells dropping to background level (i.e., the level in the "dry" (as purchased) bentonite, about 200 CFU/g) when the porewater salinity was ≤50 g/L NaCl. Higher porewater salinities were more effective at inhibiting microbial culturability; for example, when ≥60 g/L NaCl porewater was used, a water activity of less than 0.96 was achieved regardless of the bentonite dry density used (Stroes-Gascoyne and Hamon, 2008b). Studies evaluating $CaCl_2$ salinity on culturability of aerobic bacteria up to 100 g/L yielded results similar to the NaCl studies (Stroes-Gascoyne et al., 2010a).

A similar limiting water activity value was obtained by Motamedi et al. (1996) for some species of SRB in compacted pure bentonite. Pedersen et al. (2000) examined the effect of MX-80 bentonite buffer density on various microorganisms, including SRB, and their ability to produce sulfide over a 28-week incubation period, and determined that the bulk density of buffer started to exert an inhibitory effect on sulfide generation at a bulk density of ~1.5 g/cm^3. The authors postulated that microbes would be present to within a few centimeters of the container, either due to spores originally in the clay or subsequently transported by groundwater during saturation, but that the conditions within the compacted bentonite clay would be inhibitory – possibly against all microorganisms – at the time of full swelling. Ultimately, the key microbial control parameters of water activity and swelling pressure are determined by the dry density of the compacted bentonite and the porewater salinity (Stroes-Gascoyne et al., 2010b).

As suggested by pore water salinity effects (Stroes-Gascoyne et al., 2010a) described above, the performance of clay barriers would be influenced by the ambient solution chemistry; for example, as the buffer and backfill zones saturate, there will be an increase in salinity over time (Dixon et al., 2002). The concentration of total dissolved solids within the groundwater will vary depending on the surrounding environment, but will affect the swelling ability of the montmorillonite clay in the bentonite, thereby influencing the hydraulic conductivity of the matrix. Using a maximum TDS of 100 g/L salts, the highly-compacted 100% bentonite matrix would be minimally affected, with less than a one-log effect on hydraulic conductivity (Dixon, 2000). Within the backfill however, there is the possibility of microbial movement during saturation of

this zone, but the swelling properties in the compacted bentonite would remain, more or less, unchanged.

It is possible that during the saturation of the EBS buffer and backfill with groundwater, microbes could be transported to the fuel container if heat and desiccation cause radial cracking or other physical damage to the buffer integrity. Stroes-Gascoyne and West (1997) considered cell repopulation events in a series of studies involving the penetration of viable *Pseudomonas stutzeri* cells into compacted bentonite buffer plugs (50:50 bentonite:sand; dry densities of 1.2 to 1.8 g/cm^3) pre-saturated with sterile water. Their results revealed that cell mobility was restricted to less than 5 mm in all cases, the smallest sampling interval used, but that rapid movement did occur along the metallic holder-buffer interface. Their results suggested that any interfaces or zones with reduced density could provide preferential pathways for cell migration. In a similar study, Fukunaga et al. (2001) determined that *Escherichia coli* suspensions only penetrated <5 mm into compacted 70% bentonite 30% sand buffer over a 3-week period. Together, these studies provide evidence that as long as the buffer matrix remains intact, microbial regrowth or colonization of the used nuclear fuel container surface and surrounding regions will be very slow, or that these areas will remain devoid of *in situ* active microorganisms.

Given the small clay particle size, the availability of pore space in water-saturated clays is also highly-limiting, and appears to have a direct effect on microbial activity. For instance, Fredrickson et al. (1997) found no evidence of metabolic activity, as determined by anaerobic mineralization of [^{14}C]-acetate and [^{14}C]-glucose, and $^{35}SO_4^{2-}$ reduction, in intact shale cores with pore throats <0.2 µm in diameter that were collected in northwestern New Mexico. Subsequent enrichments revealed the presence of SRB and $^{35}SO_4^{2-}$ reduction in the shale after 14 days of incubation. Comparatively rapid rates of metabolic activity were found in sandstone core samples with a large percentage of pore throats >0.2 µm in diameter. From these results, the authors concluded that while viable bacteria can be maintained and stimulated in materials such as shales with pore throats smaller than the size of known bacteria, subsurface bacteria require interconnected pore throats greater than 0.2 µm diameter for sustained activity. A further observation was that the detrital organic matter in the small-pore-diameter shales is not subject to direct microbial attack. In contrast, bacteria in adjacent sandstones with a more open pore structure are likely sustained by endogenous nutrients that are slowly released from the shale. Extrapolating these observations to the near-field environment, it may be possible that similar nutrient exchange takes place between dense and more porous materials, emphasizing the need to avoid the inclusion of components that may serve as nutrients, even in dense EBS materials.

In general, there is a growing body of research (e.g., the Boom Clay at Mol, Belgium and the Opalinus Clay at Mont Terri, Switzerland) that suggests that while argillaceous matrices can host microbial populations (Stroes-Gascoyne et al., 2007c; Mauclaire et al., 2007), their activity has been limited over a longer time frame than what is currently under consideration for a DGR. Thus, the engineered barrier system is anticipated to function in restricting microbial growth and metabolism in this application.

5.2 IMPACT OF MICROBES ON THE EBS

Consideration of the potential impact of microbial activity in the context of the EBS should include two broad categories: 1) can microbial metabolism realistically have an impact on repository function; and 2) if yes, what can be done to mitigate the impact, or control microbial activity? The specific environment associated with each design and placement method,

together with the conditions of the site where they will be applied, can potentially present different outcomes in terms of microbial persistence and activity. This information should allow the development of a "microbial potential" index for different materials under conditions typical of the area at and near the used-fuel containers.

A large number of comprehensive analyses have been conducted on the diversity and activity of microbes as related to possible effects on nuclear waste repositories. Active populations of microorganisms have been found in all sites under consideration for the placement of used nuclear fuel repositories (Pedersen, 2000; Jain et al., 1997 West et al., 1985), including structural/functional materials such as bentonite clays and backfill (Stroes-Gascoyne and West, 1996, 1997; Wang and Francis, 2005) and groundwaters. Analogue sites have similarly demonstrated the presence of active microbial populations (Pedersen et al., 1996; Smellie et al., 1997; Fukunaga et al., 2005). Thus, it is a certainty that microorganisms will exist within the repository environment, particularly during construction and operations. Once present in the repository, particular organisms and functional groups of organisms will only proliferate and have potential effects to the extent that their surrounding environment will permit. Thus, nutritive (e.g., carbon, energy, micronutrients), physical (e.g., pore space, water activity, temperature, radiation) and chemical factors (e.g., pH, Eh, salinity) govern which organisms will be metabolically active and the degree to which they might influence a DGR. Site-specific and design-specific quantitative understanding of these factors can improve long-term predictions regarding microbial activity.

The various potential impacts of microorganisms on EBS components have been studied as key components of national waste-management programs (i.e., NWMO, NDA, SKB, NAGRA, POSIVA); as well as individual research projects (Stroes-Gascoyne et al., 1996, 1997b, 2010b; Pedersen, 2000; Humphreys et al., 2010). These "effects" studies include: i) direct involvement of microorganisms with radioelements through utilization of these compounds as electron donors or acceptors; ii) microbially-mediated radionuclide transport; iii) microbial reactions with metal waste containers, causing microbially-influenced corrosion (MIC) with effects on the integrity of the container; iv) impacts on major water chemical parameters such as Eh, pH, and CO_2 concentration, with resultant effects on the sorption, solubility, and mobility of radionuclides; v) production of microbial metabolites with the potential for formation of complexes with radionuclides, or biodegradation or removal of ligands with reduction of mobility of radionuclides; and vi) microbial evolution of gases such as CO_2 and CH_4 (see also Pedersen, 1996; Pedersen and Albinsson, 1992c; Pedersen and Karlsson, 1995). Integrating years of SKB research, Pedersen et al. (2008) summarized the three major effects that microorganisms could potentially exert on a used nuclear fuel repository positioned similarly to that of the Canadian concept for crystalline rock (~500 m below ground), which entailed: i) effects of microbial oxygen reduction on the repository redox conditions, with attendant changes on copper oxidation potential (with reducing conditions limiting corrosion); ii) production of sulfide, with corrosive effects on the copper fuel container; and iii) effects of microbial metabolism, including redox potential, on radionuclide mobility.

5.2.1 Impact of microbes on backfill/buffer

While there will be microorganisms in both the backfill and compacted bentonite, the backfill zone is significantly more likely to undergo microbially-mediated changes. To date, relatively few studies have specifically targeted backfill, which will contain a mixture of bentonite and crushed rock, but it is evident that the material will contain a diversity of microorganisms, which are expected to be active on saturation with water, with possible short-term benefits to the

repository redox conditions, as outlined below (Stroes-Gascoyne et al., 1997a; Stroes-Gascoyne and West, 1996).

5.2.1.1 Sources of microbes and microbial activity

Microorganisms reside in nearly all subsurface habitats, and are present on the fractures and surfaces in these habitats, as well as suspended within the porewaters and groundwaters (Ekendahl and Pedersen, 1994; Pedersen, 1993b, 1997, 1999b). The concentration of microbes present in the deep subsurface varies widely, ranging from 10^3 to 10^8 CFU per mL of water or gram of sediment (Fredrickson and Onstott 1996; Balkwill, 1989; Ghiorse and Wilson 1988) and comprises a diverse variety of predominantly Gram-negative microbes and Archaea, including SRB, methanogens and acetogens (Kotelnikova and Pedersen, 1997). Backfill and buffer materials undergo handling/processing and will not be sterile; these materials have been reported to contain various organisms (10^2 to 10^4 CFU/mL in as-bought bentonite; Haveman et al., 1995), including those with potential to impact the EBS, such as SRB (Masurat et al., 2010). The intrusion and saturation of backfill by ground or service waters could serve to both inoculate and activate organisms present in, or introduced into, the porous matrix. It is not anticipated that these microorganisms will penetrate through the compacted bentonite buffer, as the average pore throat diameter in clays is too small for bacterial cells to pass through (Chapelle, 1993); however, groundwater, and associated microorganisms, will inevitably reach the backfill and the backfill-host rock interface.

Given the presence of viable microorganisms within the EBS, the only question that remains is how active these organisms will be, and what effects their activity will have on the DGR. Following closure of the DGR, there would likely exist a strong chemical gradient between the far-field and near-field, because the repository would be oxidizing and the far-field would be relatively reducing (-225 to 0 mV; McMurry et al., 2003). Redox fronts will likely play a key role in defining the types of reactions that organisms would mediate (McKinley et al., 1997), and would create suitable conditions for lithoautotrophic (chemoautotrophic) organisms. Chemoautotrophic organisms are predominantly aerobes, and thus would benefit by being positioned at the aerobic-anaerobic interface where the conversion of Fe(II) to Fe(III) by *Acidithiobacillus ferroxidans*, for example, would yield energy for the fixation of CO_2. Alternative oxidizing electron acceptors (e.g., NO_3^-) would similarly function within a repository environment as conditions evolve from the initial oxidizing state.

A variety of microbial end products have the potential to impact a DGR, including the production of organic acids and other metabolites (e.g., formate, acetate, lactate, butyrate, nitrite, ammonia), as well as gases like sulfide, CO_2 and methane, and an assemblage of various extracellular products that might influence both the repository or radioelements. Hydrogen may also exist or be produced by microbes, but in any environment where it is produced, its evolution tends to be coupled with consumption by other organisms (i.e., SRB) such that free microbially-produced H_2 gas is not common.

In the deep subsurface, it has been speculated that hydrogen-driven ecosystems could exist (Pedersen, 1993b, 1996, 1999a; Stevens and McKinley, 1995; Kotelnikova and Pedersen, 1997). If this were the case, the presence of high amounts of sulfate would most certainly drive sulfidogenesis rather than methanogenesis, due to the higher substrate affinity of SRB for hydrogen relative to methanogens (Uberoi and Bhattacharya, 1995). However, the activity of SRB, and other microorganisms that potentially could cause corrosion, is not anticipated to occur within the buffer region near the fuel container. Accordingly, sulfide gas or other corrosive

metabolites would need to diffuse through the EBS buffer and backfill in order to reach the copper container surface, where possible stress corrosion cracking (SCC) could occur. King and Kolar (2006) previously determined through modelling that the likelihood of significant SCC occurring is very low.

In the absence of an abundance of organic materials in the repository, it is not expected that microbial fermentation will be a dominant process. Only minor quantities of fermentation end products (e.g., acetate, formate, propionate, ethanol, butyrate, lactate) are expected in a used nuclear fuel repository. Because the 100% highly compacted bentonite buffer is expected to be strongly inhibitory to microbial growth and metabolism, reduced end products resulting from any microbial fermentation which did occur, would be produced mainly in the backfill regions.

5.2.1.2 Impact of microbial production of gases

Gases could be generated as a consequence of: i) the microbial degradation of organic materials present in the backfill and buffer (e.g., yielding CO_2, hydrogen sulphide or methane); ii) the anaerobic corrosion of the metal container (evolving copper sulfides and hydrogen gas); or iii) the abiotic radiolysis of water. In general, significant quantities of microbial gas production within the compacted bentonite buffer of an EBS are thought to be unlikely for two main reasons: i) the Canadian used nuclear fuel disposal concept will incorporate minimal organic material; and ii) the low number of microorganisms and the controls on their activity within the buffer region (i.e., bentonite swelling pressure and water activity). The majority of studies on gas generation, as related to nuclear waste repositories, have been centered on the disposal of carbon-rich (often cellulose-rich) intermediate- and low-level wastes, as summarized by Humphreys et al. (2010).

The backfill region (as well as the interface between the backfill and the host rock), which has higher hydraulic conductivity (and higher amounts of trapped air initially), has been speculated to support notable microbial activity. Some organic matter will be present in the groundwater (granitic groundwater in Canada generally contains a few ppm dissolved organic matter) (Gascoyne, 2004), as well as within the EBS construction materials and clays, that could support limited microbial growth. Thus, microbial activity could generate CO_2 and methane. As discussed by Stroes-Gascoyne (1997), the availability of organic materials contained in bentonite clays would be expected to be very low, as evidenced by their extreme stability since the time of their deposition (approximately 75 to 85 Ma for Wyoming and Saskatchewan-Avonlea bentonite). The effects of exposure of the clays present in backfill to aerobic conditions with respect to recalcitrant sources of carbon are not well known, thus extrapolation of how this might contribute to *in situ* gas production remains tenuous (Stroes-Gascoyne and West, 1997; Lucht et al., 1997). Organic materials arising from EBS construction (e.g., blasting residues, drilling fluids, machine exhaust, etc.; see Section 5.1.3 on carbon availability in the repository) could also represent sources of organic carbon for microbial growth.

It is anticipated that there would be microbial activity within the backfill zone following closure of the repository, and that this would drive the gas phase from oxidizing to reducing at some point post closure; McMurry et al., 2003). Data from the long-term tunnel sealing experiment (TSX) at the AECL URL (Stroes-Gascoyne et al., 2007a) offers indirect evidence of this; samples retrieved from the 10% bentonite 90% sand backfill region of the tunnel seal contained higher numbers of SRB and a notable decrease in the number of heterotrophic aerobic, nitrate respirers and nitrate reducing bacteria compared to the 70% bentonite 30% sand highly compacted buffer material. It was proposed that the high moisture, nutrients and space

associated with this region of the backfill could stimulate aerobes, thereby depleting oxygen and creating conditions conducive for the strict anaerobes. An alternate explanation for the increase in anaerobe numbers is that the elevated temperatures obtained during the second phase of the TSX could have inhibited the aerobes and facultative anaerobes more than the strict anaerobes, which may have been preferentially stimulated. Gas analysis of samples from the isothermal test (ITT), part of a long-term (6.5 year) study on the evolution of buffer gas and microbiological parameters (Stroes-Gascoyne et al., 2002), implied that sulfide production may have only just been initiated (only about 0.02–0.5% of total available bulk sulfate was converted to sulfide), and that evolution of reducing conditions was not yet significant. Analysis of gas from the buffer material indicated that gas composition remained relatively unchanged from initial conditions (there was some slight evidence of O_2 reduction and small increases in H_2 and CH_4 levels). This may reflect a loss of viability within the resident microorganisms, as a comparison of culture-based and biochemical analysis of the buffer samples indicated that the viable population of cells was approximately 2 logs higher than what could be cultured. In general, the development of a reducing anaerobic backfill zone after the repository closure will likely retard oxidative copper corrosion, thereby serving to stabilize oxidative copper corrosion of the fuel containers over the short term (Pedersen, 2000).

Once the EBS conditions become reducing, a number of anaerobic metabolic processes could be anticipated to proceed; the most common of the microbially-derived gasses likely to be found would include CO_2, hydrogen, sulfide and methane. While CO_2 would likely be the most abundant gas initially produced given sufficient carbon, it would also be expected to be involved in subsequent microbially-mediated reactions, thereby reducing its concentration in the EBS. For example, the formation of 1 mol of methane from CO_2 and 4 mol of hydrogen would serve to reduce overall gas pressure in the EBS (Ortiz et al., 2002). Hydrogen and methane are thought to have the greatest potential for impacting the near-field EBS environment, since these are reduced compounds that may function as microbiological energy sources (and in the case of methane, a carbon source as well).

Both SRB and methanogens are well-known hydrogen utilizers, thus hydrogen consumption under reducing conditions would be expected. As indicated previously, it is likely that SRB would effectively out-compete methanogens at higher sulfate concentrations given their greater affinity for hydrogen (Lovley and Klug, 1983; Uberoi and Bhattacharya, 1995). Indeed, Sheppard et al. (1997) showed that methanogenesis would not start until sulphate levels had dropped considerably. Other organisms may oxidize H_2 using nitrate, iron, manganese, and CO_2 as electron acceptors in the absence of methanogens and SRB (Schwartz and Friedrich, 2006). Organisms that carry out these reactions are commonly found in subsurface environments, although the potential for these processes to occur is closely linked with the dominant chemical composition of groundwaters/porewaters of the DGR site, as well as the type and abundance of organic material present. In a repository these reactions will likely occur in the backfill (if conditions are conducive), not in the highly compacted bentonite buffer.

A carbon-steel used nuclear fuel container is also under consideration in Canadian EBS reference designs (Figure 2). The Belgium and Swiss designs may incorporate the use of stainless steel (e.g., AISI 316L) for high-level waste disposal, and it has been estimated that this would represent the largest gas generation source term within their EBS (Ortiz et al., 2002). The authors proposed that the hydrogen evolved due to anaerobic corrosion of the steel container surfaces would be microbially-transformed/reduced into either methane or sulfide/thiosulfate. Modelling results suggested that diffusion would not be sufficient to offset gas generation, which could create preferential pathways in the EBS. However, this is not a likely outcome due to the plasticity of clay buffer materials which would likely self-seal. In general, the

nature of the gas phase will depend on the kinetics of its production, with rapid gas evolution leading to the formation of bubbles or a separate gas phase, whereas slow evolution of gasses may simply diffuse away or be metabolized by other microorganisms at the time of production.

5.2.1.3 Effect of microbes on barrier permeability

Precipitates

Following repository closure, the presence of oxygen could stimulate aerobic metabolic processes and drive the oxidative precipitation of dissolved metals such as Fe and Mn (Tufenkji et al., 2002; Haveman et al., 2005), which could lead plugging of flow paths and pore spaces (Howsam, 1987; Goldschneider et al., 2007). Ultimately, heterotrophic metabolism would deplete the available O_2 requiring the utilization of alternate electron acceptors. Under anaerobic conditions, the reductive dissolution of Fe- and Mn-oxides present in the repository environment could then occur (Lovley, 1987; 2006). Given that these reactions tend to occur at interfaces between aerobic and anaerobic environments, or during transition from aerobic to anaerobic conditions, evolution of conditions within the repository environment, and particularly with attendant changes to backfill (primarily) permeability, are a possibility.

Biofilms

It is well known that populations of attached microorganisms and their extracellular products (biofilms) contribute to the plugging or fouling of flow paths in fractures (Wolfaardt et al., 2007; Characklis, 1990; Sharp et al., 1999). Accordingly, it has been hypothesized that bioflms could clog or plug larger pore spaces within the EBS backfill. Evidence in support of this, however, is lacking. A study by Lucht et al. (1997) at the Whiteshell Laboratories (Pinawa, MB) infused low-nutrient groundwater into columns containing different backfill preparations for a 180-day period and found no evidence to support pore clogging, based on permeameter data, even though there were 10^6-10^7 CFU/mL viable microbes and up to 30 mg/L of DOC present in the column effluent. Studies demonstrating microbially-mediated (biofilm) plugging of crushed rock and sediment/sand matrices exist; although, it must be emphasized that these study systems simulated highly-permeable environments involving the continuous flow of nutrient-rich liquid to support sufficient growth to cause plugging (Hama et al., 2001; Brydie et al., 2005; Coombs et al., 2010). Liquid flow, generally considered a stimulatory factor for biofilm formation, along with sufficient nutrient concentrations, would not be present within the backfill/buffer region of a repository. At the host-rock-EBS interface, a potential limiting role could be played by biofilms in terms of scavenging nutrients being transported from the far-field into the EBS, as well as through the direct complexation of biofilms with radionuclides that might be migrating from the repository (via adsorption, uptake or precipitation reactions; see below) (Anderson et al., 2007).

Biotransformation/biodeterioration

Due to its importance to the properties of the EBS, the integrity of the bentonite clay buffer over time is of primary significance; any functional change in the bentonite could potentially impact the ability of the bentonite to undergo swelling upon saturation and subsequently could affect its ability to control microbial growth and migration of radionuclides. The transformation of montmorillonite to illite (equation 8) has primarily been studied from an abiotic point of view (Huang et al., 1993; Wersin et al., 2007); high temperatures (~150°C), elevated pressure (100 MPa) and time are required.

$$Ca^{2+}/Na^{+} - montmorillonite + K^{+} + (Al^{3+}) \rightarrow illite + silica + Ca^{2+}/Na^{+}$$ equation 8

In general, the scarcity of illite in naturally-occurring bentonite deposits suggest that this conversion is not common (McMurry et al., 2003). Biologically-mediated changes (biotransformation) of montmorillonite (smectite) to illite, has been shown to also occur in the absence of extremes of temperature and pressure, instead relying upon the microbial reduction of Fe(III). In this case, the authors suggested that microbes dissolved smectite at room temperatures, at 101 kPa, over 14 days (Kim et al., 2004). In a more recent study by Jaisi et al. (2011), using X-ray diffraction and high-resolution transmission electron microscopy, it was demonstrated that the formation of illite from nontronite by the mesophile *Shewanella putrefaciens* and the thermophile *Thermus scotoductus* occurred at basic pH (8.4) and high temperature (65ºC). While the conversion of Fe(III) to Fe(II) is known to be mediated by various iron-reducing bacteria, as well as by SRB, it is notable that the conditions for microbial growth and activity within the compacted bentonite matrix will be severely inhibitory, as indicated elsewhere in this review. The montmorillonite to illite bioconversion is therefore not thought to be of potential significance to the functioning of the clay barrier.

A biodeterioration process of potential significance to the functioning of the repository relates to the integrity of cement-containing materials, particularly the use of cement bulkheads for the sealing of tunnels. Within the repository, cement bulk-heads play two key roles. The first role is to provide a barrier between the used nuclear fuel vault and the main tunnel system. Secondly, but perhaps more importantly, is a role in which the cement bulk-head provides a brace against which expandable clays present in the buffer and backfill may exert swelling pressure. Evidence from the Tunnel Sealing Experiment (Stroes-Gascoyne et al., 2007a) suggests that the interface between the cement bulkheads and the backfill zone is a likely region where the preferential conditions would exist for microbial growth, even possibly supporting a biofilm community of attached microorganisms. Sources for the growth of the biofilm would comprise microbes and materials contained in the backfill and groundwater, as well as those carbon compounds present in the superplasticizers that leach out of the cement. Metabolites of these biofilms would include organic acids, which could accelerate cement weathering and biodeterioration.

5.2.2 Impact of microbes on used nuclear fuel containers

The conditions under which microbially-influenced corrosion (MIC) of copper-steel or steel containers may occur is a consideration for the Canadian emplacement options. Microbially influenced corrosion of metals can occur through the direct interaction of microbes on the surface of the container, or indirectly, whereby microbes growing in another place produce chemicals (i.e., acetate, ammonia, or sulfide, as above) that diffuse to the container. Given the compelling body of research (Stroes-Gascoyne, 1997; Stroes-Gascoyne and West, 1997; Pedersen et al., 2000) indicating that the container surface and high density clay buffer surrounding the container would likely be biologically inactive (due primarily to heat, radiation and water activity effects) for a period of hundreds to thousands of years, there is little evidence to suggest that a biofilm of SRB would be able to develop provided that the EBS remained intact. Furthermore, transport studies, such as conducted by Stroes-Gascoyne and West (1997) using *Pseudomonas stutzeri*, suggest that the repopulation of the region near the container is highly unlikely so long as the clay buffer remains undamaged. The rate of SRB-induced corrosion would therefore be limited by the rate of diffusion of sulfide (HS^-; produced allosterically outside of the container zone) to the container surface (Kwong, 2011). The kinetics of this diffusion would need to be sufficient for continued MIC to be sustained. As such, the MIC

rates expected in a DGR are notably slower than when microbes are directly associated with metal surfaces (Sheng et al. 2007; Xu et al. 2009). In addition, they are dependent on the type of metal being corroded, as described below.

Corrosion of iron/steel

In short, MIC processes are facilitated by electrochemical reactions on the metal surface. Electrons produced in the oxidation reaction at the anode are consumed in the reduction reaction at the cathode because the two reactions occur at equal rates. Examples of anodic and cathodic reactions (equations 9 and 10) on ferrous metals in aerobic environments are as follows:

$Fe \rightarrow Fe^{2+} + 2e^-$ equation 9

$2H_2O + O_2 + 4e^- \rightarrow 4OH^-$ equation 10

Products of these reactions (i.e., Fe^{2+}, OH^-) will further react to produce, for example, ferrous hydroxides, in the presence of water, and ferric hydroxides in the presence of oxygen and water (equations 11 and 12):

$Fe^{2+} + 2(OH^-) \rightarrow Fe(OH)_2$ equation 11

$Fe(OH)_2 + ½\ H_2O + ¼\ O_2 \rightarrow Fe(OH)_3$ equation 12

When dehydration or partial dehydration reactions are considered, many oxides or oxyhydroxides can also be formed, including: from equation 11, FeO, and from equation 12 Fe_2O_3 and FeOOH, as well as the mixed-species Fe_3O_4, magnetite. Different atomic arrangements of the ferric species (equation 12) include haematite (α-Fe_2O_3), maghemite (γ-Fe_2O_3) and to a lesser extent β- and δ-Fe_2O_3; and goethite (α-FeOOH) and lepidocricite (γ-FeOOH), among others. In addition, when oxygen is present, the solution near the steel surface will contain quantities of Fe^{2+} and Fe^{3+}; the specific solubilities of these components is highly dependent on pH.

Following the consumption of oxygen by corrosion reactions (equations 10 and 12) and/or microbial processes, steel corrosion can continue in the presence of water, which reduces according to the half reaction shown in equation 13, to produce hydrogen gas.

$2H_2O + 2e^- \rightarrow 2OH^- + H_2$ equation 13

When coupled with iron oxidation (equation 9), ferrous hydroxides will be produced (as per equation 11); however, the lower oxidizing power of water compared to hydrogen mitigates the formation of ferric hydroxides or ferric oxides listed above (or solution-based Fe^{3+}). Only the mixed-oxide species magnetite (Fe_3O_4) will be formed that contains Fe^{3+}, owing to the high lattice energy of this oxide as a surface species. Thus, the steel surface will consist of significant quantities of magnetite and ferrous oxides/hydroxides, and an absence of ferric oxides, while the solution near the corroding surface will contain only Fe^{2+}.

While the combination of equations 9 and 10 do constitute corrosion reactions, the many iron oxides and hydroxides possibly affect the actual corrosion rates, depending on how they deposit on the surface.

Corrosion of copper

During the early phase of the DGR life cycle, when conditions are warm and oxidizing, roughening in the form of under-deposit corrosion and fast uniform corrosion of the copper containers will occur only as long as sufficient oxygen is available (Stroes-Gascoyne and West, 1996; Kwong, 2011). While corrosion is a chemical process, microbes may enhance the process by altering the local chemical environment (e.g., creation of differential aeration cells), in particular pH and Eh, and by the production of corrosive metabolites, such as sulfide which would diffuse from a remote location and may impact the durability of the metal waste containers in a repository. It was suggested by Akid et al. (2008) that up to one third (~33%) of material loss that arises from copper corrosion may be attributed to microbial activity while Kwong (2011) reported that after 1 million years MIC would contribute 1 mm of a total predicted copper container wall loss (1.27 mm; i.e., MIC would contribute ~80%; compared to a loss of 0.26 mm per 1 million years as calculated by 2010 Masurat et al. (2010b)).

In the absence of oxygen, copper corrosion (equation 14) has largely been considered to be thermodynamically unfavourable (Kwong, 2011).

$2Cu+H_2O \rightarrow Cu_2O + H_2$ equation 14

Recent work (Szakálos et al., 2007) has suggested that some corrosion of copper in pure, oxygen-free water may occur; however, NWMO, SKB and others have subsequently attempted to replicate this work with no success. Should this reaction occur, the implications would have relevance to predictions of copper container expected lifetimes. Furthermore, among many other investigations, King et al. (2001, 2010) noted that under anaerobic conditions, it is expected that copper corrosion, along with H_2 evolution (equation 15), would occur in the presence of sulfide in solution.

$2Cu + H^+ + HS^- \rightarrow Cu_2S + H_2$ equation 15

After examining HS^- effects on copper corrosion up to 167 days under anaerobic conditions using dilute $5x10^{-5}$ mol/L Na_2S in 0.1 mol/L NaCl, Chen et al. (2011), concluded that the concentration of HS^- at the copper-water interface would ultimately limit corrosion, given that the diffusion coefficient in compacted clay buffer would on the order of 10^{-7} cm^2/s. Using extreme-value calculations, based on a HS^- concentration of 3 ppm continuously being supplied by SRB to the container surface in a crystalline rock repository environment, it was estimated (King and Stroes-Gascoyne, 1995; King, 1996) that a corrosion rate of 1 nm/yr would result in a total of ~1 mm of MIC after 1 million years, a value similar to that determined over the same time frame by Kwong (1.27 mm that includes MIC as well as non-MIC corrosion effects; 2011).

Overall, conclusions from laboratory experiments suggest that the main MIC effects on copper corrosion would occur indirectly from the diffusion of sulfides produced in regions of the repository (backfill and interface), where high temperature and other conditions would not result in microbial death or inactivation (e.g., Stroes-Gascoyne and King, 2002). Diffusion of the sulfide will be limited through the compacted buffer, however, making this source of corrosion a minor component (Masurat et al., 2010a; Wersin et al. 1994, Kwong 2011, Pedersen 2010).

Corrosion of copper-iron

Smart et al. (2011) installed miniature copper–cast iron nuclear waste containers with 1 mm diameter “defects” in the outer canister shell, and exposed these to ambient granitic

groundwater in SKB's Äspö Hard Rock Laboratory in Sweden. The main aim of the study was to determine how the container would perform over time should the outer copper barrier fail, as part of a worst-case scenario study on galvanic coupling between steel and copper components. The authors came to three general conclusions: i) water analysis showed that there were compositional differences between water from inside the support cages compared with the external borehole water, and these differences were attributed to enhanced corrosion of iron components in their experimental system, as well as increased microbial activity inside the cages that surrounded their experimental container assemblies; ii) microbiological analysis showed that SRB were active in the boreholes and the support cages, with the microbial activity higher inside the support cages compared with the boreholes outside the support cages; and iii) over time, the electrochemically-measured corrosion rates for both iron and copper increased in the experimental systems containing low density bentonite, while increased rates were observed only for iron in the absence of bentonite. It was suggested that microbially-produced sulfides were responsible for the increased rates. However, the authors noted that this effect has not yet been confirmed in fully-compacted bentonite, and that the cause of the increased microbial activity reported in point ii above could have been stimulated by the presence of bentonite and corroding iron. Furthermore, because hydrogen may be generated by the anaerobic corrosion of the used fuel containers, as well as by the radiolysis of water, there would seem to exist the potential for these processes to fuel further SRB growth and sulfide generation, thereby accelerating overall corrosion.

5.2.3 Impact of microbes on radionuclide fate

In the early years following the DGR closure, it is expected that any viable microorganisms within the clay buffer immediately surrounding the used nuclear fuel containers would almost completely be inactivated as a consequence of the combined effects of heat, low water activity, limited pore space, and radiation (Motamedi et al., 1996; Stroes-Gascoyne et al., 2010b). On cooling, the near-field region around the containers would eventually become saturated with groundwater, at which time conditions might enable surviving cells to once again become active; however, the swelling pressure exterted by the bentonite buffer is expected to inhibit bacterial growth.

In the event of container failure, microbes can interact with both toxic and non-toxic radionuclides dissolved from the used nuclear fuel. The reaction of microorganisms with inorganic metals is well understood, and includes: metal oxidation/reduction (redox) reactions, biosorption of metals to cell surfaces and extracellular components, intracellular accumulation of metals, and extracellular precipitation (West et al., 2002; Suzuki and Banfield, 2004; Frazier et al., 2005; Vieira and Volesky, 2000; Merroun et al, 2005, 2006; Merroun and Selenska-Pobell, 2008). These reactions may occur in order to detoxify these compounds, generate energy, or occur spontaneously due to the metabolic products produced by the cell. Consequently, microbe-radionuclide interactions may affect the mobility of the radionuclide by mediating its immobilization on, or in, the cell, or by altering its solubility.

5.2.3.1 Microbial-radionuclide redox reactions

A variety of microorganisms are known for their abilities to involve metal elements, including redox-sensitive radionuclides (i.e., uranium, plutonium, neptunium, technetium), in a wide range of redox reactions. Much of the available literature centers on the application of microbial biotechnology for the remediation of uranium-contaminated sites associated with the shallow

subsurface (e.g., Lovley et al., 1991; Abdelouas et al., 1998; see also review by Anderson and Lovley, 2002). Because toxic metals cannot be degraded, remediation strategies have focused on stabilizing these elements so that they become less mobile and thus less biologically-available. This includes using microorganisms to reduce soluble-phase actinides (e.g., U(VI)) to their insoluble (i.e., U(IV)) state, as proposed by Lovley et al. (1991) as a strategy for preventing the movement of soluble U into groundwater systems. An adequate supply of organic carbon, or hydrogen, as electron donors is necessary for these anaerobic reactions to proceed; indeed, the creation of anaerobic environmental conditions through stimulation of microbial metabolism is the initial phase of this approach. A variety of organisms, which mediate similar metal reduction reactions, include SRB (*Desulfovibrio* spp.) and Fe-reducing bacteria (*Geobacter and Shewanella* spp.) as well as others. In typical reactions of uranium, U(VI) behaves as an electron acceptor (dissimilatory U reduction) with the production of low-solubility uranium minerals, thereby decreasing the concentration of soluble U(VI).

It is noteworthy that this reaction may proceed in the opposite direction upon interaction of reduced uranium compounds under oxidizing environments (Anderson and Lovley, 2002; Merroun and Selenska-Pobell, 2008), a microbially-mediated process that has been exploited to recover uranium via bioleaching from low-grade ore deposits (Brierley, 1978). In these reactions, addition of Fe(III), under acidic conditions, functions as a U(IV) oxidant and yields the more-soluble U(VI). The re-oxidation of the reduced Fe(II) is performed by *Thiobacillus ferroxidans* (reclassified as *Acidithiobacillus ferroxidans* in 2000), which use reduced iron as an electron donor for energy production during CO_2 autotrophy.

More relevant to DGR environments, naturally-occurring redox cycling by anaerobic microbes has been demonstrated by Wilkins et al. (2006, 2007) to influence the speciation and mobility of radionuclides associated with low-level radioactive waste at the Drigg low-level storage site in the UK. In that system, the removal of both soluble U(VI) and Tc(VII) from groundwaters [via reduction to their respective insoluble forms, U(IV) and Tc(IV)] was correlated with microbial Fe(III) reduction in microcosms constructed with sediment. Wilkins et al. (2007) further confirmed that the potential for nitrite reduction to re-oxidize and hence re-mobilize these elements was radionuclide-specific (i.e., uranium underwent oxidative re-solubilization, whereas technetium did not).

While microbial U(VI) and Tc(VII) reduction results in the formation of less-soluble species, it has been reported that microbial reduction of plutonium, under certain circumstances, may lead to the formation of the more soluble form Pu(III) (Renshaw et al., 2007, 2009; Humphreys et al., 2010). While the authors demonstrated that both *Geobacter sulfurreducens* and *Shewanella oneidiensis* could reduce Pu(IV) to the more soluble Pu(III), there was no increase in soluble phase Pu concentration (Renshaw et al., 2009). It was speculated that the interactions between the organisms and Pu(IV) initially involved sorption to the cell surface, followed by gradual reduction. Microcosm studies indicate that despite the microbially-mediated formation of Pu(III), no increase in solution-phase Pu was observed. It should be noted that the solubility of Pu species is pH-dependent; for example, Pu(III) will precipitate as Pu_2O_3 at pH values greate than 7.0. Similarly, Pu(IV) (or Pu(III)) can dissolve as hydroxide containing cation or anion; again, depending on pH.

It is clear that microbially-mediated redox reactions have the potential to influence the speciation and mobility of radioelements that may be released from a breached fuel container; however, the exact reactions will likely be difficult to predict. While anaerobic conditions necessary for these reactions to occur seem assured, a significant source of electron donors to drive actinide reduction is thought to be limiting.

5.2.3.2 Microbial influences on the movement of radionuclides

The high sorption capacity of clay-based buffer and backfill materials is key to the retardation of radionuclide migration. The extreme conditions in compacted bentonite (radiation, water activity, swelling pressure and temperature) make it unlikely that microbes would be in any condition to participate in the diffusion of radionuclides out of the zone nearest the used nuclear fuel container. However, there is an increased possibility that fractures within the outer regions of the backfill zone, or at the interface between the backfill and the host rock, could be colonized (or recolonized) by microorganisms, which could then potentially affect actinide mobility. One consideration is that soluble radionuclides or radionuclides sorbed to bentonite colloids could diffuse out of the repository and reach attached biofilm communities (West et al., 2002), which could function to retard colloid-facilitated nuclide diffusion by sorbing either the clay-radionuclide complex or free radionuclides. Subsequently, biofilm cells with sorbed clay/radionuclides could themselves be transported through fissure conduits along the backfill-host rock interface, mobilizing radioactive materials from near-field into the far-field (Kurosawa and Ueda, 2001). Ohnuki et al. (2010) examined interactions between U(VI) and Pu(VI), *Bacillus subtillus* and kaolinite clay. The authors determined that both U(VI) and Pu(VI) sorbed to bacterial cells in the presence of kaolinite, but that U(VI) became directly sorbed to the cells; whereas, Pu(VI) underwent sequential reduction to Pu(VI), Pu(V) and then Pu(IV) before sorbing to cells. The microbial cells were determined to compete directly with clay colloids for radionuclide binding, and that similar mechanisms utilized by attached biofilms on rocks *in situ* may be expected to retard radionuclide migration.

Such a mechanism could explain the stability of uranium species (i.e., no chemical evidence suggesting a conduit for radionuclide migration) associated with the Cigar Lake UO_2 deposit, despite the existence of large fractures spanning the clay dome overlying the UO_2 deposit (Brown and Sherriff, 1999; Smellie et al., 1997). While evidence that microbes sorb radionuclides along with a variety of other metal elements is abundant (Merroun and Selenska-Pobell, 2008), there are also reports (Anderson et al., 2007) that biofilms in fractured subsurface (granite surfaces) decrease the capacity for adsorption of mobile radionuclide compounds. Using neutral pH anaerobic groundwater conditions in a microcosm, Anderson et al. (2007) determined that biofilms suppress the capacity for the fluid-rock interfaces to act as barriers (i.e., to sorb) to specific nuclear material migration offsite (e.g., ^{60}Co(II), ^{241}Am(III), ^{234}Th(IV), but not trivalent species such as Pm and Am). Overall, the effects that microbes and adherent biofilms may have on free actinides released from the repository, versus those associated with migrating colloidal clay particles into the fractures, have not been fully elucidated.

Evidence for the indirect facilitated transport of radionuclides via the metabolic products of microbial cells also exists. Frazier et al. (2005) reported that bacterial siderophores promoted the dissolution of UO_2 at 5-fold greater net dissolution rates than by simple proton-promoted dissolution. Siderophores normally function to sequester iron under limiting conditions, where low solubility iron exists as iron oxides (Boukhalfa and Crumbliss, 2002). The fact that normally-stable tetravalent actinides (e.g. U(IV)), held under anaerobic conditions may in fact be mobilized by soluble microbial organic ligands, offers an additional route for radionuclide transport. Siderophore-coupled accumulation of radionuclides (Pu(IV)) was also observed for *Microbacterium flavescens* (JG-9), which produces the siderophore desferrioxamine-B (DFOB) (John et al., 2001). In that study, it was shown that only living and metabolically active *M. flavescens* were observed to take up Fe(III)-DFOB and Pu(IV)-DFOB complexes.

Earlier work by Stroes-Gascoyne et al. (1999), examining the potential for microbial cells to penetrate buffer material (50% sand, 50% bentonite), revealed that migration of *Pseudomonas stutzeri* cells into dry, compacted buffer with a dry density of 1.6 g/cm^3 was limited to ~5 mm (the smallest sampling distance used in the study); whereas, movement of these bacteria along the interface between metallic surface and the buffer occurred more rapidly. The authors concluded that interfacial zones, as well as regions within the backfill with reduced dry densities, could provide preferential conduits for microbial transport. However, in order for microbial cells or their components to bridge the distance from the backfill zone to the fuel container, it is required that the repository become entirely or partially compromised. Specifically, the limiting pore size provided by the high swelling pressure of expanded clay would need to fail so that conduits sufficiently large for cells to pass are formed. It follows that as long as the buffer remains intact, one can expect that both microbially-mediated and free migration of radionuclides would both be severely hindered.

Overall, the potential for microbes to influence radionuclide migration, while poorly understood, is expected to be dependent on the conditions of the system (e.g., pH and Eh), and therefore reversible. In light of the uncertainties associated with contaminant transport effects elicited by microorganisms, generic Canadian crystalline host rock safety assessments have utilized a conservative approach, such that sorption and retardation of radionuclides by microbes is assumed negligible (Garisto et al., 2004).

6. RECOMMENDATION OF A PATH FORWARD FOR NWMO'S NEAR-FIELD MICROBIOLOGY RESEACH PROGRAM

A literature-based review has been conducted related to the impact of microorganisms on repository engineering and postclosure safety assessment of a deep geological repository. This analysis was conducted in support of the NWMO's implementation of Adaptive Phased Management (APM). Specifically, microbial distribution, activity and survival in the near-field environment have been reviewed with specific focus on their potential impact on the EBS performance, and relevance to Canadian repository design options. The anticipated impacts that microbes have on the near-field repository environment, as well as recommendations for ongoing and future research components, are provided below.

6.1 SUMMARY OF POTENTIAL MICROBIOLOGICAL IMPACTS WITHIN THE NEAR-FIELD ENVIRONMENT

A substantial body of work conducted by international nuclear research programs has provided evidence that a diverse community of microorganisms can be expected in the near-field repository environment. Findings to date further suggest that there will be a substantial gradient effect with respect to zones of inhibition within an EBS, being most inhibitory at, or near, the container surface, and extending into the compacted bentonite clay buffer. The primary controls on microbial growth in this highly inhibitory zone will be water activity, clay swelling pressure, temperature and radiation. Evidence is present to suggest that these factors will create a biologically inactive zone extending from the container tens of centimetres into the compacted clay buffer. Microbes positioned further from the container, particularly in the backfill region with lower density clay, will not be subjected to the same inhibitory factors, and hence could remain viable. The extent of their activity will be governed, in part, by the temperature, availability of nutrients, as well as the mass transport conditions dominant within the backfill, and enclosing

geologic formations.

Interfacial regions that exist between the various EBS components, primarily the buffer- and backfill-host-rock interfaces, as well as interfaces between cement components and the backfill, may support greater microbial activity and hence larger populations than the highly compacted bentonite buffer. Water movement along these interfaces will be governed by the saturation rates imposed by the enclosing low permeability far-field. Microbes positioned at interfaces would be expected to produce greater amounts of soluble and gaseous end-products than elsewhere in the repository. If gases are produced in sufficient quantity, so as to accumulate more rapidly than they dissolve, diffuse or are consumed, this could potentially impact the integrity of the EBS.

While SRB-mediated MIC will probably not occur in the oxidizing phase of repository evolution, allochthonously-produced HS^- could migrate to the container surface once conditions become anoxic, after which indirect corrosion could result at a rate limited by sulphide diffusion through and precipitation (as FeS) in the buffer. In the event of used fuel container failure, other potential microbially-mediated processes, including microbial movement to and from the container, as well as transport of microbe-radionuclide complexes or radionuclide-siderophore complexes, would similarly rely on the integrity of the compacted bentonite surrounding the used fuel containers. Water activity within the compacted bentonite is expected to remain sufficiently limiting so as to prevent significant microbial activity. The current understanding of the potential for microorganisms to impact the near-field environment includes:

i. The EBS environment will not be sterile. Microorganisms will be introduced to the EBS during construction and operation (via people, machines, drill and process water, and EBS components) or from the far-field via groundwater. In either case, a diverse community of microorganisms with differing metabolic capabilities, including those tolerant of repository conditions (including aerobes, anaerobes and extremophiles), will initially inhabit the buffer, backfill, etc. In addition to the introduction of microorganisms, DGR construction will also introduce various nutrients and conditions necessary for microbial growth (i.e., oxygen and nitrogen).

ii. After placement of used nuclear fuel containers, conditions within the near-field environment will initially be inhospitable to microbial growth and activity. Two broad zones of inhibition can thus be envisioned within the EBS: i) the microbicidal (lethal) zone closest to the used nuclear fuel container; and ii) the habitable (non-lethal) zones further away from the container. Within the lethal zone, evidence suggests that microbial inactivity will result due to the combined effects of heating, water limitation and radiation. Within the non-lethal zone, a functionally diverse, metabolically-active community of organisms could be (at least initially) established.

iii. The activity of the viable organisms present within the non-lethal regions of the EBS will serve to consume nutrients using oxygen as a terminal electron acceptor, and help drive redox conditions from oxidizing to reducing within the repository. Ultimately, a combination of biological and chemical reactions is expected to result in the creation of a stable anaerobic environment.

iv. Upon establishment of anaerobic repository conditions, a variety of organisms with the capabilities to utilize alternate electron acceptors have the potential to become active. Such activity could generate various soluble and gaseous end-products with limited

potential to impact both the physical and chemical aspects of the repository. Gas generation could create preferential pathways in the EBS and generated sulfide could diffuse to the used fuel container where it would cause corrosion effects, as governed by the limited rate of sulphide diffusion within the repository and by its precipitation as FeS.

v. Lastly, in a container failure scenario, released radionuclides could interact, to varying degrees, with microorganisms, their soluble extracellular products, or the clay buffer. Under these circumstances, the potential for either retardation or facilitated transport of radioactive material has been hypothesized, as described in Section 5.2.3. Transport effects should therefore be evaluated on a site-specific basis (Bass et al., 2002).

6.2 IDENTIFICATION OF KNOWLEDGE GAPS AND POTENTIAL FOR FURTHER RESEARCH

The various agencies (e.g., NWMO, SKB, POSIVA, NAGRA, NDA) tasked with the long-term management of used nuclear fuel have been conducting a broad range of experiments addressing key aspects of potential microbiological effects in future DGR facilities. To further refine the understanding of how microbiological processes will interact with EBS performance and long-term DGR safety, additional research efforts could be developed and/or monitored. Consistent with international best practice, these microbiological investigations could draw upon laboratory, *in situ* and modelling exercises, as described in this section.

Recommendation 1

Over the last 20 years, significant advances in subsurface microbiology in the context of a DGR for used nuclear fuel have been made. With respect to the repository near-field, research has generally focused on two broad questions: 1) can microbial metabolism realistically have an impact on repository function; and 2) if yes, what can be done to mitigate the impact, or control microbial activity? Considerable investigation has been conducted on microbial viability in compacted bentonite buffer and the identification of bulk densities capable of suppressing microbial activity in laboratory microcosm experiments (Stroes-Gascoyne et al., 2007b, 2010; Stroes-Gascoyne and Hamon, 2008, 2010). Importantly, the specific environment associated with each design and placement option, together with the host rock formation for the repository, can potentially present different outcomes in terms of microbial persistence and activity (Humphreys et al., 2010). As such, it is important that as repository designs are refined, microbial implications should be reassessed to ensure previous performance assumptions remain valid.

Continued use of laboratory microcosms to experimentally test repository designs relevant to the Canadian DGR program is recommended. Such experiments could further evaluate microbial processes in the bentonite and interfaces present in the EBS. Archaea have often been overlooked when considering key components of complex microbial systems and this represents a significant gap in understanding. It is thus recommended that archaea and prokarya both be examined with equal emphasis. The potential for halophiles, methanogens, xerophiles and thermophiles to function within the repository context is not a new consideration; however, the analysis and recognition of the potential role these microbes may play justifies more intense study, including the potential for other corrosive agents (e.g., ammonia, acetate) to be produced as a result of microbial metabolism. Laboratory experiments could be developed to test the different environmental conditions present during repository evolution.

Recommendation 2

Less attention has been given to understanding interfaces and transition zones in the repository relative to the compacted bentonite buffer matrix. Interfaces could potentially support increased microbial diversity and metabolic activity (Stroes-Gascoyne and West, 1997; Stroes-Gascoyne et al., 2010b). Furthermore, the ability to predict the degree to which biofilms might develop at these interfaces during saturation of the repository is lacking. The extremely low rates of nutrient and metabolite flux in the consolidated materials necessitate extended time frames of experimentation and require the development of innovative experimental set-ups.

Biofilms, in the conventional sense (i.e., thick layer of adherent bacterial cells and their exopolysaccharides), are not expected to develop at EBS interfaces. However, the boundaries between the host rock and the backfill could represent a zone with an active and sustained/stable microbial population. Laboratory based experiments evaluating biofilm formation at realistic flow rates and coupled with contemporary culture-independent methods, is recommended. Furthermore, while biofilm studies to-date have been performed almost exclusively at solid-liquid interfaces, mostly solid-air interfaces will initially be available for microbial colonization in the EBS. The conventional biofilm model is therefore not entirely applicable in zones other than fractures, which leaves the question as to whether or not characteristics often attributed to biofilms can realistically form the basis for conceptual and experimental models to predict microbial behavior in the bulk of the EBS. Therefore, the fundamental question of whether biofilms indeed form at these interfaces should be addressed, and if so, the behavior of microbes and their potential activity at solid-air interfaces should be assessed.

Recommendation 3

A diverse community of microorganisms with differing metabolic capabilities could be present in the repository environment. Microorganisms will be introduced to the EBS near-field during the open phase of repository construction or from the far-field via groundwater. The numbers, distribution and activity of microorganisms in the host rock provide a realistic scenario of the endpoint towards which these characteristics may evolve in the near-field, with the probable exception of the area directly adjacent to the containers where localized biological inactivation may occur.

Continued monitoring of the literature on deep subsurface microbiology is recommended as new research continually improves our understanding of the diversity and activity of life in the deep subsurface. For example, the existence of bacteriophages (bacterial viruses) in deep groundwaters has been shown, although their impact on microbial communities in consolidated materials and in groundwater is unknown.

Recommendation 4

While analytical methods evaluating microbial biomarkers and genetic material can generate information about the microbial species present and their potential metabolic capabilities, they do not yield information about the rates of microbial activity. Upon establishment of anaerobic repository conditions, a variety of organisms with the capabilities to utilize alternate electron acceptors have the potential to become active. Of particular interest are methanogenesis and sulphate-reduction processes, which could potentially create preferential gas migration pathways in the EBS and produce sulphide which could lead to microbially-influenced container corrosion, respectively. Hydrogen released during container corrosion can potentially stimulate

either process, and as such, an improved understanding of these biogeochemical processes in the context of a DGR for used nuclear fuel can improve long-term performance assessments. Continued participation in rock laboratory studies of microbiological processes and their effects on EBS components (i.e., bentonite) are therefore recommended to test repository designs relevant to the Canadian DGR program.

Recommendation 5

Understanding of microbiological processes is also typically taken into account implicitly while developing qualitative arguments in favour of safety (Humphreys et al., 2010). Metabolic activity in the deep subsurface may scarcely meet the requirements of cell preservation, let alone proliferation (Fredrickson and Onstott, 1996). One would expect that given sufficient time following used nuclear fuel emplacement, the repository would eventually return to those far-field conditions where these low potentials for microbial growth and activity dominate. As such, questions that require consideration are: i) to what extent will biotic reactions rates increase as a consequence of the disturbances that would accompany construction of a DGR, and ii) how long will it take for microbial rates to return to the original levels? However, the understanding of cellular energetic requirements for maintenance and ultimately growth in the subsurface is limited, hindering the ability to predict biogeochemical processes.

Through experimental and literature based studies, continued research into the energy requirements of cells, and the maximum potential rates of energy supply for the chemoautotrophic microorganisms to change the geochemistry in different zones of the EBS is recommended. Different scenarios of the flux of nutrients to the heterotrophic populations, and their specific requirements to move from a state of preservation to one of active metabolism, need to be better defined to enable realistic predictions of potential effect during modelling studies.

Recommendation 6

The materials that may be used in a repository (e.g., containers, buffers, backfills) contain potential nutrient and energy sources for microbial use (Humphreys et al., 2010). Organic carbon in the clay components of the repository (buffer and backfill) may provide a substrate for microbial growth, along with other materials left behind during the construction process (e.g., fuels, detergents, lubricants, wastes from human activities) (Hallbeck, 2010). Similarly, container corrosion can provide electron donors (H_2) for microbial metabolism through an array of terminal electron accepting (redox) processes. Thermodynamic modelling of redox processes can predict the consumption of electron acceptors (e.g. O_2, CO_2 and SO_4^{2-}) in the repository, and it is important that future efforts focus also on describing *in situ* rates. Of particular importance is sulphate reduction, which produces sulphide that can cause microbially influenced corrosion. There is the need to build on current knowledge (e.g., Pedersen, 2010) to expand on describing sulphate reduction potentials under relevant repository conditions so that the EBS can be designed to inhibit or minimize microbial growth.

More detailed investigation into the kinetics of sulfide generation from materials present in the buffer and backfill is necessary to provide greater confidence in source terms for use in modelling exercises. Building on the earlier work of e.g. Stroes-Gascoyne (1989) and Hallbeck (2010), additional modelling exercises could provide a better prediction of MIC in the repository given the carbon and nutrient sources present. Identification of limiting nutrients in the repository can ensure they are controlled during construction operations.

7. SUMMARY

The potential for microbial activity in low permeability crystalline and sedimentary rock formations considered in the Canadian DGR concept, is low. In a review in Scientific American, Fredrickson and Onstott (1996) stated that average population doubling times within subsurface environments may be so slow (e.g., 100 years or more; see Section 3) and that realistically, metabolic activity would scarcely meet the requirements of cell preservation, let alone proliferation. In some subsurface environments, there are questions surrounding whether viable cells may even be recoverable, and that perhaps cells present in those environments, while metabolically functional, may have lost the ability to replicate. One would expect that given sufficient time following nuclear waste emplacement, the repository would eventually return to those far-field conditions where these low potentials for microbial growth and activity dominate. As such, questions that require consideration are: i) To what extent will biotic reactions rates increase as a consequence of the disturbances that would accompany construction of a DGR, and ii) How long will it take for microbial rates to return to the original levels? Based on these questions, it follows that DGR design and operations should have goals to: i) Minimize increases in biotic reaction rates, and ii) Ensure a return to the original biotic reactions rates in the shortest time possible. Current EBS designs function to contain and restrict migration of radionuclides to diffusional levels, and to inhibit or minimize microbial growth rates.

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