Far-field Microbiology Considerations Relevant to a Deep Geological Repository – State of Science Review

NWMO TR-2011-09 **December 2011**

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ABSTRACT

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Abstract

This report presents a state of science review of international literature and knowledge on the role of microorganisms in relation to the key issues affecting the design and performance of a deep geological repository (DGR) for used nuclear fuel, with a focus on far-field microbial processes. The 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. The report is aimed at those who are familiar with the area of long-term nuclear waste management, but who are not experts in microbiology and the geochemistry of the subsurface.

The literature for international programs on far-field microbiology, as reviewed in this report, typically reported several general findings:

1. It is recognized that indigenous microbes exist in a broad range of geologic environments and it cannot *a priori* be assumed that a subsurface geologic environment is sterile.
2. The review of international programs shows that microbiological characterization programs can be integrated with the geologic/hydrologic/geochemical investigations to ensure feedback between these approaches.
3. The presence, diversity and activity of indigenous microbial populations in the far-field is controlled by a number of factors, including principally: geologic (physical) and chemical (including mineralogical) properties of the host rock; transport properties of the host rock; geochemistry of the associated groundwater; hydrogeologic properties; and both geologic and geochemical history and evolution of the site.
4. Hydrology, geochemistry and resident microbial populations may be sensitive to changes or perturbations in the system. Many systems possess geochemical buffering capacity to counter the effects of perturbations.
5. Microbiological far-field investigations should incorporate techniques that eliminate, to the degree possible, any contamination due to sampling/drilling, but should also control for contamination by characterizing not only the indigenous geochemistry and microbiology, but also the geochemical and microbiological properties of any potential contaminant end-members.

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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 far-field microbial processes in relation to the key issues affecting the characterization, design and performance of a used nuclear fuel repository. 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 (and biosphere) beyond the near-field. The 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. The report is aimed at those who are familiar with the area of nuclear waste management, but who are not experts in microbiology and the geochemistry of the subsurface.

1.1 OBJECTIVES

The report addresses the relationships between environmental conditions of the deep subsurface (salinity, porosity, water activity, temperature, geochemistry) and microbial activity. The review considers all rock types that are potentially suitable as host rocks for a DGR in Canada, and highlights differences for crystalline and sedimentary rocks where relevant. Specifically, with regard to the far-field microbiological considerations relevant to a DGR for used nuclear fuel in Canada, the following is included: i) the diversity and activity of microbial life at depth in low permeability rocks with high total dissolved solids (TDS) and high ionic strength; ii) the effects of microorganisms on the geochemistry of the far-field, with particular emphasis on the role of microbes in developing and maintaining reducing conditions; iii) microbial effects on radionuclide migration, including effects from colloids or biofilms; and iv) the effects of microbial gas production.

The report comprises the following sections:

1. Introduction to the scope, objectives and structure of the report.

2. Review of the diversity and activity of microbial life at depth (including sampling and analytical methods).

3. Review of the implications of far-field microbiology on DGR performance, including the effects of far-field microbiology on: geochemistry, gas production, transformation of radionuclides, and radionuclide transport in the far-field.

4. Review of international and Canadian research programs.

5. Recommended strategy for moving forward that is consistent with approaches taken by other international nuclear waste management organizations in their far-field microbiology programs.

The report outlines a suggested approach for an investigation strategy and for development of a microbiology technical program that could be conducted as part of detailed site characterization activities. Consideration was given to sampling methods that could be used during site investigation (e.g., core versus water sampling, contamination prevention measures, surface

exploration versus underground exploration, etc.), experimental work needed in preparation for site investigation, and analytical approaches (e.g., culture-based or molecular-based) that could be used.

2. DIVERSITY AND ACTIVITY OF MICROBIAL LIFE AT DEPTH

2.1 MICROORGANISMS IN THE SUBSURFACE

2.1.1 A Brief History of Subsurface Microbiology

Chemolithotrophic communities, or microbes drawing their energy for life from geologically produced chemical species rather than from the products of photosynthesis, were discovered in the late 1970's at mid-ocean ridge hydrothermal vents. The discovery sparked a revolution in our understanding of the range of possible mechanisms for sustaining life, and hence in our concept of the extent of the planet where life could be found. Since that time, our understanding that life is not simply a thin veneer on the earth's surface, but may permeate deep into the subsurface of this planet, has evolved rapidly. Serpentization of ultramafic rocks and alteration of basaltic ocean floor have been invoked as key mechanisms by which geochemical processes of water-rock interaction may provide energy and reducing power for chemoautotrophic microbial communities on the seafloor (Charlou et al., 2002; Bach and Edwards, 2003; Kelley et al., 2005; Takai et al., 2005). Chapelle et al. (2002) and Spear et al. (2005) reported the presence of H_2-utilizing chemoautotrophic microbial communities in continental volcanic hot springs, while Stevens and McKinley (1995) suggested that H_2 autotrophic microbial ecosystems might be important at 1.3 km depth in continental flood basalts.

A major gap remains in our understanding of microbial life beyond the deep hot biosphere. Investigations, particularly in the continental or terrestrial deep subsurface, are recognizing that chemosynthetic communities are not restricted to the high temperature hydrothermal vents and springs, but can be sustained under lower temperature regimes by similar types of water-rock reactions, albeit at much slower metabolic rates (Sherwood Lollar et al., 1993a; Shock and Schulte, 1998; Pedersen, 2000a; Chapelle et al., 2002; Kelley et al., 2005; Spear et al., 2005; Lin et al., 2006).

Heterotrophic microbial communities are similarly recognized now to play a much larger role than previously imagined, both in the deep marine biosphere and in anaerobic environments in the deep continental subsurface. Biodegradation was once thought to be an important process only in shallower aerobic environments, but it has become clear that biodegradation of crude-oil and coal reserves under deeper, anaerobic conditions also generates these increasingly sought-after natural gases (Head et al., 2003; Jones et al., 2008). The factors controlling methane and natural gas formation - in particular the nature of the subsurface microbial community and the rates of anaerobic biodegradation - are undergoing significant re-evaluation (Head et al., 2003; Zhou et al., 2005; Zhou and Ballentine, 2006; Jones et al., 2008).

The implications of this conceptual evolution in our understanding of deep subsurface microbiology are profound, as it suggests much larger volumes of the Earth's subsurface may be habitable. In just 30 years, science has moved away from the misconception that life consists of a thin veneer on the surface of the planet driven by the sun's energy alone, to recognition of the deep biosphere oases at vents and springs, to the possibility that the Earth's biosphere extends to kilometers depth over the planet's entire circumference. Whitman et al.

(1998) has suggested that the combined biomass of this deep biosphere may exceed the mass of biota on the Earth's surface. This suggestion is, as yet, unverified, but is the focus of significant interest and research activity in recent years.

2.1.2 Microbial Classification and Diversity

A microbe is a single cell that contains everything needed to thrive and pass on its genes. The smallest microbes can be viewed only under a microscope (sub-micron size), but the largest single-celled organisms can be visible to the naked eye (up to 1 mm). Because of the small size of most of these organisms, DNA sequences are used to classify them based on their genetic relatedness. This phylogenetic approach has generated a tree of life consisting of three domains (Woese et al., 1990), namely the Eukarya, the Bacteria and the Archaea. A further classification system breaks microbial life down into Prokaryotes and Eukaryotes by examining the structure of individual cells (see Figure 1).

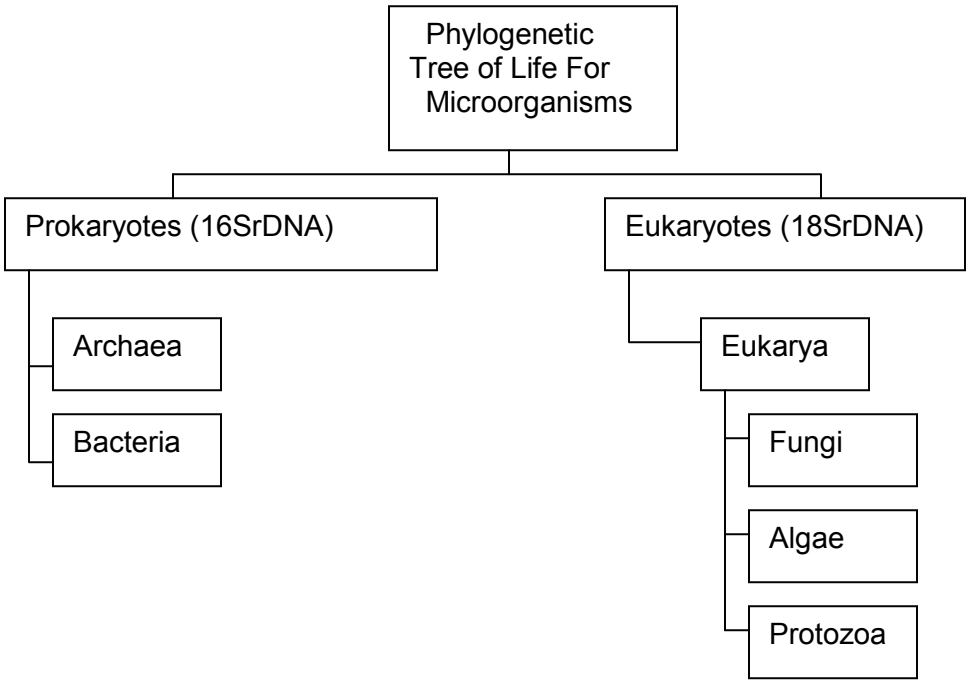

Figure 1. Classification of Microorganisms (Modified after Humphreys et al. 2010)

Prokaryotes are typically single-celled organisms whose DNA is not contained within a nucleus, whereas the eukaryotes do house their DNA in a nucleus and can be either single-celled or multi-cellular. The Bacteria and Archaea are all prokaryotes whereas the Eukarya are eukaryotes. The domains Bacteria and Archaea exclusively contain microorganisms, while the domain Eukarya contains both microorganisms and larger organisms (e.g., protozoa).

Algae, fungi and protozoa are eukaryotes whose cells are typically more complex than those of the related prokaryotes, in that they contain a distinct nucleus in which their genetic material is contained.

Viruses represent a special case not included in the universal tree of life. Viruses are extraordinarily small (typically 0.01-0.1 μm) infectious agents. They are non-cellular, cannot reproduce independently and have no independent metabolism. A virus is simply a submicroscopic particle of nucleic acid (DNA or RNA) wrapped in an exterior coat of protein called a capsid.

Many text books provide a more detailed treatment of the Universal Tree of Life and related classification details (e.g., Chapelle, 2001). This report focuses exclusively on subsurface prokaryotes.

2.1.2.1 Bacteria

Bacteria have a relatively simple cellular structure. The sac-like interior (cytoplasm) of the cell contains a single molecule of DNA (chromosome) containing typically between 2,000 to 10,000 genes, protein assembly structures called ribosomes, and may also contain smaller circles of DNA called plasmids. Bacteria are separated from the environment by a cell wall. Bacteria are further classified as Gram-positive, those which have a thick cell wall outside the cell membrane, and Gram-negative, those with a thin cell wall and a further membrane outside the cell wall. To survive, especially in dry places, Gram-positives can form spores. Bacteria are typically robust and have adapted to and colonized a wide diversity of environmental conditions.

2.1.2.2 Archaea

Microorganisms in the domain Archaea were thought to be bacteria until molecular data revealed that they differ from all other bacteria, plants, animals and fungi. What unites Archaea organisms is their ability to tolerate extreme conditions. Many Archaea function optimally at around 100 ^{o}C, while some, *Pyrolobus*, for example, can survive up to 113 ^{o}C. The current demonstrated upper temperature limit for life (approximately 121^{o}C) is that of Strain 121 that was isolated from the NE Pacific near a black smoker hydrothermal vent and is closely related to *Pyrolobaculum* and *Pyrodictium* (Kashefi and Lovley, 2003). Other Archaea are similarly adapted to extremes of pH (Pedersen et al., 2004), alkalinity and pressure.

According to Chapelle (2001), the Archaea comprise three kingdoms. Chrenarchaeota are largely thermophilic (heat-loving); Euryarchaeaota are divided into those which produce methane from carbon dioxide and hydrogen (methanogens), those which thrive in salt brines (extreme halophiles), and those which live at extreme levels of high heat or low pH (thermoacidophiles). A third Kingdom, Korarchaeota, has been proposed based on the 16S rRNA sequences found in microorganisms collected at geothermal sites such as Yellowstone National Park and the 9^{o}N East Pacific Rise deep-sea hydrothermal vent. The methanogens have the most influence on the geochemistry of groundwater. They require no oxygen, needing only hydrogen and carbon dioxide. As a result, they are among the most prevalent microbes found in the anoxic subsurface environment.

2.1.3 Microbial Metabolic Processes

In addition to the above genetic-based evolutionary relationship classification, microorganisms can be classified based on their metabolic capabilities (energy sources, ability to generate energy, nutritional requirements and metabolites). From the point of view of far-field microbiology relevant to a deep geological repository (DGR), such classification can be more useful because it provides information on the relationship of the microbiology to the geologic environment, and on potential microbial impacts on the geology and geochemistry of the subsurface.

Chapelle (2001) defines metabolism as "the complex series of energy-utilizing chemical reactions carried out by the cell." A cell can perform two types of metabolic processes. It can produce energy by breaking down organic compounds, a process called catabolism, or it can do the opposite, by using energy to build organic compounds, called anabolism. The overall functions of the cell include energy extraction and storage, cell construction, maintenance and repair.

Looking at life from the energetic perspective, there are two sources of energy: oxidation-reduction (redox) and light. Those who turn light into energy, including some prokaryotes and some eukaroyes, are called phototrophs. Because there is no light in the subsurface, at least in the far-field or where undisturbed by other anthropogenic activities, photosynthetic-based life is not directly addressed in this report.

Redox-derived life includes both organotrophs, which generate energy by the oxidation of organic compounds (originally produced by the photosphere), and lithotrophs, which generate energy by the oxidation of inorganic compounds.

A further classification of microorganisms is based on the differing sources from which they draw the carbon required to build cellular components. Autotrophs obtain carbon from carbon dioxide, while heterotrophs require more complex organic carbon sources. Autotrophy is commonly, but not exclusively, associated with lithotrophic and phototrophic energy strategies.

Energy release involves the transfer of electrons from electron donors (such as organic substrates in the case of organotrophs, or sulphides, CH_4 or H_2 for example in the case of lithotrophs (Table 1)) to electron acceptors. Electron acceptors are those inorganic compounds that can accept electrons and allow complete oxidation of organic substrates and reduced inorganic species. The most common acceptors are oxygen, nitrate, ferric iron, sulfate, and carbon dioxide. Manganate, selenate, uranyl ions and other oxidized compounds can also act as electron acceptors. The combination of the two processes, the oxidation of organic chemicals and the reduction of external electron acceptors, is called respiration.

Table 1: Typical Microbial Lithotrophic Processes Relevant to Subsurface Microbiology (Adapted after Humphreys et al. 2010)

Electron Donor	Electron Acceptor					
	O_2	NO_3^-	Mn (IV)	Fe (III)	SO_4^{2-}	CO_2
Sulphides (Mineral/Dissolved)	✓	✓				
Elemental Sulphur	✓	✓				
Ferrous Iron	✓	✓				
Uranium	✓	✓				
Ammonia	✓	✓				
Methane	✓	✓	?	?	✓	
Hydrogen	✓	✓	✓	✓	✓	✓

Note: Processes that are suspected but not proven are indicated by "?"

Cells have electron transport systems, which physically carry electrons from organic molecules and reduced inorganic species to electron acceptors. The most important role of these systems is to synthesize ATP (adenosine triphosphate) in order to conserve some of the energy from redox reactions.

The relevant metabolic processes for a given subsurface environment depend on the native microbial population, which is largely controlled by the energy sources and electron acceptors and donors present. Energy can be derived from many sources in a possible repository environment, including organic carbon, methane and reduced inorganic molecules such as hydrogen. While oxidizing energy sources, microbes typically use electron acceptors in this order: oxygen, nitrate, manganese, iron, sulfate, sulphur, and carbon dioxide. At the same time, the metabolizing microorganisms may use hydrogen and short-chain organic acids produced from fermentation, a process that does not require electron acceptors.

Anaerobic environments usually dominate at depth in the subterranean environment, due to the low solubility of O_2 and the rapid utlilization of available O_2 by aerobic microorganisms in the shallow subsurface. Table 2 provides examples of the typical impacts of microbial processes on the subsurface environment.

Table 2: Possible Effects of Microbial Processes (Adapted from Hallbeck and Pedersen, 2008b)

Metabolic groups of microorganisms	Activity	Effect on the environment
Aerobic respiration	Oxidation of organic material or inorganic compounds by oxygen reduction	Depletion of oxygen and organic material Increase in alkalinity Lowering of redox potential
Anaerobic respiration	Oxidation of organic material along with the reduction of compounds other than oxygen	See below for each specific group of bacteria
Nitrate-reducing bacteria	Oxidation of organic material along with nitrate reduction	Depletion of organic material and nitrate; increase in nitrogen gas and/or nitrite and alkalinity; lowering of redox potential
Iron-reducing bacteria	Oxidation of organic material along with ferric iron reduction	Depletion of organic material and ferric iron Increase in ferrous iron concentration and alkalinity Lowering of redox potential
Manganese-reducing bacteria	Oxidation of organic material along with manganese (IV) ion reduction	Depletion of organic matter and manganese (IV) Increase in manganese (II) concentration and alkalinity Lowering of redox potential
Sulfate-reducing bacteria	Oxidation of organic material or carbon dioxide along with sulfate reduction	Depletion of organic matter and sulfate Increase in sulphide concentration and alkalinity Lowering of redox potential
Methanogenesis		
Heterotrophic methanogens	Convert short-chained organic material to methane and carbon dioxide	Depletion of organic material Increase in methane gas and carbon dioxide (alkalinity) concentrations Redox not influenced
Autotrophic methanogens	Oxidation of hydrogen gas and reduction of carbon dioxide to methane gas	Depletion of hydrogen gas and alkalinity Increase in methane gas concentration Redox lowered
Acetogenesis		
Heterotrophic acetogens	Convert organic material to acetate	Depletion of organic material other than acetate Increase in acetate concentration Redox not influenced
Autotrophic acetogens	Oxidation of hydrogen gas along with reduction of carbon dioxide to acetate	Depletion of hydrogen gas and alkalinity Increase in acetate concentration Redox lowered

2.1.4 Extreme Environments

Microbial growth in subsurface environments was formerly thought to be limited by the scarcity of nutrients such as nitrogen and phosphorous. But, in an environment with a limited energy supply, it is unlikely that scarce nutrients will limit microbial growth and/or metabolic activity. There are sources for both nitrogen and phosphorous in groundwater. Microorganisms use phosphorous in the form of phosphate, which is found in low amounts dissolved in groundwater. Ammonium and nitrate, which are found in limited amounts even in deep groundwater (Silver et al., 2011), are typical sources of the reduced nitrogen needed to synthesize amino acids and nucleotides. Microorganisms can also incorporate nitrogen into the cell through nitrogen fixation. As this ability is widespread in microbial groundwater populations, nitrogen availability will rarely limit growth.

In fact, the past four decades of research have shown that many environments once considered to be "extreme" or limited for life, are in fact populated by specialized microbial populations that have adapted to these exotic ecological niches. The tolerance of microbes to environmental conditions is much broader than once thought and has been recently summarized in Table 3.

Table 3: Tolerance of Microbes to Extreme Environments (From Humphreys et al., 2010)

Condition	Example of organism	Limit of growth
High temperature	'Black smoker' bacteria	Reported to 113^{o}C
Low temperature	*Sporotrichum carnis*	-20^{o}C
High pH	Nitrifying bacteria	12
Low pH	*Thiobacillus ferrooxidans*	0
High salinity	*Halobacterium halobium*	50% salt by weight
Low salinity	*Salmonella orianenburg*	70 ppb dissolved salts
Radiation	*Deinococcus radiodurans*	Single dose 5000 Gy
Chemical toxins e.g. $PbCl_2$	*Aspergillus niger*	67 mg mL^{-1}
High pressure	*Desulfovibrio desulfuricans*	180 MPa

It is now widely recognized that virtually no subsurface environments can be assumed to be sterile *a priori*. Instead, subsurface environments may host microorganisms, which may be viable but inactive, even over geological time scales (Onstott et al., 2010).

Any number of conditions (lack of energy or food, reduction in water activity) can cause microbes to die or go dormant in one of several ways. Different species deal with these difficulties in different ways. Endospores formed by some gram-positive and sulfate-reducing bacteria are particularly hardy. They can show no sign of life for years and then, within a matter of a few hours, spring to life as actively growing cells if they enoucnter an improvement in their environmental conditions.

Microbes resist environmental constraints in many other ways. Many microbes achieve a level of metabolic activity low enough to allow them to survive for years, all but lifeless, at a fraction of their former size. Thus it is possible for a microbe to survive the extreme conditions that might be found in a repository (radiation, dryness, heat or high pH, decreasing water activity or decreasing pore space), only to revive when conditions improve. However, if conditions are such that no life forms survive, or the environment is insufficiently porous to allow microbes to travel and repopulate the affected areas, then it is possible that the formation, once the original

microbial community has disappeared, may remain sterile. DGR sites are typically chosen, in part, based on low porosity and permeability and low water activity.

Microbes indigenous to the Yucca Mountain, on the Nevada test site, have remained viable (but non-culturable) after exposure to gamma radiation up to 10^4 Gy (at 1.63 Gy min^{-1}) and could be later brought to culturable form (Pitonzo et al., 1999). Billi et al. (2000) demonstrated that dessication tolerant cyanobacteria could return to viable cells after exposure to 15kGy of ionising radiation (X-rays), but not after 20kGy. The bacteria studied came from dry and hypersaline environments, and their resistance to radiation was thought to stem from an enhanced ability to repair damaged DNA that evolved as a response to those extreme environments.

The Yucca Mountain project also supplied data on the effects of temperature on microbe survival and migration. Two test isolates in a block of tuff heated to 142 ^oC not only tolerated the heat but moved through the tuff itself to a distance of 1.5 m from their injection point (Chen et al., 1999). Also, some Yucca Mountain isolates survived repeated exposure to 120 ^oC heat, probably by generating spores (Horn et al., 1998).

Microbes in alkaline groundwater in Jordan were found to tolerate pH 12 and above (Linklater, 1998). In experiments where sulfate-reducing bacteria (SRB) were grown over a range of pH and Eh values, activity at pH 8 to 10 was enhanced by decreasing Eh (Fukunaga et al., 1995). Simulations of repository conditions in the UK (Gardiner et al., 1997) have shown that bacteria can survive a wide range of pH values and a temperature as high as 90 ^oC. SRB from other sites have been found to have a joint temperature and pressure tolerance of up to 80 ^oC and 310 bars, and a separate radiation tolerance of up to 10^3 Gy over 40 hours (West, 1995).

In summary, research on the limits to life and the diversity of organisms that can survive in what are anthropomorphically referred to as “extreme conditions” is rapidly evolving as investigators target not only terrestrial life, but also the possibility for life elsewhere in the solar system. As such, it is recommended that periodic reviews of the literature on assumed limits for life include not only literature from the deep subsurface microbiological community, but literature from the field of astrobiology and planetary exploration. For instance, the Report of the COSPAR Mars Special Regions Colloquium (Kminek et al., 2010) provides an excellent review of the rapidly evolving research concerning the effect of salinity, or more specifically water activity, on subsurface microorganisms.

It has long been known that halophile bacteria can thrive in salt brines (20-30%) (Grant and Larsen, 1989). The effect of solutes in solution is more commonly evaluated using the term water activity (a_w), the partial pressure of water in equilibiurm with the salt solution, or the effective water content expressed as its mole fraction (Grant, 2004). The a_w of pure water is 1.0 and factors that decrease a_w include solute content, dessication and sub-zero (^oC) temperatures (Kminek et al. 2010). The most relevant effect in the context of this report is solute content, although in the subsurface, additional parameters such as capillary forces between grains, and surface-binding of water films can have additional, and typically less well understood, indirect effects (Grant, 2004; Kminek et al., 2010) on water activity.

Studies from food science concerned with food preservation provide some of the most extreme examples. A food spoilage fungus (*Xeromyces bisporus*) has been shown to grow at the lowest water activity documented (a_w=0.61) in a sucrose solution (Grant, 2004; Kminek et al., 2010). More relevant to this report, diverse microorganisms, including Bacteria, Archaea and Eukaryotes, have been demonstrated to grow even in NaCl saturated solutions (~5 M NaCl)

with an a_w of 0.75 (Grant, 2004; Kminek et al., 2010). Even lower water activities can occur in systems where other salts (KCl, $CaCl_2$, $MgCl_2$) dominate, such as the Dead Sea brine (Hallsworth et al., 2007) and a Ca-Cl_2-rich lake in the Antarctic Dry Valley where solute concentrations approach 50% (wt./vol.) and a_w of approximately 0.45 (Kminek et al., 2010). Based on such findings, Kminek et al. (2010) suggests that a threshold value a_w of 0.5 is a conservative lower limit that is below the lowest known a_w of 0.62 demonstrated to support the growth and reproduction of microorganisms (Beaty et al., 2006). Kminek et al. (2010) notes that the limit for maintenance of metabolic activity and long-term survival of microorganisms cannot be definitively stated at this time (Navarro-Gonzalez et al., 2003; Kminek et al., 2010).

2.2 MICROBIAL METHODS

This section describes the methods used in the literature cited in this report.

2.2.1 Sampling Methods

A review of nuclear waste management international programs indicates that the acknowledged best practice is to integrate any microbiological characterization programs at the same time as geologic, hydrogeologic and geochemical investigations, early in the site characterization process (Humphreys et al., 2010). The characterization and sampling program itself, however, can disturb the *in situ* geochemical conditions and microbiological community. To minimize this impact, and to minimize any contamination of the *in situ* microbiological samples with organisms introduced by drilling and/or sampling, a variety of approaches are taken by the international community.

2.2.1.1 Minimizing Perturbation and Contamination during Sampling

Sampling of subsurface waters requires drilling, using a range of techniques that vary significantly depending on whether the groundwater is in unconsolidated, sedimentary or crystalline rock. Drilling approaches vary as well as a function of depth, sediments, desired sample (core and water; water alone; water and gas, etc.). A review of drilling technology is not within the scope of this. Pedersen (Pedersen, 2000a; Pedersen et al., 2008) provides a brief outline of the techniques applied within the SKB program at Äspö, and POSIVA program at Olkiluoto, including a comparison of downhole sampling devices and the recovery of groundwater samples via pumping water to the surface. A review should include a review of hydrogeologic best practice for obtaining representative groundwater samples in low permeability and/or fractured rock, where rates of groundwater flow into a borehole may be slow relative to possible pumping rates. As well, flow rates may vary substantially from one formation to another and, hence, any bulk water sample will preferentially reflect the contribution from the higher permeability formations and dilute the characteristics of the low permeability units.

While all international programs emphasize the need to minimize and control for contamination, most recognize that the processes of drilling and sample collection inherently mean that no sample can necessarily be considered pristine (Pedersen, 1993; Pedersen et al., 2008). While sampling equipment can be sterilized (see below), contamination of boreholes by drilling equipment and circulation of drill fluids is inherently a risk. International best practice controls for contamination typically by adding chemical tracers or dyes (Pedersen, 2000a). Natural tracers can also be used to monitor the extent of contamination. For instance, if drill waters contain dissolved oxygen (DO) or tritium reflecting their surface origin, monitoring these

parameters until anaerobic and non-tritiated conditions are re-established can serve as an indication that *in situ* groundwaters have flushed out contamination. This assumes a well characterized hydrogeologic and hydrogeochemical environment and evaluation of the potential for *in situ* groundwaters to contain the proposed tracers (DO, tritium, etc.). The appropriate tracer techniques, and time to flush out contamination, will differ as a function of the specific geologic, hydrogeologic and geochemical conditions and selected drilling fluids.

A similar series of approaches are relevant to microbiological sampling. Sterilization of sampling equipment is a necessary first precaution. Various approaches exist specific to the nature of the materials used in construction of the equipment. Pedersen (2000b; 2008) and Pedersen et al. (2008) describe a common approach that immerses sampling equipment in 11ppm chlorine dioxide solution for several hours, followed by flushing several times in sterile water. Other groups have used a combination of such rinses and sterile washes with auto-claving (Stroes-Gascoyne and West, 1996; Moser et al., 2002; Lin et al., 2006; Onstott et al., 2006; Stroes-Gascoyne et al., 2007; Hallbeck and Pedersen, 2008c). These references also provide information on best practice for storage and shipment of microbiological samples.

2.2.1.2 Comparing and Contrasting End-members

International best practice shows that despite the above attempts to minimize contamination, additional steps are needed. The key concept is that microbiological investigations should be carried out not only on the target groundwater but on all other end-members that may have impacted the system, including: drilling fluids and muds; and volatiles that may have entered the system due to drilling or excavation; as well as any other groundwater systems that may have been intersected while drilling to the target depth. Gihring et al. (2006) provides an excellent overview of this approach for microbial investigations in the deep gold mines of South Africa and demonstrates how these various end-members can be differentiated based on microbiological and geochemical characteristics. Pedersen et al. (2008) provides an additional example of how this comparison and contrast approach was critical to evaluating the reliability of various downhole sampling devices as compared to sample collection via pump (Pedersen et al., 2008). In the scientific literature, there is a large and extensive heritage on this approach for the deep subsurface, including, but not limited to: Kieft et al., 1999; Takai and Horikoshi, 1999; Pedersen, 2000a; Smith et al., 2000; Onstott et al., 2003; Ward et al. 2004; Moser et al., 2005; Smith and D'Hondt, 2006; Finster et al., 2009; Cockell et al., 2010.

2.2.2 Analytical Methods

Analyzing the geochemical context of a site can reveal significant information about its potential microbiology. Determining both the potential terminal electron accepting processes (TEAP) and the available nutrients at the site will provide an understanding of the potential microbial impacts (West et al., 1992; West, 1995; West et al., 1998; Jolley et al., 2003; West et al., 2006; Tochigi et al., 2008). If the geochemical contextual evidence reveals the likelihood of significant microbial activity, then a more detailed characterization should proceed predicated on both culture-based approaches and molecular microbiological techniques.

2.2.2.1 Culture-based Approaches

2.2.2.1.1 Total Number of Cells (TNC)

The TNC method simply and directly determines the living biomass in a system by determining the total number of cells, as these parameters are linearly related (Eydal and Pedersen, 2007). Water captured under sterile conditions is preserved by adding formaldehyde to a concentration of 2-4% (v/v). Samples are filtered onto a black polycarbonate filter with a pore size of 0.2 µm. Applied fluorescent dyes bind to the cells' RNA and DNA, which can then be precisely counted under a fluorescence microscope. Direct viewing also offers a clear picture of the cell size and the number of dividing cells. Total (live and dead) cell counts can be done by acridine orange (AO), 4'6-diamidino-2-phenylidole (DAPI), etc. These are dyes that bind to DNA but cannot distinguish between live and dead cells. Live cell counts can be done with other dyes, such as 5-cyano-2,3-tolyl-tetrazolium chloride (CTC), that bind to molecular structures that are involved in respiration or metabolism (Hallbeck, 2009).

2.2.2.1.2 Culturable Cell Counts

This is an indirect method of identifying culturable cells in a sample. The cells are distributed on a plate of solidified agar to number between 30 and 300 per plate. The composition of the medium can vary from sample to sample. The energy, carbon, and nutrients added to the medium should mimic the environment at the sample's source. After incubation, bacterial colonies appear as small dots of growing cells on the agar plate. Anaerobic microorganisms can be grown with the same technique, with the plates incubated in an anoxic atmosphere. Because this technique produces mostly fermenting and nitrate-reducing organisms, it is crucial to select the most appropriate substrate and carbon source and to ensure the cultures receive enough nitrates. The culturable count method has many disadvantages. It is impossible to use only one growth medium for a sample containing a variety of microorganisms. As well, significant information about the geochemical conditions must be obtained in order to select appropriate culture conditions (Hallbeck, 2009).

2.2.2.1.3 Microcosm Enrichment Cultures and the Most Probable Number (MPN) Method

Microorganisms from sites and materials associated with nuclear waste disposal have been extensively isolated and enumerated via culture-based techniques (e.g., Francis and Dodge, 1988; Vreeland et al., 1998; Farkas et al., 2000; Horn et al., 2004; Fukunaga et al., 2005; Hallbeck and Pedersen, 2008a). This is accomplished by growing organisms from the samples in a variety of solid and liquid media, with the composition of the media and the incubation technique varied to isolate particular physiological groups of organisms. Estimated numbers of culturable organisms can then be achieved either by directly counting the organisms on solid media or using liquid media and statistical approaches such as the Most Probable Number (MPN) technique. This approach is now common and relatively easy to undertake. However, very few environmental microorganisms (less than 1%) are culturable in this way, with microorganisms recovered onto solid media having the lowest recoveries (often less than 0.1% (Hallbeck and Pedersen, 2008a). It is important to note that many bacteria already identified through molecular biology have not yet been cultured (Rappe and Giovannoni, 2003), which suggests culture-based techniques may be isolating only the most culturable organisms within

an environment, and not necessarily the most important ones. Recovery significantly improves with the combined use of liquid media, specifically tailored to the potential site, and an MPN approach (Hallbeck and Pedersen, 2008a). Successful recovery requires mirroring the nutrient and cultural conditions at the original site and long incubation times (Tanner, 2007).

It should also be noted that if the conditions under which a group of organisms are isolated and enumerated differ critically from those on site, organisms in culture may be unable to perform their customary roles in an environment. Culture-based techniques, then, may be too imprecise to provide anything more than a rough guide to the relative proportions of the organisms at a site. Clearly a large number of culturable organisms will indicate an active population, but their absence should not be presumed to indicate the absence of an active population. An absence of evidence for life does not always mean that no life is present (Humphreys et al., 2010).

Culture-based techniques do allow isolated organisms to be used for further investigation through the development of so-called enrichment cultures and microcosm studies. Microcosm studies contain microorganisms from the site of interest and the environmental conditions they are exposed to can change to meet the experiment's objectives, as summarized in Humphreys et al. (2010). These studies can target a single bacterial species or focus on either defined or undefined communities of microorganisms. To concentrate on either a single species or a defined community, the microorganisms employed must be from the site in question or possess particular characteristics of interest. Single species experiments have been frequently used to investigate direct bacterial interactions with radionuclides (e.g., Boukhalfa et al., 2007; Icopini et al., 2007; Nedelkova et al., 2007; Renshaw et al., 2007). Microcosms can be run as sealed batch systems or as flow-through systems accepting added nutrients. Flow-through systems are commonly run as columns and have been used to investigate radionuclide release (Kelly et al., 1998), bioclogging (Nikolova et al., 2001), and the influence of biofilm in mineral precipitation and corrosion (West et al., 1998; Hama et al., 2001; Tuck et al., 2006).

Microcosm enrichment cultures are used to investigate specific functional groups within an environment. Samples are incubated under conditions which vary to select and promote the growth (enrichment) of specific desired organisms. Because the process may allow certain organisms, which may have been only common in the source environment, to become dominant in the enrichment culture, this technique prevents the enumeration of microbial groups in the sample. As with all other culture-based techniques, success depends on achieving the precise culture conditions which will promote the growth of the organisms of interest. Once again, absence of growth due to inappropriate culture conditions can be too easily mistaken for an absence of microbes if not correlated with other approaches described herein (e.g., see Sections 2.2.2.1.1 and 2.2.2.1.2 (Humphreys et al., 2010)). As Humphreys et al. (2010) notes, enrichment cultures have been used widely, for instance to investigate the possibility of specific terminal electron-accepting processes within sites (Beadle et al., 2001), the impact of iron reduction on uranium behaviour (Fox et al., 2006), the recovery of SRB and acetogens from bentonite (Fru and Athar, 2008), microbial growth potential in Yucca Mountain rock samples (Kieft et al., 1997), and cellulose degradation under WIPP conditions (Vreeland et al., 1998).

2.2.2.2 Molecular Biological Techniques (Culture-independent)

Culture-based methods rely on the ability of microorganisms to grow under laboratory conditions or in *in situ* experimental flow chambers. The results can be limited by the organisms' ability to grow under conditions that wholly or partially mimic those of the original site. Culture-independent techniques provide a means to overcome some of these limitations.

In brief, molecular microbiological approaches provide a picture of the microbial community's DNA, with the 16S RNA gene being most frequently used. Because the methods do not require the organisms to be cultured, this approach should broaden the percentage of the microbial community that can be targeted. Limitations include the potential for contamination, extrapolation of the sampled biomass to possible spatial and temporal variability in the *in situ* population, and the fact that this enumerative information does not easily provide information about levels of activity or rates of microbial processes.

However, innovative new techniques using nucleic acids, such as quantitative polymerase chain reaction (qPCR), have given researchers numerical data on the copy number of both functional genes and 16S RNA genes in microorganisms associated with contaminant degradation. The methods and limitations of these techniques have been explored, including those techniques pertaining specifically to the subsurface (see Weiss and Cozzarelli, 2008 and references therein).

The question under consideration and the nature of the research environment will influence the choice of techniques, as discussed by Weiss and Cozzarelli (2008). Investigators requiring a simple estimate of the size and diversity of the microbial population may perform assays, such as direct counting, plating of samples onto a general media, phospholipid fatty acid (PLFA) analysis, DNA fingerprinting or amplification of DNA with universal primers. Investigating a more complex biogeochemical process, for instance sulfate reduction, might require specific culture media or primers targeting either certain groups of sulfate reducers or the dissimilatory sulfate reductase gene. It may then require a combination of stable isotope and PLFA analysis to identify the organisms employing this process. The specific geochemistry of the site under investigation will determine which technique is most able to detect and characterise certain groups of organisms (Weiss and Cozzarelli, 2008).

2.2.2.2.1 Methods

As briefly described by Hallbeck (2009), obtaining samples for molecular microbiological investigations involves the following steps. DNA is first extracted from the source sample. If little DNA (i.e., few cells) exists in the sample, that DNA must be amplified using polymerase chain reactions (PCR). This process amplifies the desired part of the DNA, ideally in the same proportions as found in the original sample (although potential for bias in amplification does exist). This results in a mixture of DNA from all organisms in the sample. A further step sorts the DNA, either by cloning or gel electrophoresis. Hallbeck (2009) summarizes the next steps as follows.

Cloning: In cloning, a vector is used to introduce one copy of the 16S rRNA gene into an *E. coli* cell. The vector is often a plasmid, a small ring of DNA which can integrate the 16S rRNA sequence. The plasmid is then taken up by the one *E.coli* cell, which is then grown into colonies on agar plates. The plasmid is copied into all new cells such that the single 16S rRNA

gene is multiplied millions of times. The desired DNA sequence can be extracted from the *E. coli* and sequenced.

Sequence comparison: The acquired sequences are then compared with samples in public databases such as Genebank. The database sequences can be derived from pure sources or from the cloning of environmental samples. Of the two, pure source material provides richer evidence of the organism's metabolism and requirements.

Restriction enzyme digestion and gel electrophoresis: The restriction fragment length polymorphism (RFLP) technique is initiated by extracting and amplifying the 16S rRNA gene by PCR with a fluorescent label at one end of the sequence. PCR products from a range of samples are then digested by a variety of restriction enzymes, which cleave the DNA fragment into specific base-pair combinations. The result is DNA fragments of different lengths and, therefore, different weights. Gel electrophoresis targeted to specific weights separates the fragments. The differing patterns of the fragments are the focus of comparisons between samples. This technique does not identify specific organisms.

2.2.2.2.2 Genomics

The data that has been collected since all the molecular techniques described above came into common use have broadened into the field of study known either as genomics or metagenomics. It uses sequence-based and functional analysis to study a microorganism's genome. Its techniques center on isolating DNA, cloning it into a suitable vector, and screening it for transformation before it is analyzed through high-throughput sequencing (Weiss and Cozzarelli, 2008). Large numbers of clones can be screened rapidly and sequenced under whole-genome shotgun sequencing (Chen and Pachter, 2005), an approach used in sites as varied as the Sargasso Sea (Venter et al., 2004), an acid mine drainage site (Tyson et al., 2004), and isolated fracture waters from a South African gold mine at 2.8 km depth (Chivian et al., 2008). With data collected in this way, researchers can detail a community's diversity and composition, estimate the metabolic roles of individual organisms (Handelsman, 2004), and assemble near-complete genomes of the most prevalent strains (Weiss and Cozzarelli, 2008).

Metagenomics also includes the use of microarrays, a hybridization technique, whereby probes detect the relative presence of organisms and expression of multiple genes (Gentry et al., 2006; Weiss and Cozzarelli, 2008). Microarrays can be combined with sequencing studies to screen and identify clones. DNA microarrays have been used to detect 16S rRNA genes of *Geobacter Chapellei* and *Desulfovibrio desulfuricans* in soil extracts, to track gene expression of *G. sulfurreducens* under varying conditions, and to study functional genes such as those associated with methanotrophy in landfills (Stralis-Pavese et al., 2004).

2.2.2.2.3 DGGE and TGGE

PCR fragments may also be separated by electrophoresis techniques such as Denaturing Gradient Gel Electrophoresis (DGGE) and Temperature Gradient Gel Electrophoresis (TGGE) as discussed by Humphreys et al. (2010). In DGGE, amplified DNA fragments are denatured by chemicals, allowing the separation of DNA fragments which are the same size but with different base pair sequences. In TGGE, the denaturing is done through temperature rather than chemicals. In both cases the result is a banding pattern on the gel which reflects the microbial diversity of the sample analyzed. DGGE and TGGE can be used to profile communities and to

separate fragments for sequence analysis, but their application to environmental microbiology have revealed potential shortcomings, as summarized by Liu and Stahl (2007). The methods cannot precisely discern the exact number of bands under examination (the problem of "band overlap") or identify multiple bands which may have been created by one organism. Moreover they cannot separate fragments greater than 500 base pairs, limiting the information to be gained from the sequencing of an excised band.

2.2.2.3 Biological and Physiological Markers

Beyond microscopic investigations, biochemical markers offer another picture of the microorganisms in a site's geological environment. The co-enzyme Adenosine Tri-Phosphate (ATP) is key to the transfer of energy between cells. The presence of ATP can indicate active microbial populations. Eydal and Pedersen (2007) compared ATP analysis with viable and direct microbial counts of both shallow and deep groundwaters, and found ATP to be a reliable indication of microbial activity, with a detection limited of 2×10^3 cells mL^{-1}. ATP levels correlated with total cell counts, and the ratio of ATP levels to cell counts, provided an indication of the metabolic activity of the groundwater environment under investigation.

As described by Humphreys et al. (2010), phospholipid fatty acid (PLFA) analysis is yet another technique to assess viable microbes within an environment. PLFAs are microbial membrane components, which are rapidly degraded on cell death, and consequently represent the living microbial community present within a sample. Because specific lipids indicate specific groups, PLFA analysis goes beyond simply estimating overall biomass levels and, in addition, provides insight into the overall microbial community structure. In contrast, the breakdown products of phospholipids, as well as neutral and glyco-lipids, can be used as markers for cell debris. PLFA analysis has been used to provide insight in the microbial ecology of a wide range of subsurface habitats (Ringelberg et al., 1997; Green and Scow, 2000; Weiss and Cozzarelli, 2008). While complementing both molecular and biochemical profiling, PLFA analysis may, in fact, more effectively detect changes in microbial communities. For instance, PLFA in organisms that are starved or otherwise under environmental pressure are different from organisms that are well-fed (Ramsey et al., 2006).

Physiological characterisation, which examines both the consumption of substrates and the generation of metabolic end products, has been used to reveal the presence and activities of organisms from a wide range of sites and experimental programs. This physiological profiling has been used to characterise bacteria from some radioactive waste disposal sites (Vreeland et al., 1998; Nedelkova et al., 2007) and to investigate the subsurface degradation of organic pollutants and associated bacteria (Garland et al., 2007). Stable isotope probing (SIP) uses a ^{13}C-label to investigate the subsurface degradation of organic compounds (Madsen, 2006) and can be an alternative to radio-labels such as ^{14}C. SIP allows researchers to "track the flow of atoms in isotopically enriched molecules through complex microbial communities into metabolically active microorganisms" (Madsen, 2006; Weiss and Cozzarelli, 2008). Specifically, after an environmental sample has been exposed to stable isotope-enriched substrates, the labelled biomarkers recovered (DNA, RNA or PFLA) help identify the microorganisms using the added substrate.

3. IMPLICATIONS FOR A DEEP GEOLOGICAL REPOSITORY

There are two sources for microorganisms in the far-field subsurface around a potential repository. First, indigenous microorganisms in the rock, which may have existed since the rock's formation or been introduced by inflowing groundwater; second, exogenous microorganisms entering from the near-field of a repository.

As described in Section 2, microorganisms in the subsurface exploit a variety of metabolic processes depending on the nature and abundance of the available energy sources, electron acceptors and nutrients (see Table 1). The extent of microbial activity (whether simple maintenance, growth and/or reproduction) also depends on environmental conditions - in particular the presence of water, water activity, temperature, pH, and redox potential, the last being a measure of the potential for electron transfer in a given system. The corollary clearly also holds true - microbial activities themselves have an impact on their surroundings through the depletion and transformation of reactants and through the cycling and accumulation of redox reaction end-products and by-products (see Table 2).

As outlined in Section 2, microorganisms can be classified as autotrophs (those that use carbon dioxide (gas) or dissolved inorganic carbon (DIC) as their carbon source for synthesis of biomass), or as heterotrophs (those that use the carbon present in organic compounds). Both are prevalent in subsurface microbiological populations. However, while microorganisms at the Earth's surface contain large numbers of phototrophs (those that use the sun's light via photosynthesis), subsurface microbial populations are dominated by chemotrophs - organisms which can metabolize without light, using chemical sources of energy available at depth.

As discussed by Bass et al. (2002), chemotrophs are further classified by the type of electron donor: organotrophs use organic compounds as donors, whereas lithotrophs use reduced inorganic materials. The same prefixes are used to define particular metabolic types. For instance, chemo-organotrophs (or chemo-heterotrophs) degrade organic compounds; chemo-lithotrophs (or chemo-autotrophs) oxidize, for instance, reduced nitrogen, and photo-lithotrophs oxidize, for instance, reduced sulphur and capture light energy.

There are two kinds of subsurface ecosystems: detrital and productive (Bass et al., 2002). Detrital systems use carbon fixed initially at the surface by photosynthesis. The resulting organic material was then buried under sediment and metabolized by subsurface chemo-organotrophs. This depletes the organic matter, but it is unlikely to exhaust it due to the presence of non-degradable material, or because compaction, cementation and decreased porosity have impeded microbial access to it. Photosynthesis is impossible in the lightless subsurface. Here, carbon and energy found in inorganic material is processed by chemo-lithoautotrophy. Many redox couples can support chemo-lithoautotrophs. A self-sustaining ecosystem may be created when dependent chemo-organotrophs come into contact with organic material generated by subsurface primary producers. It is likely that such a community will generate enough energy only to maintain itself, but not to proliferate (Bass et al., 2002).

3.1 INDIGENOUS POPULATIONS OF MICROORGANISMS IN THE SUBSURFACE AND EFFECTS ON THE GEOCHEMICAL ENVIRONMENT

3.1.1 Sediments and Sedimentary Rocks

Bacteria understood to be associated with deep subsurface environments were characterised a century ago, yet science has only recently accepted the existence of a deep subsurface biosphere. Original scepticism was based on the notion that it could not be guaranteed that the organisms detected in samples did indeed come from the deep subsurface. More rigorous collection and analysis have lessened many of those concerns.

The modern era of subsurface microbiology was launched in June 1986 at the Savannah River Plant (SRP) in South Carolina, with seed money from the U.S. Department of Energy (DOE) Subsurface Science Program (Onstott et al., 2010). Three wells were cored to depths of up to 200 m using chemical and biological tracers to constrain contamination. During the decade that followed, samples of the diverse microbial communities observed in subsurface aquifers were recovered at depths as great as 2.7 km over a wide range of environments and rock types (Onstott et al., 2010 and references therein). The microorganisms were predominantly saline-tolerant, thermophilic, fermenting, Fe(III)-reducing and sulfate-reducing bacteria compatible with pressures to 32 MPa and *in situ* temperatures of 76^{o} C (Onstott et al., 1999). In Europe, while some microbial studies of sedimentary systems were also carried out in the mid-1980's (summarized in Cristofi and Philp, 1997), the period of greatest activity was in the 1990's. This work focused first on marine seafloor sediments (Section 3.1.1.1) and then on microbiological investigation in subsurface petroleum reservoirs. While these environments are not considered as potential host rocks in the Canadian repository program, they are summarized herein to provide context on characterization approaches and potential geochemical processes that should be considered in continental sedimentary rock environments (Section 3.1.1.2)

3.1.1.1 The Deep Marine Biosphere

For decades, the seafloor was seen as an uninhabitable desert. Then the Ocean Drilling Program (ODP) of the 1980s and 1990s began to drill deeper than ever before, obtaining good data with little disturbance. As discussed in detail in Parkes et al. (1994), all sediment studies cause some sediment disturbance and contamination, however ODP coring and sampling procedures produced microbiological data which was sufficiently consistent with geochemical analyses to indicate negligible contamination. "Smearing" during coring could not have caused the noted increase in bacterial life in deeper layers, as the core passed through regions of lower bacterial activity on its way down. As well, good core recovery (99%) and shipboard measurements indicated minimal disturbance to the seafloor. This approach to assessing potential contamination is consistent with the approach described in this report (Section 2.2.1.2).

Parkes et al.'s seminal papers (1994; 2005) demonstrated that bacterial distributions and activities are commensurate with geochemical changes in viable bacterial populations in sediments at Pacific Ocean sites to depths of >500 m. Bacterial profiles with depth were remarkably consistent, and deviations could be linked to specific environmental factors. For example, their work provided microbiological and geochemical evidence for sulfate reducers and both H_2/CO_2-utilizing and acetate-utilizing methanogens, and for methane-consuming

organisms, presumably anaerobic CH_4 -oxidizers (D'Hondt et al., 2002; Parkes et al., 2005), fermenters, and Mn- and Fe-reducers.

Archaea were found to be far less diverse than bacteria. As in other subsurface sediments, all depths were dominated by the diverse miscellaneous crearchaeotic groups. Only one methanogen was detected and this was from the methane zone. However, methanogen-specific genes (mcrA) were observed at all four depths, a finding consistent with methane production rate measurements. Diversity was limited to *Methanobacteriales* and *Methanosarcinales* (taxa using H_2/CO_2 and/or acetate respectively), which is consistent with methane generation from both these substrates. Diversity is similarly limited in other deep sediments. There were no sequences for anaerobic methane oxidizers. The rate of sulfate reduction indicated that the reducing bacteria reached a maximum proportion of 0.02% of the overall population at 30 m and 0.002% at 60 m. Significantly, this work demonstrated that prokaryotes directly involved in sulfate reduction and/or anaerobic methane oxidation, while quite active, might represent only a small proportion of the total population. This illustrates a continuing challenge of deep subsurface microbiology (PCR and other related techniques may underreport small proportions of the population). It underscores the necessity of coupled geochemical investigations to identify processes (Parkes et al., 2005).

Many of the tracer methods developed by DOE during the late 1980's and early 90's were adopted by the Integrated Ocean Drilling Project (IODP) as it undertook microbial studies of the sub-seafloor biosphere. Many of the past decades' advances come from the molecular analysis of samples recovered on IODP cruises. These include, as summarized by Onstott et al. (2010):

- the recognition of Archaea as a significant, if not dominant, microbial domain in sub-seafloor sediments;
- the use of RNA analyses to identify truly active microbial cells (only active cells have RNA);
- a better understanding of subsurface metabolic functions through sequencing of complete metagenomes and application of microarrays designed to detect functional genes;
- the discerning of acetogenic from acetotrophic activity on site by applying stable carbon isotope analyses to volatile fatty acids; and,
- the use of ion microprobe analyses to identify the carbon substrates for subsurface microorganisms.

In 2005, Schippers et al. (2005) showed the presence of intact membranes and ribosomes - the first conclusive evidence of thriving bacteria - in 16-million-year-old sediments more than 400 m deep. Recently, Roussel et al. (2008) reported metabolically active microbes in 111-million-year-old sediments 1.6 km below the seabed. The sole scientific drilling mission dedicated to this biosphere was mounted in 2002, but activity is increasing, with 3 to 4 more missions scheduled through the IODP by 2013, and with the National Science Foundation's (NSF) support of new initiatives such as the C-DEBI Research Coordination Network led by K. Edwards and J. Amend of the University of Southern California (USC) to develop international collaborations in deep marine biosphere research and exploration. Initiatives include installing sub-seafloor laboratories known as circulation obviation retrofit kits (CORKs), which seal scientific instruments inside deeply drilled boreholes and measure life in the deepest marine subsurface (Mascarelli, 2009).

Soils and surface sediments can contain billions of prokaryotic cells per cubic centimetre, but their abundance drops off exponentially with increasing depth (Parkes et al., 1994). Although

the number of cells per unit of deep subsurface sediments is relatively small, the vast extent of the sediments (estimated at 5×10^{25} cm^3) means that total prokaryote cell numbers in all environments are of the order of 10^{30} (Whitman et al., 1998). Extrapolating from direct counts of sedimentary microorganisms at a small number of ODP sites, it has been estimated that prokaryotes constitute anywhere from one-tenth to one-third of the earth's biomass (Parkes et al., 1994; Whitman et al., 1998). Despite this large apparent mass, the magnitude of its metabolic activity is still largely a mystery.

3.1.1.2 Continental Sedimentary Systems

Even though deep biosphere exploration could be said to have begun in a continental setting with the DOE's Savannah River Project and other research carried out in the 1980's and 90's through the DOE Subsurface Science Program, continued exploration of continental sedimentary rocks and sediments has lagged behind deep marine research (Amy and Haldeman, 1997). The single exception to this is with regard to petroleum reservoirs.

An increased understanding that subsurface microbes play a role in the production and alteration of oil and gas deposits has led to much debate about just how much subsurface life exists and how influential such life may be. Many other factors, such as the role of basin and petroleum system formation and fluid circulation among them, are being examined to see how those factors could influence the deep biosphere by controlling the formation and distribution of organic carbon; the delivery of nutrients, electron donors and acceptors; and the distribution and flow of groundwater (see reviews in Head et al. 2003; Sherwood Lollar and Ballentine, 2009).

The fact that hydrocarbon degradation occurs rapidly only under aerobic conditions hindered scientific acceptance of the idea that anaerobic processes might play a significant role in degradation of organic compounds at depth (Connan et al., 1996). Significant progress has been made in this area in recent years, however, because anaerobic microorganisms and metabolites have been identified in many petroleum-rich sedimentary basins worldwide (Head et al., 2003 and references therein). This is consistent with the finding that oxygen is rapidly consumed due to aerobic processes in the shallow subsurface and regional groundwaters rapidly become anoxic with depth.

Anaerobic processes are slow relative to aerobic degradation and are thought to be linked to iron reduction, methanogenesis, and microbial sulfate reduction if H_2S concentrations are not too high (Head et al., 2003 and references therein). Because nitrogen sources are common in petroleum reservoirs, it is unlikely to be a limiting factor on microbial activity, although phosphorous may still be a limiting nutrient (Manning and Hutcheon, 2004; Oldenburg et al., 2006). Ultimately, the nature and extent of groundwater circulating in these sedimentary systems plays a key role, controlling mineral dissolution and the release of N and P from minerals (Head et al., 2003).

There is some evidence that pore size limits microbial activity in sedimentary rocks. Frederickson et al. (1997) evaluated 24 shale and sandstone cores collected from a site in northwestern New Mexico at depths >165 m and found no evidence of metabolic activity in core samples with pore throats <0.2 μm using ^{14}C labelled acetate and glucose mineralization and ^{35}S labeled SO_4^{2-} reduction. However, sulfate reduction was detected in enrichment cultures in some small pore throat samples, suggesting that bacteria remain viable despite the restrictive pore throat diameters.

Some of the most extensive work on microbial activity in low permeability continental sedimentary systems has been carried out in the Opalinus Clay formation at the Mont Terri Rock Laboratory, Switzerland (more details are provided in Section 4.3). Clay-rich formations, such as the Opalinus, have been the focus of significant international attention as possible host formations for nuclear waste repositories due to their low permeability, diffusion-dominated transport regimes, geochemical stability and capacity for self-sealing (Stroes-Gascoyne et al., 2011; Wersin et al., 2011). Reviews of a 5-year investigation of microbial processes indicated that contamination during drilling promoted a thriving community of NO_3-, Fe- and SO_4- reducers and methanogens (Stroes-Gascoyne et al., 2011; Wersin et al., 2011). In contrast, undisturbed Opalinus Clay contained a microbial population, limited not only in biomass but also in viability - an inactive or dormant state attributed to restricted pore throat sizes and limited water availability (Stroes-Gascoyne et al., 2007; Poulain et al., 2008).

3.1.2. Crystalline Rocks

Investigations of the deep biosphere in crystalline rocks (largely igneous, but in some cases metamorphic) have focused on either research related to deep geologic repositories (for low, intermediate and high level radioactive waste) or research for more general science objectives conducted in excavations (exploration boreholes, deep mines). Occasionally, excavations were developed originally for scientific purposes, but more commonly for opportunistic projects (i.e., excavations originally associated with the mining and exploration sector). Microbiological investigations carried out in the first 1000 m depth are summarized in Section 3.1.2.1. To provide context on characterization approaches and potential geochemical processes that should be considered in crystalline rock environments, research in deeper settings (>1000 m are also discussed in Section 3.1.2.2. Finally, a summary of the controls on microbial diversity in crystalline settings is provided in Section 3.1.2.3.

3.1.2.1 The Deep Biosphere - The First 1000 m

Two of the largest international subsurface microbiology research projects were associated with the Stripa Mine in Sweden (since 1976) and the Underground Research Laboratory (URL) in Whiteshell, Manitoba, Canada (since about 1985). While both projects have been closed, they provided new information on subsurface life between 500-1000 m (see reviews: Pedersen, 1993; Stroes-Gascoyne and Sargent, 1998; Pedersen, 2000a) and informed and inspired a series of international research programs that are summarized in Section 4.

Likely the best-characterized deep subsurface environment, at least down to 440 m, is the Äspö Hard Rock Laboratory, one of the first dedicated underground laboratories for microbiological research related to high-level radioactive waste storage. Taken together, the research from the Fennoscandian Shield, Canadian Shield (Whiteshell), and research to 1400 m depth in fractured basalt in the Columbia River Basalt near the U.S. Hanford site (Stevens and McKinley, 1995) established that even in crystalline rock aquifers, once thought likely to be sterile, microorganisms live and grow both as sessile populations and as biofilms attached to mineral and fracture surfaces (Pedersen, 2000a). At these depths, the results of molecular microbiological studies indicate both several novel microbial species (e.g., *Desulfovibrio aespocensis*, a mesophilic sulfate-reducing bacterium; *Methanobacterium subterraneum;* and *Methylomonas scandanavica*, a methanotrophic psychrotropic bacterium) and substantial

biodiversity (Pedersen, 2000a and references therein). Overall, the microbial populations included both heterotrophic organotrophs, consuming either organic compounds originally derived from the photosphere (typically up to a few mg/L TOC in shallow (<500-1000 m) granitic bedrock (Pedersen, 1996a; Pederson 1996b; Hallbeck, 2010)) or compounds derived from microbial degradation of higher molecular weight organic compounds (including simple organic acids and gas byproducts such as CH_4 and CO_2 (Pedersen, 2000a)) and chemoorganotrophs. Typically, across the Fennoscandian Shield, the most abundant microbial populations (based on MPN) are found at depths of 300-500 m, ranging from 10^3 to 10^7 cells/mL (Hallbeck and Pedersen, 2008a). Cell densities in the range 10^3 to 10^5 cells/mL have been reported for similar depths in Canada (Stroes-Gascoyne and West, 1997; Bass et al., 2002). Acetogens are often the dominant component of the populations, followed by iron-reducing and sulfate-reducing bacteria and methanogens (using either dissolved inorganic carbon or acetate).

Evidence has been developed for deep H_2-driven ecosystems in these environments as well (Stevens and McKinley, 1995; Pedersen, 1996a; Pedersen, 2000a; Stevens and McKinley, 2000; Bass et al., 2002; Ward et al., 2004; Sherwood Lollar et al., 2006). Tests at many of these sites have revealed micromole per litre (μM) concentrations of hydrogen, methane and carbon dioxide. It is assumed that at the base of these ecosystems are three kinds of organisms: autotrophic acetogens producing acetate by reacting hydrogen with carbon dioxide; autotrophic methanogens producing methane from hydrogen and carbon dioxide; and acetoclastic methanogens, which convert the acetate product of the autotrophic acetogens into methane. The very slow metabolic rates expected under non-disturbed conditions are difficult to quantify on the short-term time scale of the site investigations (Pedersen, 2000a; Lin et al., 2006; Chivian et al., 2008).

3.1.2.2. The Ultra-Deep Biosphere - Beyond the First 1000 m

As discussed in Section 2, depth in and of itself is not a limiting or controlling factor on deep subsurface life. The nature and diversity of life in the subsurface is influenced by a multitude of factors, including temperature, pressure, water activity, availability of nutrients and electron acceptors, and geologic setting. A key parameter that is occasionally under-investigated is the hydrologic setting.

The presence of water is critical to habitability. The degree to which subsurface water can flow or mix (hydraulic conductivity) and hence provide a continuous supply of the requirements for life, or remove constituents that limit or poison life, is an equally important parameter. Unlike marine sediments or unconsolidated surface sediments, in which groundwater flow is dominated by advection and diffusion, crystalline rock is, to a large degree, dominated instead by fracture-controlled flow. Storage and flow of groundwater is constrained along irregular, anisotropic three-dimensional fracture networks. In such systems, the distribution of life will similarly be patchy and irregular, rather than a smooth continuum controlled by depth from surface.

Another important dimension to consider in the deep biosphere is time. Fracture networks are not static, but open and close on geologic time scales as a function of tectonic changes, changes in stress – even in tectonically quiescent settings (Sleep, 2007) – and geochemical changes. For instance, a new fracture can rapidly be resealed by precipitation of fracture minerals such as quartz and calcite. Generations of mineral layers in fractures can attest to the cyclicity of this opening and subsequent re-sealing of the system and therefore to the temporal variation in the supply lines for life and the potential for microbial communities to undergo

transport and mixing. It is important to note that the nature of this hydrogeologically isolated fracture network may also be what sustains habitability. Unlike sediments or hydrothermal vents, where fluid transport and mixing is rapid and the potential for advective and diffusive loss of the products of water-rock reaction is high, the hydrogeologically isolated fracture network products accumulate over geologic time scales accounting for H_2 levels as high as 10 mM in these fluids (Lin et al.,2006; Sherwood Lollar et al., 2007).

Not surprisingly, the microbiological findings in Section 3.1.2.1, in particular the evidence for the existence of ubiquitous and diverse microbial communities, with a significant component of heterotrophs and chemo-organotrophs, are a function of the degree to which regional groundwater flow, although slow, nonetheless maintains the connection to the surface carbon cycle. The geochemical interactions that drive these systems are not a function of hydrothermal and magmatic fluid inputs mixing with seawater, such as seen at the deep sea vents, but are a function of transport of regional meteoric and paleometeoric groundwaters, which, in the case of elevated plateaus, can penetrate from hundreds of metres (e.g., the Fennoscandian Shield) to kilometers in depth (e.g., Witwatersrand Basin, Columbia River basalts) (Onstott et al, 2010). For instance, in the Witwatersrand Basin, fracture waters to depths of 1-1.5 km have residence times derived from noble gases of <1 Myr to a few Myr, and hydrogen and oxygen isotope values that reflect a paleometeoric water origin (Lippmann et al., 2003, Ward et al., 2004). Fracture waters in mines to depths of 0.5 km in Sweden (Pedersen, 2000a) and to 1.4 km in South Africa (Ward et al., 2004; Lin et al., 2006) have heterogenous microbial populations. Geochemical and stable isotope data indicate significant populations of rapidly metabolizing SO_4-reducing and methanogenic bacteria, including Euryarchaeota clone sequences highly similar to cultivated methanogens such as *Methanosarcina, Methanolobus, Methanosaeta, Methanospirillum and Methanobacterium* (Ward et al., 2004). Carbon isotope values and geochemical signatures indicate that methane is produced by microbial reduction of CO_2 (Sherwood Lollar et al., 2006). Rates of microbial methanogenesis - derived from carbon geochemistry, stable isotopes and ^{4}He measurements - are more rapid, at around 100 nM yr^{-1} (Onstott et al., 2006).

Such rates and biodiversity are low compared to shallow subsurface ecosystems. While typical uncontaminated soils, for instance, may have thousands of unique prokaryotes per gram, an investigation of 39 samples from between 0.72-0.33 kmbls (kilometers below surface) in the Witwatersrand Basin demonstrated overall low biodiversity (on average 16 bacterial and archaeal OTU's (operational taxonomic units per gram)), but, nonetheless, a range of biodiversity with depth (Onstott et al., 2006). In addition to the archaeal taxa (Euryarchaeota and Chrenarchaeota) mentioned above, bacterial taxa were dominated by alpha-, beta- and gamma-Proteobacteria (67%) and Firmicutes (15%) (Onstott et al., 2006; Gihring et al., 2006).

As fracture waters become deeper and more saline, several significant changes occur. Long residence times on the order of 10-25 Myr have been found for the deepest high-salinity fracture waters in the South African crystalline rocks (Lippmann et al., 2003; Lin et al., 2006). Correlations of increasing salinity with highly altered hydrogen and oxygen isotope signatures for the waters, together with increasing concentrations of radiogenic noble gases, suggested that fracture waters were subject to extensive water-rock reactions over geological time, and have been hydrogeologically isolated (Sherwood Lollar et al., 2007). Although these fracture waters contained large concentrations of methane and higher hydrocarbon gases, carbon and hydrogen isotope data indicated that the gases were the result of local geochemical reactions and were not primarily the products of microbial activity (Sherwood Lollar et al., 2006; Sherwood Lollar et al., 2007).

Available electron donors for energy production were restricted to abiotically-produced H_2, hydrocarbons (Lin et al., 2005b; Sherwood Lollar et al., 2006) and small organic acids, with growth supported mainly by H_2 oxidation (Onstott et al., 2006). The availability of terminal electron acceptors was also restricted and O_2 was absent from fracture fluids. Significant nitrate was characteristic only in mine-impacted fluids, and metal reduction was inhibited by high pH (Onstott et al., 2006) and low Fe^{3+} concentrations in the rock strata (Kieft et al., 2005). Typically, the only favourable terminal electron accepting reactions predicted by thermodynamic modelling were anaerobic sulfate and CO_2 reduction. These thermodynamic predictions were supported by aqueous geochemistry and stable isotopic evidence, indicating that these reactions occurred in most of the fracture systems, albeit at much slower rates than in the overlying paleometeoric waters. For instance, rates of microbial methanogenesis, estimated at 100 nM/yr up to depths of 0.5 km, did not exceed 0.01 nM/yr in the ultradeep waters (Onstott et al., 2006). Fluids from borehole DR938H3 harboured only two types of taxa, a predicted sulfate reducer (*Desulfotomaculum* sp.) and a methanogen (*Methanobacteria* spp.), consistent with findings indicating that the oxidation of H_2 coupled with the reduction of sulfate or dissolved inorganic carbon were the only reactions predicted as thermodynamically favourable for growth (Moser et al., 2005). Environmental genomic studies of one such 15-25 Myr fracture-water system revealed a microbial ecosystem of extremely low biodiversity dominated by a SO_4-reducing chemoautotrophic thermophile that used hydrogen produced by radiolysis to survive at maintenance levels only 2.8 km below the surface (Lin et al., 2006; Chivian et al., 2008). These deep groundwaters are the most isolated from any photosynthetic sources of organic carbon and nutrients based on noble gas residence time estimates on the order of tens of Ma and a significant component of >2 Ga neon (Lippman-Pipke et al., 2011).

3.1.2.3 Controls on Diversity

It has been proposed that extreme environments result in low diversity, and a main factor controlling microbial biodiversity is the number of environmental niches or the diversity of growth limitations (Kassen and Rainey, 2004). Temperatures of fracture fluids in the studied South African mines were mainly within the range of 25 to 60^{o}C, indicating that these environments likely would select for mesophilic to moderately thermophilic microorganisms. Fracture water pH was generally mildly alkaline (pH 8 to 9), which would have limited the growth of obligately, neutraphilic organisms or acidophiles. With the exception of the boreholes at the shallowest levels, Eh was characteristic of anoxic environments, which would have limited the growth of any obligate aerobes. Because the *in situ* pressures ranged from ~20 to 250 bars, only the deepest regions would select for barophiles or barotolerant species. Only in the case of the deepest fracture waters was the salinity high enough (i.e. slightly greater than seawater) to impose a selective pressure for halotolerant species (Gihring et al., 2006).

Despite these environmental pressures, and the selection for different taxa that they drive, in fact none of these parameters are actually extreme from a geomicrobiological perspective. The greatest controlling factor limiting the biodiversity is thought to be the very limited availability of electron donors and acceptors in the waters of these fracture zones, which are hydrogeologically, physically and chemically isolated (Gihring et al., 2006; Lin et al., 2006; Onstott et al., 2006; Chivian et al., 2008).

3.1.3 Microbial Populations and Activity in the Near-Field to Far-Field Transition

The preceding two sections (3.1.1 and 3.1.2) discussed indigenous microbial populations and their *in situ* activities in potential host rock formations being considered in Canada for a DGR, i.e., sedimentary rock and crystalline rock. This section discusses microbial populations and activity in the transition zone from the near-field (the DGR) to the far-field (the intact host rock).

3.1.3.1 Redox Transitions

As discussed, as the transition occurs from surface to shallow subsurface to the deep subsurface, there is decreasing availability of oxidants and photosphere-derived organic compounds, slower rates of regional groundwater circulation and longer groundwater residence times, and typically less readily available nutrients, electron donors and acceptors. Aerobic communities give way to microbial populations dominated by anaerobes. These anaerobes can be either facultative (i.e. can respire aerobically or anaerobically) or obligate (i.e. can only respire anaerobically). The objective of describing these natural biogeochemical, hydrogeological and microbiological gradients in detail is that similar trends and gradients are likely to develop at the boundary between the near-field of the DGR and the far-field environments (which are the subject of this report). Excavation and development of a DGR inevitably results in aeration of some zones above and below the DGR and circulation of more fluids and gases that may have been in contact with the surface. Steep redox and oxygen gradients are likely to develop that, on a relatively short time and spatial scale, will promote aerobic microbiological processes including the activity of iron-, manganese-, sulphur- and methane-oxidizers (Bass et al., 2002). The near-field to far-field transition will likely be characterized by the reversal of this process and the gradual reestablishment of natural far-field geochemical and microbiological characteristics (Bass et al., 2002). Surface (exogenous) microorganisms introduced during excavation and DGR development (which can grow to significant density; e.g., 10^8-10^9 microorganisms/gram in the excavation damage zone (Grant et al., 2000)) would likely have limited potential to migrate to and survive in the anaerobic far-field, although that possibility cannot be ruled out (Bass et al., 2002).

In addition to colonization and migration of surface microorganisms, changes to the distribution and composition of native microbial communities may occur at the near-field to far-field transition. For instance, when anaerobic groundwater with high dissolved loads of ferrous iron, manganese (II) and reduced sulfur interfaces with more aerobic zones, chemolithoautotrophic bacterial communities may bloom in response to the steep redox gradients (Bass et al., 2002). Given the prevalence of H_2-utilizing SRB and methanogens in the deep subsurface, any H_2 produced, for instance, by corrosion of metal around the repository could be utilized as readily as native subsurface H_2 (Sherwood Lollar et al., 2007). If far-field microbiological communities have been limited by available organics (typically dissolved organic matter is present, at highest, at a few mg/L (Pedersen, 1996a) and may be vanishingly scarce (μM) in fracture waters at kilometres depth (Gihring et al., 2006; Lin et al., 2006)), high organic contents migrating from the near-field may promote temporary opportunistic growth of acetoclastic methanogens and heterotrophs (Bass et al., 2002) at the near-field to far-field transition until such substrates are depleted. Hallbeck (2010) provides a review of the principal organic materials that would be introduced during the construction of a DGR for used nuclear fuel and discusses their implications on the near-field microbial activity.

3.1.3.2 Microbiological Impacts on pH in the Far-field

As has been discussed, the organic and inorganic products of a wide variety of microbial processes that occur in the far-field could potentially alter pH. A repository's surrounding far-field geosphere would likely be anaerobic (low in redox potential), except near the ground surface, and, for a short period, close to the excavation walls. Therefore, aerobic reactions are not likely to be the most common generator of acids. Under anaerobic conditions, fermentation processes can produce short-chain fatty acids, including acetic acid, most commonly from the degradation of organic material. Acetate may also be produced from H_2 and DIC utilizing-reactions. These weak organic acids would have a limited effect on pH, and they are generally metabolized quickly.

Microorganisms metabolize nitrogen and sulphur to produce inorganic acids. In the case of nitrogen, the process is a part of the global nitrogen cycle, whereby a variety of chemical, physical and biological activities convert nitrogen through its various oxidation states. The acidification effect results from the dissolution of nitrogen dioxide and leads to the production of nitric and nitrous acids. In nitrification, reduced nitrogen compounds are oxidized by many different groups of microorganisms. Two groups of chemo-lithoautotrophs are prevalent. One group obtains its energy from the oxidation of ammonia to nitrite (*Nitrosomonas, Nitrocystis, Nitrospira*) and the other from the oxidation of nitrite to nitrate (*Nitrobacter, Nitrococcus and Nitrospira*) (Bass et al., 2002). Novel lineages of *Nitrospira* spp. have been detected in the deep mines of South Africa (Gihring et al., 2006).

Sulfate reduction, leading to the generation of hydrogen sulphide, is particularly important in most of the deep subsurface environments discussed in this report, and microbial reduction is the only process that can bring about the reduction of sulfate at temperatures relevant to a DGR environment.

Reduction of dissolved inorganic acids by methanogens consumes H^+ by the following reaction:

$$4H_2 + HCO_3^- + H^+ \rightarrow CH_4 + 3H_2O \qquad [1]$$

Studies suggest that the conversion of hydrogen and carbon dioxide to methane can be energetically more favourable than the route to acetate. Despite the documented occurrence of methanogens, the likely extent of these microorganisms is unknown, as is their potential to alter the pH of the far-field.

Fermentative bacteria generate organic acids and their activities render half the substrate oxidized and the other half reduced. The major products of this process are partially reduced organic materials, such as low-molecular fatty acids, simple organic acids, such as acetate and acetate ethanol, and reduced inorganic materials (i.e., carbon dioxide and hydrogen). These products are used in further processes that result in the formation of carbon dioxide, hyrdrogen sulphide and methane (Bass et al., 2002).

While the net effects of microbial activity on pH in the far-field are difficult to predict, changes in pH can have a significant impact on solubility, sorption and transport of radionuclides, and will be addressed in Sections 3.3 and 3.4. Overall, reduction in pH due to microbial activity is likely to be limited by the overall slow rates of microbial activity in the far-field (Bass et al., 2002) and by the buffer capacity of the host rock.

3.1.3.3. Degradation of Organic Compounds

As summarized by Pedersen (1996b), organic carbon in groundwater can be divided into dissolved, particulate and colloidal organic matter. Low-molecular-weight (LMW) organic acids often comprise the largest fraction (i.e., 50%) of organic matter in groundwater while high-molecular-weight (HMW) organic acids, such as humic substances, comprise about 15%. LMW organic acids are produced by microorganisms during fermentation or are formed during decomposition of higher molecular weight organics originating from ground surface.

As discussed, levels of dissolved organic material in naturally occurring far-field environments are low, ranging from up to several mg/L reported for granitic rocks in Fennscandia to ~500 m in depth (Anderson et al., 2006) to μm levels in km-deep saline fracture waters in the Witwatersrand Basin (Lin et al., 2006). Depending on the concentrations, rate of transport, and rate of degradation within the near-field, organic compounds introduced or generated in the near-field could be further degraded by anaerobic microbial populations in the far-field (Hallbeck, 2010 and references therein). The most important implication of this is its impact on gas production, and on the transport of radionuclides, which will be discussed in detail in Sections 3.2 and 3.3, respectively.

3.2 MICROBIAL ACTIVITY IN THE FAR-FIELD AND ITS EFFECTS ON GAS PRODUCTION

3.2.1 Sedimentary Rock Environments

For years, the thinking of petroleum geologists about subsurface petroleum degradation focused on aerobic processes (Horstad et al., 1990). However, even where deep regional groundwater contains a significant meteoric component, dissolved oxygen is likely to have been rapidly lost to oxidation of minerals and organic matter by near-surface aerobic microbes. Head et al. (2003) and Roling et al. (2003) have highlighted the increasing emphasis on the role of anaerobic degradation processes.

Petroleum reserves can occupy many permeable rock types, but are most commonly found in porous sediments such as sandstone and limestone in the presence of water and dissolved solutes. It has been observed that over geological timescales, biodegradation is significant in reservoirs with temperatures below 80 ^{o}C (Connan, 1984), with evidence of biodegraded oils at depths of up to 4 km. It has been suggested that some low temperature reservoirs, which appear to have suffered no biodegradation, have either been recently charged with fresh oil or were uplifted from deeper subsurface regions too hot for microbial communities to persist (Wilhelms et al., 2001).

Microbial investigations of petroleum-bearing sedimentary rocks have reported isolates ranging from mesophilic to hyperthermophilic (Magot et al., 2000), which are capable of exploiting the major terminal electron accepting processes (aerobic respiration; nitrate-, iron-, sulfate-reduction; methanogenesis and fermentation).

As noted previously in this report, culture-based techniques have a significant bias and are unlikely to represent the entire microflora. Nonetheless, there is abundant indirect evidence for microflora capable of anaerobic degradation of organics. As discussed by Head et al. (2003),

estimates of first-order alkane degradation rate constants of about 10^{-6} yr $^{-1}$ have been modelled assuming degradation occurs adjacent to oil-water contact. Beyond the hydrocarbons, both hydrogen and organic acids, especially acetates, are alternate sources of electron donors in petroleum reservoirs. As electron donors are clearly not limited, biodegradation is probably acceptor limited by the availability of electron acceptors and/or nutrients. There is, regrettably, very little data on the availability in oil reservoirs of electron acceptors, and of nutrients such as phosphorous and nitrogen. However, evidence for the existence of a deep biosphere in petroleum reservoirs continues to be identified (Roling et al., 2003 and references therein), starting with the common presence of microbes in reservoirs of biodegraded petroleum. As described by Head et al. (2003), where significant microbial biodegradation of organic compounds (including hydrocarbons in petroleum reservoirs) has occurred, microbially produced methane will mix with any pre-existing natural gas, which may contain both thermogenic methane and higher hydrocarbon gases, such as ethane, propane and butane. Typically isotopically light methane (lighter in ^{13}C than thermogenic methane) is indicative of this secondary biogenic methane (Schoell, 1988).

Methanogens need CO_2 or short-chain organic acids, alcohols and hydrogen to grow (Connan, 1984) and fermentative bacteria are required to supply them with electron donors. A large number of fermentative organisms have been detected in petroleum reservoirs (Magot et al., 2000). These strains can enhance their growth by using thiosulfate or elemental sulphur as electron acceptors (Ravot et al., 1995). Without fermentative organisms, methanogens would need alternative electron donors, such as hydrogen, from several possible sources: thermal degradation of organic matter, hydration of igenous rocks at depth, or, as suggested (Roling et al., 2003), from the oil during degradation.

3.2.2 Crystalline Rock Environments

In organic-depleted groundwaters in crystalline rocks, thermogenically derived gases are seldom encountered, and gases are typically derived from geological water-rock reactions such as radiogenic decay (e.g., helium, neon, argon); radiolysis of water; serpentization reactions that produce H_2; and, mineral-water reactions producing abiogenic hydrocarbons under what are predominantly highly reducing anaerobic conditions (Sherwood Lollar et al., 2002; Lippmann et al., 2003; Fruh-Green et al., 2003; Lin et al., 2005a; Lin et al., 2005b; Bradley and Summons, 2010). Microbial communities both consume the gaseous products of these reactions (e.g., H_2 utilization by SRB and methanogens (Pedersen, 1993; Pedersen, 2000a; Pedersen et al., 2000; Lin et al., 2006; Sherwood Lollar et al., 2006; Humphreys et al., 2010) and potential anaerobic oxidation of methane (Gihring et al., 2006)), and can also produce biochemical gases that contribute to the total gas load (e.g., microbial methanogenesis).

3.2.2.1 Gas Content

Groundwater studies in the Fennoscandian Shield reported high amounts of dissolved gases at some locations. The groundwater of Olkiluoto, Finland, contains more than 1,000 mL of dissolved gases per litre at 800 m (Pitkanen and Partamies, 2007). In general, groundwater in western Finland contains high volumes of dissolved gases (100-500 mL per litre) at a depth of 100-500 m (Sherwood Lollar et al., 1993b; Pedersen, 2001). Groundwater in the Swedish Äspö site contains fewer dissolved gases, typically 20-60 mL per litre. However, this data set extends only to depths of 500 m (Hallbeck and Pedersen, 2008c). Results from deeper settings (to 2.7

km in Canada and 3.3 km in South Africa) document dissolved gas contents up to 30,000 mL of gas per litre of water (Lippmann et al., 2003; Ward et al., 2004; Sherwood Lollar et al., 2007; Sherwood Lollar et al., 2008).

3.2.2.2 Gas Composition

In crystalline Precambrian Shield Rock, groundwater typically contains dissolved gases such as nitrogen, hydrogen, carbon dioxide and methane, as well as ethane, propane and the noble gases helium, neon, argon, krypton and radon. Small traces of oxygen were found only at shallow depths. Nitrogen is often the dominant gas in most samples examined at <500 to 1000 m (Sherwood Lollar et al., 1993a; Hallbeck and Pedersen, 2008c). Some of the nitrogen may have been dissolved from air in rain and surface waters that infiltrate as groundwater. Because nitrogen values exceed solubility, other sources of dissolved nitrogen must exist as well, and possible sources, such as degassing of basement rocks and fluid inclusion leaching, have been variously discussed (Sherwood Lollar et al., 1993a; Hallbeck and Pedersen, 2008c; Silver et al., 2011). Microbial nitrate reduction has also been suggested as a possible source of dissolved N_2 in these waters (Jain et al., 1997; Silver et al., 2001; Hallbeck and Pedersen, 2008a).

Carbon dioxide concentration generally decreases with depth. Active organisms degrade organic material and produce carbon dioxide, and other autotrophic organisms transform carbon dioxide to organic carbon. These organisms may influence the concentration of the gas and therefore affect the carbonate system, pH, and mineral precipitation and dissolution (Pedersen, 2000b).

The content of hydrogen and methane varies considerably between the studied sites, but typically H_2 levels increase in deeper, more saline fracture waters (Sherwood Lollar et al., 2007). Between 2 µM and 1600 µM of hydrogen in groundwater from Canadian Shield and Fennoscandian Shield rocks have been reported (Sherwood Lollar et al., 1993a; Sherwood Lollar et al., 1993b).

Studies of deep Swedish granite reported evidence of on-going methane-generating processes (Pedersen, 2000b), with up to 720 µM methane detected at 440 m depth at the Äspö HRL (Kotelnikova and Pedersen, 1997). Between 1 µM and 18,600 µM of methane have been reported in Canadian Shield and Fennoscandian Shield groundwaters (Sherwood Lollar et al., 1993a; Sherwood Lollar et al., 1993b). Similar levels were identified for the Witwatersrand Basin (Lippmann et al., 2003; Ward et al., 2004; Lin et al., 2006).

Studies of methane and higher hydrocarbon gases in both Canadian and South African rocks identified two major gas types. Paleometeoric waters were dominated by hydrocarbon gases, with compositional and isotopic characteristics consistent with production by methanogens coupling H_2 oxidation to CO_2 reduction and H_2-utlizing SRBs. In contrast, the deepest, most saline fracture waters contained gases that did not resemble the products of microbial methanogenesis, and were dominated by both high concentrations of H_2 gas and CH_4, as well as higher hydrocarbon gases, with isotopic signatures attributed to abiogenic processes of water-rock reactions in these high rock/water ratio and hydrologically-isolated fracture waters (Sherwood Lollar et al., 2008). The relative contributions of microbial versus geologic gases can be evaluated by an integrated use of various parameters, including gas composition and isotopic analysis (Sherwood Lollar et al., 2002; Sherwood Lollar et al., 2006; Sherwood Lollar et al., 2008).

3.2.2.3 Microbial Transformation of Gas

As described in this report, H_2-driven microbial ecosystems are typical of the deep subsurface. Hydrogen-consuming microbial communities have been found over a wide range of geological environments (Stevens, 1997), including Swedish granite aquifers (Pedersen, 2000b; Hallbeck and Pedersen, 2008a; Hallbeck and Pedersen, 2008c), Belgian Boom Clay (Ortiz et al., 2002), the Columbia River Basalt Group (Stevens and McKinley, 1995), the Lidy Hot Springs (Chapelle et al., 2002), and the South African Witwatersrand Basin (Ward et al., 2004).

In these settings, a wide variety of microorganisms can oxidize hydrogen using several terminal electron acceptors, including nitrate (Smith et al., 1994; Vasiliadou et al., 2006; Harris et al., 2007), ferric iron (Lovley, 1991; Harris et al., 2007), sulfate (Matias et al., 2005; Moser et al., 2005; Harris et al., 2007), and carbon dioxide (Kotelnikova and Pedersen, 1998b; Moser et al., 2005; Harris et al., 2007).

There are two potentially competing reactions when carbon dioxide is used as a terminal electron acceptor for hydrogen oxidation, acetogenesis and methanogenesis. In Swedish deep granitic host rocks, methanogenic hydrogen consumption rates ranged up to 5.6 μM h^{-1} (Pedersen, 2000b). Acetogenic hydrogen consumption, by contrast, was much higher, up to 0.13 M h^{-1} (Kotelnikova and Pedersen, 1998a). However, acetogens do not dominate all hydrogen-driven subsurface environments. The Lidy Hot Springs site (Chapelle et al., 2002) is dominated by a methanogenic hydrogen-consuming community. In the Boom Clay environment, hydrogen metabolism is coupled to sulfate and thiosulfate reducers, as well as to methanogens (Ortiz et al., 2002). The reaction schemes for these processes are outlined below, after Humphreys et al. (2010).

$5H_2 + 2NO^-_3 + 2H^+ \rightarrow N_2 + 6H_20$	Hydrogen oxidation via nitrate reduction	[2]
$H_2 + 2Fe(III) \rightarrow 2Fe(II) + 2H^+$	Hydrogen oxidation via iron reduction	[3]
$4H_2 + SO_4^{2-} + 2H^+ \rightarrow H_2S + 4H_20$	Hydrogen oxidation via sulfate reduction	[4]
$4H_2 + CO_2 \rightarrow CH_4 + 2H_2O$	Methanogenesis	[5]
$4H_2 + 2CO_2 \rightarrow CH_3COOH + 2H_2O$	Acetogenesis	[6]

Humphreys et al. (2010) concluded that, as hydrogen-oxidizing microbes exist in many different subsurface environments, it is likely that hydrogen oxidation will feature in the far-field of any geological disposal site.

Reviews of gas production in the near-field have pointed out that gas production can impact a DGR due to pressure increases, fracturing, and impacts on the integrity of buffer and backfill materials (Stroes-Gascoyne and West, 1996; Bass et al., 2002). In terms of transport, migration rates in groundwater may increase due to colloid attachment to gas bubbles or gas-phase release of radionuclides (whereby microbial production incorporates ^{14}C or ^{3}H, for instance, onto metabolic products such as CH_4, H_2S or H_2) (Stroes-Gascoyne and West, 1996; Bass et al., 2002). In the far-field, as discussed above, gas production can impact the geochemical and microbial environement, but impacts on radionuclide transport are likely to be indirect, as described in the following section.

3.3 EFFECTS OF FAR-FIELD MICROBIOLOGY ON TRANSFORMATION AND TRANSPORT OF RADIONUCLIDES

3.3.1 Interaction with Groundwater Chemistry

The characteristics of radionuclides in waste will be determined by near-field conditions, but may change if migration to the far-field introduces new conditions. Redox potential is a key condition, particularly for redox-sensitive radionuclides such as, uranium, plutonium, neptunium, selenium and technetium. Microbial processes can change the oxidation state of certain radionuclide ions, possibly affecting their transport by affecting their solubility and sorption. Higher oxidation states mean increased solubility and, therefore, mobility (Bass et al., 2002).

Bass et al. (2002) provide the following examples:

1. At high values of Eh (such as near the surface) U (VI) is thermodynamically stable.
2. At the low Eh values expected at depth, U(IV) would be stable with a mixture of U(IV) and U(VI) at intermediate levels.
3. Conversion between U(VI) and U(IV) is a slow process.
4. Similarly under low Eh conditions at depth in the far-field, other radionuclides would be expected to be stable in their more reduced forms (e.g., Pu (III), Np (III), TV (IV) and SE (II)).

Less is known about the reduction of radionuclides during biotransformation under the type of anaerobic conditions likely to prevail in the far-field, as discussed by Bass et al. (2002). Uranium can be transformed from U(VI) to U(IV) by iron-reducing (e.g., *Geobacter metallireducens, Shewanella putrafaciens*) and sulfate-reducing (e.g., *Desulfovibrio desulfuricans*) bacteria and precipitated (Lovley, 1991; Lovley, 1995). Several different bacteria, including sulfate reducers, can reduce selenate (SeO_4^{2-}) and selenite (SeO_3^{2-}) to elemental selenium (Bass et al., 2002). Sulfate reduction can take place when sulfate and formate, or CO_2, are present. In either type of reduction, the reduced compounds are less soluble than their parent compounds.

As discussed in this review, the effect of microorganisms on geochemical conditions has implications for radionuclide fate because both pH and Eh can have significant impacts on solubility, sorption and transport of radionuclides. Microbial impacts on groundwater Eh and pH were not independently modeled in the generic Canadian case-study safety assessment, but rather are modeled in conjunction with several organic and inorganic processes that determine the overall chemistry of groundwater in the far-field (Garisto et al., 2004). 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. As such, anaerobic microbial processes prevailing in the far-field would typically promote reduction of radionuclides to less soluble forms, resulting in slower radionuclide transport (Bass et al., 2002). However, biofilm growth that covers mineral surfaces can also potentially block access to sorption sites and decrease sorption (Bass et al., 2002) (See also Section 3.3.2). In addition, 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). As such, site-specific investigations are recommended for safety assessment (Bass et al., 2002). Consequently, in light of these uncertainties associated with contaminant transport effects elicited by microorganisms, generic Canadian crystalline case-study safety assessments have utilized a conservative approach,

such that sorption and retardation of radionuclides by contaminants is assumed negligible (Garisto et al., 2004).

3.3.2 Interaction with Biofilms and Impact on Pore Spaces

It is now understood that deep subsurface microbiological communities consist of both planktonic (free floating in water) and attached (sessile) communities, termed biofilms. Biofilms are matrix-enclosed bacterial populations, which adhere to each other and/or to surfaces or interfaces. Included among them are microbial aggregates and floccules, extracellular polysaccharides (EPS), and also communities which adhere to the pore spaces of porous media. EPS is exuded by those cells which actively grow, and enhances their adhesion while promoting rapid growth.

Onstott et al. (2010 and references therein) discuss the way in which variations in physical substrates provide different chemical conditions that select for specific types of microorganisms. As well, interfaces between solids, liquids and gases also engender steep chemical gradients, which create more diverse ecological niches than can be found in bulk water. As a result, there is generally higher diversity and activity in surface-attached microbial communities than in those suspended in water. It is important to examine these sessile communities because it is likely that they are directly involved in the alteration and precipitation of diagenetic mineral phases and in changing the chemical and physical properties of the rock.

Biofilms have been shown to promote propagation and dispersion of bacteria by shedding cells to the surrounding groundwater. When nutrients are scarce, daughter cells can shrink and, hence, penetrate deeper into porous media. How these biofilms are structured, and how they develop, may alter the pattern of radionuclide mobility. For example, a reduced bacteria (perhaps due to starvation) may enter and block small pores (Bass et al., 2002).

Biofilm growth and pore blocking can be important in sedimentary rock where fluids may migrate through the bulk rock (porous media) matrix. In the matrix of fractured crystalline rock, the effects may be even more significant due to low permeability and pore throats that are often physically too small to allow passage of bacterial cells. As such, biofilm growth is preferentially along mineral and fracture surfaces. In both settings, the impacts can be physical (for instance, altering porosity) and/or chemical (such as changing pH and redox conditions). Physical and/or chemical changes will cause intracellular or extracellular mineral formation or degradation (Bass et al., 2002). Biofilms can also immobilize radionuclides via cell surface metal adsorption (Haas et al., 2001). All of these processes have the potential to affect the transport of radionuclides in the far-field.

Experiments carried out in batch reactors and columns, using crushed Äspö granite, demonstrated rapid (over days) reduction in permeability and groundwater flow due to pore clogging by biofilms (Hama et al., 2001; Tuck et al., 2006). It was suggested that the bacteria active in filamentous biofilm could survive in low nutrient conditions, consistent with previous hypotheses that bacteria can concentrate relevant chemical species for mineral formation, or may accelerate clay formation. This implies that the introduction of new nutrients by, for instance, links to surface water or migration from the near-field, can actually change the localised hydrological regime of a granitic environment. Minerals (both biogenic precipitates and trapped in biofilms) are more stable than biofilm, and can persist in pores long after biofilm has decayed or been removed.

Microbial communities are expected to be more diverse where biofilms form, for example in open fractures (Stroes-Gascoyne et al., 2000 and references therein). The availability of carbon sources, nutrients, terminal electron donors and terminal electron acceptors is flow dependent and will control biofilm growth and community composition (Stroes-Gascoyne et al., 2000). In an *in situ* biofilm reactor study in the Stripa Mine in Sweden, Pedersen and Ekendahl (1992) found that attached bacteria counts were several orders of magnitude greater than unattached bacteria in flowing groundwater. Similarly, Stroes-Gascoyne et al. (1999) found that bacterial concentrations in biofilms were approximately 100 times greater than in groundwater. 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 also can alter pH, redox, groundwater chemistry, and rock surfaces (Coombs et al., 2010). Ongoing research and model development evaluating the effects of biofilm development under a range of different geologic settings (Coombs et al., 2008) will lead to improved understanding of biofilm formation and its implications for groundwater transport modelling (Coombs et al., 2010). While biofilms will still be important in the far-field, the slow rates of microbial growth is expected to support less biofilm development than in the near-field (Lucht et al.,1997; Bass et al., 2002). Ongoing research is addressing developments of qualitative models to evaluate the effects of biofilm development under a range of different geologic settings (Coombs et al., 2008).

3.3.3 Interaction of Radionuclides with Biofilms and Sorption

In addition to the physical effects of pore blocking and permeability reduction, microbes can interact with radionuclides via sorption and desorption. The passive sorption of metal species to the surface of bacteria has been widely observed for a variety of metals including iron, nickel, chromium, uranium, plutonium, neptunium and others. A microorganism's net negative surface charge causes reactions at its surface, which will compete with reactions with local rocks. The binding characteristics of metals are further altered by the microbial production of extracellular polysaccharides, which is crucial to biofilm formation. Little is known about the nature of these binding events, and most of what is known is site-specific (Bass et al., 2002).

In addition to sorption to microbial cells and biofilms themselves, microbial activity may alter the mineralogy of the host rock and have resulting changes in sorption characteristics. The investigations by Ferris et al. (1999; 2000), of the sorption of dissolved metals onto bacteriogenic iron oxides in deep groundwater in granitic environments, suggested that those oxides are an important sink for dissolved metals. However, the apparent influence of bacterial organic matter in the solids led to an increase in iron oxide and a concomitant decrease in metal distribution coefficients (K_d). This has implications for the transport of dissolved metals in groundwater where iron oxides and bacteria comingle, typically in transition zones between anaerobic and aerobic environments (Humphreys et al., 2010). In summary, by providing sorption sites for radionuclides, microbial populations and biofilms may lead to greater sorption and reduced transport of radionuclides. Conversely, if abundant biofilm growth were to cover most of the mineral surfaces and increase pore clogging (as discussed in Section 3.3.2), the overall access to sorption sites may be reduced. In fractured rocks, extensive biofilm development may restrict exposure of the rock matrix itself, and by reducing the number of sorption sites, lead to more rapid transport of radionuclides (Vandergraaf et al., 1997; Stroes-Gascoyne et al., 1999; Bass et al., 2002; Anderson et al., 2006, 2007). There are significant uncertainties about the net impact of microbial activity and biofilms, and therefore site-specific investigations are recommended (Bass et al., 2002).

3.3.4 Colloids

Particles between 10^{-3} to 10^{-6} mm are termed colloids and include both mineral particles (in particular clays) and biological particles, such as microbial cells, viruses or organic matter (Hallbeck and Pedersen, 2008c). They are suspended in the migrating groundwater and are sufficiently small so that interfacial forces are significant controls on their transport and fate. Radionuclides can adhere to colloidal particles, or can be incorporated within and transported with them. It is important, therefore, to determine the occurrence and formation of these colloids in groundwater and the period of time during which they remain stable. Such an investigation should be part of any site investigation. Microorganisms vary greatly in size. Many found in groundwater are $\leq 10^{-3}$ mm and may be considered colloids. In addition, the surface of many microorganisms may host negatively charged groups, such as hydroxyl and carboxyl, which allow positively charged ions and compounds in groundwater to bind. Many different types of microbial viruses were present in recently studied groundwater in the Äspö Hard Rock Laboratory, suggesting a possible biological origin for a fraction of the colloids (Kyle et al., 2008). There was a strong negative correlation between salinity and the number of viruses, similar to that between colloids and salinity.

Mobile microbes may uptake and then transport radionuclides. If the movement of cells is sufficiently slow compared to groundwater movement, then the net effect is a retardation of the radionuclide. However, due to the size exclusion effect, it is possible that microbes (and any sorbed radionuclides) may move more rapidly than average groundwater flow (De Marsily, 1986). There would be no net effect for a non-sorbing radionuclide, while sorbing radionuclides would react similarly to organic complexants. Such effects are site- and compound-specific and can be evaluated with groundwater transport and flow models (Bass et al., 2002).

The flux of microbially borne radionuclides is in proportion to both the concentration of microbes and the extent of their radionuclide uptake. It has been shown that with low concentrations of mobile microbes, the microbially mediated flux of radionuclides is likely negligible compared with a typical transport flow (Bass et al., 2002). Since large planktonic microbial populations and fracture controlled flow are unlikely in the far-field (both discussed in previous sections), the effects of such processes in the far-field is likely to be small (Bass et al., 2002).

3.3.5 Chelating and Complexing Agents

Microbial activity can potentially encourage faster transport through groundwater by producing chelating agents, which bind to some migrating radionuclides and reduce their sorption. It is not known how significant this process is. It is, however, unlikely to affect ^{36}Cl and ^{129}I because, under relevant conditions, chlorine and iodine do not customarily form complexes with typical chelating agents (Cotton and Wilkinson, 1980).

Chelating agents, known as siderophores, are used by many species to acquire iron in places, such as groundwater, where metals are in short supply. The evidence for the acquisition of radioactive metal species by such siderophore-employing microorganisms is sparse. Some studies indicate the process may occur in the subsurface (Bass et al., 2002).

Microorganisms also produce many less-specific chemicals, which may bind metal species and affect their transport, such as low-molecular-weight acids and alcohols, which should be evaluated for their ability to complex metals. The significance of complexing agents depends on environmental conditions, the radionuclides of interest, and groundwater flow rates. Their

importance is still unclear but their effect is likely to be transient as the agents themselves will be consumed by other bacteria (Bass et al., 2002).

3.4 SUMMARY

In summary, microbial activity (usually as biofilms) may influence the transformation and transport of radionuclides in the far-field, with flow characteristics possibly changed in both fractured crystalline rock and sedimentary rock. Additionally, if a host rock offers appropriate redox and mineralogical conditions, it appears biofilms influence radionuclide sorption onto mineral surfaces. Microbes may also influence the radionuclide transport via cell surface adsorption, mineral precipitation, and the formation of bacteriogenic iron oxides.

This area continues to be an active field of research, and future advances should be followed closely to assess their implications for far-field microbiology (Humphreys et al., 2010). Bass et al. (2002) conclude that all of the following areas require additional investigation for both crystalline and sedimentary systems to determine the role of microorganisms in:

(a) modification of redox states, leading to altered solubility and sorption;

(b) pore blocking and modification of sorption because of the physical presence of microbial cells and biofilms;

(c) modification to mobility of radioactive materials because of the microbial production of chelating agents or the presence of motile microorganisms that may take up radioelements;

(d) microbial modification to pH, potentially leading to altered solubility and sorption of materials;

(e) microbial degradation to organic complexants, which are either native to the far-field or are derived from the near-field;

(f) gas production, which may affect gas or groundwater migration in the geosphere; and,

(g) microbial halogenation, where microorganisms may incorporate radioactive chlorine and iodine into organic materials and retard their travel times in the geosphere.

4. COUNTRY PROFILES

4.1 SWEDEN

Recent research instigated by the Swedish Nuclear Fuel and Waste Management Company (SKB) has focused largely on characterization of two potential host repository sites (Forsmark in the municipality of Osthammar, and Laxemar in the municipality of Oskarshamn) leading up to the selection of Forsmark as the host municipality for a deep geological repository for used nuclear fuel.

Prior to these siting-related investigations, research focused on generic studies of microbiology in Swedish granitic rocks and experimental and characterization studies at the Äspö Hard Rock Laboratory located outside Oskarshamn. Early research conducted by Pedersen (1987), assessed microbial numbers in deep groundwaters in Swedish granitic rock environments in preparation for future studies assessing microbial effects on a high level nuclear waste repository. Pedersen (1989) also studied the effects of microbes on radionuclide migration in deep granitic Swedish groundwaters.

Kotelnikova and Pedersen (1998b) studied the microbial consumption of O_2 in open ponds in the Äspö tunnel, concluding that O_2 reduction by microbes occurs in groundwater and that CO_2 is produced concurrently with O_2 consumption, supporting conclusions that CO_2 is of biogenic nature. The information obtained was used to predict microbial activities after O_2 is introduced to a repository.

Pedersen (2000b) reported further on microbial processes in radioactive waste disposal and summarized the 1987-1999 work from SBK's microbiology research program. One of the report's goals was to determine the effects of microbes on high level waste (HLW) repositories. The report summarized geosphere research conducted in support of the Swedish far-field microbiology research program, but also reviewed near-field microbiological implications, including microbially influenced corrosion, and other effects such as the production of sulphide, carbon dioxide, organic carbon and methane, and the reduction of oxygen.

Hallbeck and Pedersen (2008b; 2008c) provided a series of reviews of microbiology, colloids, groundwaters, and gases in granite terrains, with a focus on microbial modelling. The major goals of these studies were to understand undisturbed conditions at two sites, the Laxemar-Simpevarp site, and the Forsmark site, particularly the causes of copper corrosion and bentonite loss, and to determine how hydrogeochemical conditions would change during the construction and operation of a repository. Models took into account pH, Eh, sulphur species, iron (II), manganese (II), carbonate, phosphate, nitrogen species, total dissolved solids, colloids, fulvic and humic acids, DOC, dissolved gases and microorganisms.

At Laxemar (Hallbeck and Pedersen, 2008c), the highest number of microorganisms, based on most probable numbers (MPNs) between about 100 and 900 metres below sea level, was found between 300-600 m. Acetogens were the most dominant, with significant populations of iron-reducing and sulphate-reducing organisms, with the latter more prominent at greater depths. Iron-reducing bacteria (and some Mn-reducing bacteria) were thought to derive their energy, electrons and carbon through degradation of organic matter originating from surface. Colloids were found to be present on the order of 10^6 mL^{-1}, similar to the total cell count numbers, and hence the report suggested that many of the colloids were probably of biological origin. As discussed in the earlier section on biofilms (Section 3.3.2), work by Anderson et al. (2006; 2007) suggested that development of biofilms may suppress the capacity for subsurface radionuclide absorption since the biofilm coats the rock and can reduce radionuclide diffusion to the rock absorption sites. The degree of suppression depends on the chemical nature of the specific radionuclides, and is least important for trivalent species (Anderson et al., 2007). The main components of dissolved gases were N_2, followed by CO_2 in the shallower waters, trending towards increasing dissolved concentrations of helium, methane, argon and higher hydrocarbons (up to propane) in the deeper waters. While the studies concluded more data (including isotopic studies) were required to determine the origins of the gases in the groundwater at this site, it suggested that samples at 300-400 m contained biologically produced methane, while samples from other depths contained abiogenic methane.

Similar to the Laxemar site, acetogens dominate at the Forsmark site, followed by sulphate reducers or iron/manganese reducers (Hallbeck and Pedersen, 2000b). Colloids at Forsmark ranged from 10^5 to 10^8 mL^{-1}, many of which are likely of biological origin. Gas concentrations generally increased with depth. Biologically produced methane was only likely in one sample at 445 m depth. Other gases included helium, hydrogen, nitrogen and argon. Additional studies were recommended to clarify the origin of methane, helium, argon and nitrogen using isotopic studies.

Hallbeck and Pedersen (2008a) summarize the work conducted by the Swedish program regarding the development and testing of several methods for estimating the total numbers of microorganism groups, their amounts and diversity in groundwater and the rates of microbial processes. The enumeration and cultivation methods were tested and evaluated on groundwater from boreholes at 450 m depth in the Äspö Hard Rock Laboratory and at Forsmark and Laxemar. Importantly, excellent reproducibility of the methods between duplicate samples and over time was shown. At all sites, nitrate-, iron-, manganese and sulphate-reducing bacteria and acetogens and methanogens were found in numbers up to approximately 87,000 cells/L in groundwater.

4.2 FINLAND

Finland's used nuclear fuel will be deposited into a deep geological repository at Olkiluoto, where a research tunnel called Onkalo is currently under construction. Several microbiology studies have been conducted by Posiva Oy as part of site assessment activities to determine the suitability of the Olkiluoto site for nuclear waste disposal (Pedersen, 2007; Pitkanen and Partamies, 2007; Itavaara et al., 2008; Pedersen, 2008).

Pedersen (2007) studied the microbiology of shallow and moderately deep (4 to 14.9 m) groundwaters in the bedrock of Olkiluoto to determine the geochemistry, biomass and the diversity of microbial life. One of the goals was to determine whether there is a transition to deep subsurface-type microbial life within the shallower depths. Pedersen (2007) found that at shallower depths, aerobic bacteria were dominant and microorganism populations varied seasonally. During the summer and fall, especially, these aerobic bacteria consumed all available oxygen and act as a barrier to its transport to the deeper subsurface. The study suggests the transition to anaerobic groundwaters at Olkiluoto typically occurs between 15-25 m depth.

Pedersen (2008) analyzed shallow and deep groundwater and described sixty datasets collected over a three-year period (2004-2006) to present a picture of site conditions prior to the construction of Onkalo. Sixty analytical data sets on the microbiology of Olkiluoto groundwater were collected from two regions – one from a set of boreholes between 3.5 to 24.5 m, and another from boreholes extending from 35 to 450 m depth. Oxygen reduction in shallow groundwater was confirmed. Microbiological and geochemical evidence exists for anaerobic microbial oxidation of methane (ANME) to a depth of 300 m in Olkiluoto; however, definitive proof of these microbes is required. Below 300 m depth, ANME is suggested to be limited by a lack of sulphate. It was also suggested that rates of sulphide production by ANME in the shallower groundwaters might be limited by the rate of methane transport from deeper layers.

Pitkanen and Partamies (2007) looked at the origin and implications of dissolved gases in the groundwater at Olkiluoto from 40 m to 956.5 m depth, in order to understand the

hydrogeochemical properties and evolution of the groundwater. The dissolved gases in groundwater were investigated because these gases make up a large portion of the dissolved species at the Olkiluoto site and because they also control hydrogeochemical evolution and provide information on both palaeohydrogeology and recent hydrology. It was determined that nitrogen and methane were the major gases in the groundwater, while gas compositions depended on redox conditions in the strata. Methane was found in deeper, saline water below 300 m, derived either from abiogenic hydrocarbons (found in the deepest parts), or from bacterial methane, which increases at depth. Carbon dioxide reduction was proposed as the pathway for the production of biogenic methane. As noted, only trace amounts of methane were found in sulphate-rich waters above 300 m due to ANME.

Pitkanen and Partamies (2007) indicated that, below 800 m depth, groundwaters were saturated with methane and that hydrogen levels increased. There is no evidence for infiltration of O_2 to these depths in recent geological history. Hydrocarbon concentrations were high at this site, which is common for rocks with low oxygen fugacity during crystallization. Stable isotope analyses of the water and hydrogeochemical models of the Cl (salinity) and methane suggest that saturation of the waters with methane takes place slowly, requiring tens of thousands to hundreds of thousands of years. The report called for more detailed studies of helium isotopes at this site and more data for hydrogen, methane, higher hydrocarbons, DIC, fracture calcites, and microbes from greater depths, especially for the most saline groundwaters. In Itavaara et al. (2008), sulfate-reducing bacteria (SRB) were studied using quantitative PCR (qPCR) to determine the potential for corrosion by sulfide produced by SRB. Sulfate-reducing bacteria were found in all samples. However, establishing a clear relationship between their number and geochemistry, and their relationship to methanogens, was highlighted as an area requiring more study (Pitkanen and Partamies, 2007; Itavaara et al., 2008).

4.3 SWITZERLAND

4.3.1 Mont Terri Underground Research Laboratory

The Mont Terri Project is an international underground research laboratory (URL) located in the Jura Mountains in north-west Switzerland. The URL is located in the Opalinus Formation, a Mesozoic clay deposit that is more than 100 m thick and is a candidate host rock for a used fuel repository in Switzerland. The goal of the Mont Terri Project is to characterize the hydrogeological, geochemical and geotechnical characteristics of the Opalinus Clay argillaceous formation.

The microbiology of the Opalinus Clay Formation has been investigated to assess whether microbial metabolism and its products could affect the physical and geochemical conditions of a DGR for used nuclear fuel (Mauclaire et al., 2007; Stroes-Gascoyne et al., 2007; 2011). Early investigations suggested that up to 10^8 bacteria per gram were present in the Opalinus Clay (Mauclaire et al., 2002). These numbers were later revised to 10^5 to 10^8 cells/g (Stroes-Gascoyne, p. comm., 2011).

A multi-laboratory research program was conducted to further evaluate microbial occurrence and activity in the clay formation (Stroes-Gascoyne et al., 2011). Inside the URL, a 15 m long borehole was drilled with compressed N_2 using aseptic techniques and using yellow-green fluorescent microspheres as a particle tracer to assess and correct for any contamination (Stroes-Gascoyne et al., 2007). Core samples were distributed to five laboratories and analyzed using microscopy, culture-based, and molecular-based techniques. PLFA analyses

yielded 64 ng PLFA per gram of clay, including biomarkers for anaerobic sulphate reducing bacteria, and several culturing attempts indicated viable bacteria (Mauclaire et al., 2007; Stroes-Gascoyne et al., 2007). However, there was a consistent failure to extract PCR-amplifiable DNA from the core samples, possibly due to the strong binding of DNA to the core matrix (Stroes-Gascoyne et al., 2007). In addition, cells were not evident using microscopic techniques. Overall the microscopic, cell culture and molecular results suggested only a small viable *in situ* microbial community. Given the small pore sizes and low water content in the formation, very limited growth and activity of microbial communities in the intact host rock is expected (Stroes-Gascoyne et al., 2007). Results of the Porewater Chemistry experiments (summarized in Stroes-Gascoyne et al., 2011) indicated drilling-related disturbances, particularly contamination with organic matter, fostered a temporary bloom of anaerobic microbes, including NO_3-, Fe- and SO_4- reducers and methanogens. Significantly, Wersin et al. (2011) concluded that, due to the large buffering capacity and diffusion-dominated nature of the clays, the effects of drilling and excavation disturbances will be temporary and spatially limited.

Current experiments of microbiological interest underway at the Mont Terri Underground Laboratory include the Microbial Activity (MA) and Hydrogen Transfer (HT) experiments (Swiss Geological Survey, 2009). The MA experiment will evaluate the effects of geomechanical and geochemical perturbations associated with the excavated damage zone of a borehole or gallery, which can provide favourable conditions for the growth of microbes, either indigenous or introduced when more space and water become available. The HT experiment aims to evaluate diffusion of hydrogen in the Opalinus Clay, and will measure hydrogen consumption processes in the experimental borehole and identify the role of microbial activity on these processes. The experiment involves the release of H_2 in a gas circulation experiment in an experimental borehole.

4.3.2 NAGRA

Microbiology research conducted by the Swiss National Cooperative for the Disposal of Radioactive Waste (NAGRA) has focused on: 1) describing microbial processes in HLW repositories that may affect repository conditions; and 2) developing quantitative models for microbial growth.

Papers from NAGRA have investigated microbial processes, such as biodegradation of repository materials, groundwater chemistry alteration, and organic complexant concentration. In Watkins and Grogan (1995), the effects of microbes on biodegradation, speciation, solubilisation, precipitation, complexation, sorption, bioaccumulation, and adhesion are explored for two cases: the HLW (high level waste) Swiss repository system and the low and intermediate level waste (L/ILW) repository system. A computer program called EMMA (Estimation of Maximum Microbial Activity) was developed and applied to both HLW and L/ILW repositories. Chemical electron transfer reactions are used to calculate energy requirements of microbial populations. Microbial biomass estimations are calculated by combining biomass calculations and nutrient inventories for carbon, phosphorous, nitrogen and sulfur.

Both McKinley et al. (1985) and Watkins and Grogan (1995) conclude that microorganisms will be present in the repository environment, and that they will drive biodegradation of organics, change groundwater chemistry, and sorb radionuclides (McKinley et al., 1985). Watkins and Grogan (1995) demonstrated the validity of microbial growth modelling based on both energy and mass balances (McKinley and Grogan, 1991; Capon and Grogan, 1993). Both reports determined that available energy was the major constraint to the accumulation of microbes,

rather than availability of nutrients. The authors were also in agreement that complexation of radionuclides with organic complexing agents would have the largest influence on repository safety. Watkins and Grogan (1995) suggested that microbial activity is unlikely to have a negative impact on repository performance for HLW.

These NAGRA reports recommended various actions. A literature review regarding the development of biofilms and the properties of biofilms, which will allow for determination of their contribution to radionuclide mobility and the degree to which the development of biofilms may protect and promote the development of microbial communities was recommended (Watkins and Grogan, 1995). The reports have promoted an enhanced sharing of information and models, and the concept that further studies on microbial activities should be conducted in simulated repository conditions, as opposed to laboratories. They also recommended more site-specific studies to determine the availability of nutrients, migration properties, biodeterioration, bioenergetics and tolerances (McKinley et al., 1985). Both studies emphasized that experimental studies should be performed under realistic simulated repository conditions because laboratory studies may overestimate metabolic potential in normally nutrient-poor environments.

4.4 UNITED KINGDOM

Microbial investigations relevant to nuclear waste disposal in the United Kingdom were carried out originally by Nirex (originally the Nuclear Industry Radioactive Waste Executive) and are now carried out by the NDA (Nuclear Decommissioning Authority). Reviews of early studies on the significance of microbial activity for intermediate and low level waste disposal include West et al. (1984), West and McKinley (1984), Christofi (1991), and Rosevear (1991).

In addition to the Nirex work on the role of microorganisms in the near-field, Bass et al. (2002) provided a review of processes involving microorganisms within the far-field geosphere around a deep repository. This report emphasized that two potential sources of microorganisms exist in the geosphere, both the indigenous microorganisms, and organisms introduced into the near-field that might subsequently migrate to the geosphere over time. The report summarizes the various ways in which microbial processes can affect groundwater flow and the transport of radionuclides. In the report, it is acknowledged that more work needs to be done to determine: i) microbially induced changes in redox states and alteration of solubility and sorption of metals; ii) how microbes can block pores and change the sorption and retardation of radionuclides, iii) how microbes change mobility of radioactive materials due to chelation; iv) microbial modification of pH and thereby of solubility or sorption; v) microbial degradation of organics; and vi) microbial gas production and halogenation. While acknowledging the need for additional study, the report concluded that the impact of all of these processes on the far-field likely would be small.

A Nirex Technical Note (2006) describes the processes that determine migration or non-migration of radionuclides in the geosphere, focusing on intermediate level waste in low permeability fractured crystalline rock in Sellafield. The Nirex (2006) report outlines areas of future research and suggests that the effects and implications of microbiological activity on a DGR for HLW are well known, but that site-specific research is required before a repository is operated.

In 2010, NDA published the review, "Microbial Effects on Repository Performance", which summarizes the UK research program and provided a critical review of the international literature on microbial effects in and around a DGR for HLW (Humphreys et al., 2010). A large focus of the report is on near-field microbiology. Regarding the far-field, the report identifies several important processes: i) the potential for microbial transformation of organic complexing agents to reduce radionuclide migration in the geosphere; ii) the potential for generation of gases, such as hydrogen and methane, and the subsequent further transformation by microbial populations, given that hydrogen-oxidizing microbes have been found to be common in the far-field; and, iii) the potential for microbial populations and biofilm formation to impact solute transport processes and influence radionuclide migration in the far-field, although the effects will be site-specific (i.e., subject to local hydrogeochemical and hydrogeological settings). Humphreys et al. (2010) emphasize the role of indigenous bacteria in maintaining reducing conditions in deep groundwaters or returning groundwaters to reducing conditions after aeration in the near-field as a result of the establishment of a DGR. The authors note that much more work has been done on granitic rock compared to other geologic settings.

4.5 JAPAN

The Mizunami Underground Research Laboratory (MIU), located near Tono, Japan, is part of a major research project being carried out by the Tono Geoscience Center (TGC) and the Japan Atomic Energy Agency (JAEA). The MIU is comprised of a main shaft, a ventilation shaft and sub-stages, and the site is characterized by two major types of groundwater chemistry: Na-Ca-HCO_3 type (in shallow sedimentary formations) and Na-(Ca)-Cl type (in deep sedimentary formations and in the granite basement rock). The major goals of this project are to determine techniques that are appropriate for studying geological environments, to collect data from these environments, and to develop engineering which can be applied to the deep subsurface.

Working at MIU, Fukuda et al. (2010) analyzed the geomicrobiological properties of groundwaters found from 1148-1169 m depth in granitic rocks at the laboratory. Fukuda et al. (2010) concluded that microbial populations in the deep groundwater at MIU are phylogenetically and physiologically different from those in other deep groundwater environments discussed in this report. The deep groundwaters at this site contain only moderate concentrations of organic matter, methane, and hydrogen, and were depleted in all major electron acceptors other than CO_2. Both sulphate reduction and methanogenesis were thought to be negligible at the site based on culture-based experiments and the fact that no PCR amplification, from primer sets specific for these processes, was successful. Total cell counts (based on acridine orange staining) were on the order of 10^4 cells/mL and the most common phylotypes identified based on 16S rRNA sequence analysis were related to *Thauera spp.* When cultured, *Thauera spp.* can utilize dissolved organic compounds such as aromatic and aliphatic hydrocarbons, possibly with nitrate or Fe(III) as electron acceptors.

4.6 UNITED STATES

In October 2000, Atomic Energy of Canada Limited (AECL) published a comprehensive review of international literature on microbial responses to abiotic environmental factors in the context of the proposed United States HLW repository at Nevada's Yucca Mountain (Meike and Stroes-Gascoyne, 2000). The AECL report was largely a literature review outlining the physical,

chemical and biological perturbations of the natural environment that could affect microbial activity and repository performance at the Yucca Mountain site, which is characterized by a thick unsaturated zone in volcanic tuff deposits, arid conditions, and a significant thermal load. The report examined microbial states, phases and growth requirements, cell death, survival strategies, and adaptability to environmental changes. Taking into account factors that affect microbial growth (for instance, pH, salinity, high temperature), the goal was to estimate the expected amount of microbial activity at Yucca Mountain, an important element affecting design decisions.

Kieft et al. (1997) completed a study of the far-field microbiology around the Yucca Mountain site to quantify the microorganisms, identify factors that might limit microbial growth and activity, and determine the potential for mineralization of organic compounds. Direct microscopic cell counts, as well as phospholipid fatty acid concentrations, confirmed that total biomass was near detection limit at all 9 sites sampled around Yucca Mountain (10^4 to 10^5 cells/g dry weight). Based on laboratory microcosm amendments, water appeared to be the primary limiting factor on microbial growth and it was determined that microbial activity following the addition of water resulted in significant increases in cell counts and in uptake of radiocarbon labelled CO_2 (Kieft et al., 1997). Because the addition of N and P did not significantly change activity, when compared to addition of water alone, the study concluded that N and P are not limiting factors and are likely present in sufficient quantities at the site.

4.7 CANADA

Atomic Energy of Canada Ltd. (AECL) initiated research at the underground research laboratory (URL) in the Archean age granite batholith called Lac du Bonnet in the early 1980s. This early work consisted of microbial surveys (Mayfield and Barker, 1982a; Mayfield and Barker, 1982b) and near-field modelling (Stroes-Gascoyne, 1989). Brown and Hamon (1994) enumerated planktonic organisms (10^3 to 10^5 cells/mL) and noted decreasing cells with depth and with increasing salinity of the groundwaters. Stroes-Gascoyne and West (1994) and Haveman et al. (1995) confirmed the presence of microorganisms in seven boreholes approximately 240 m below surface at URL and Stroes-Gascoyne et al. (1994b) described changes in the microbial concentrations with borehole flushing.

AECL researchers also pioneered characterization of far-field microbiology in granitic rocks with studies on natural analogues (e.g., Cigar Lake uranium deposit) (Stroes-Gascoyne et al., 1994a). Stroes-Gascoyne and West (1996; 1997) provide reviews of the first decade of research in near-field and far-field microbiology. In boreholes from the URL, using scanning electron microscopy and acridine orange staining (Jain et al., 1997) and API ® test strips (Haveman et al., 1995), active microbial populations were identified, of which 0.1 -10.2% of the total cell count were culturable aerobes, and 0.01 - 7.4% of the total cell count were culturable anaerobes (Jain et al., 1997). *Pseudomonas* and related organisms were dominant with denitrifying, N_2-fixing, sulfate-reducing and iron-precipitating bacteria present as well. Conversely, no methanogens or iron-oxidizers were identified in these studies. Laboratory microcosms successfully cultured both aerobes and anaerobes, although cell numbers were an order of magnitude lower for the latter (Stroes-Gascoyne et al., 2001). The results suggested DOC and N were co-limiting in the URL groundwaters. In contrast, P did not appear to be a limiting factor despite its low concentrations since addition of P did not have an effect on the incubations (Stroes-Gascoyne et al., 2001).

Based on the URL results, there has been some suggestion that methanogens may be rare or absent in Canadian Shield groundwaters (McMurry et al., 2003) compared to studies undertaken on the Fennoscandian Shield (Pedersen, 2000a; Pedersen, 2000b). Methanogens have been successfully cultured at other sites on the Canadian Shield (Doig, 1994; Doig et al., 1995) and in similar settings in gold mines in South Africa (Ward et al., 2004). While it was once thought, based on studies at Whiteshell Laboratory, that levels of methane and hydrogen were lower on the Canadian Shield compared to other Precambrian Shield hosted groundwaters (McMurry et al., 2003), investigations at a wider range of Canadian Shield sites have shown gas levels and compositions to be comparable to other sites (Sherwood Lollar et al., 1993a; 1993b; 2007; 2008). Evidence of microbially-generated methane, based on gas geochemistry and isotopic signatures, suggests that methanogens may be much more prevalent on the Canadian Shield than previously thought. Determining the role of methanogens requires site-specific investigation of geochemical indicators, as well as culture-based and molecular microbiology (Sherwood Lollar et al., 1993a; 2006).

In recent years microbial research in the Canadian context has expanded beyond granitic environments to include microbial analysis of limestone and shales (Stroes-Gascoyne and Hamon, 2008). Samples from the Queenston shale and Cobourg limestone were examined for biomarkers, specifically phospholipid fatty acids (PLFA), neutral lipid fatty acids (NLFA) and diglyceride fatty acids (GLFA), as well as cultured in both dilute growth media and media modelled on the *in situ* saline porewaters from these formations. While methodological developments are required to verify the results for waters of very low water activity, some viable microorganisms (and larger numbers of dead cells) were found in the shale samples. In contrast, results for the limestone samples were consistent only with contamination by common aerobes during drilling or sample handling. Culture results indicated the presence of common non-halophilic, mostly facultative anaerobic spore formers, but the indigenous nature of these organisms remains to be investigated (Stroes-Gascoyne and Hamon, 2008). Other studies of microbial biomass and activity in shales and sandstones from New Mexico suggest that sustained microbial activity likely requires interconnected pore throat thickness >0.2 μm in diameter (Fredrickson et al., 1997). However, bacteria may persist in some small-pore-throat samples or be capable of renewed viability, if nutrients are slowly released from the rock over geologic time to areas where more space is available, or added in laboratory amendments (Fredrickson et al., 1997).

5. RECOMMENDED STRATEGY FOR MOVING FORWARD

The goal of this review was to summarize the international literature and the state of knowledge on far-field microbial processes relevant to a deep geological repository for used nuclear fuel. A site for a used nuclear fuel DGR has not yet been selected in Canada, and so there are a variety of possible geological environments relevant to Canada discussed in this report.

5.1 GENERAL FINDINGS

The literature for international programs on far-field microbiology relevant to a DGR, as reviewed in this report, typically reported several general findings:

1. It is recognized that indigenous microbes exist in a broad range of geologic environments and it cannot, *a priori,* be assumed that a subsurface geologic environment is sterile (as this report is focused on far-field microbiology, this does not consider sterilization close to a DGR and HLW itself). It was once thought that igneous or metamorphic rock might not contain indigenous organisms because, unlike sedimentary geologic formations, microorganisms could not have survived in igneous/metamorphic rocks since their formation (Bass et al., 2002). It remains possible that extreme conditions of temperature or pressure (e.g., deep burial, magma intrusions) have the capability to "sterilize" a given setting to a certain extent (Section 3). However, recent research, as reviewed in this report, has demonstrated that: i) organisms can occupy a far wider range of temperature, pressure and water activity than previously understood (Section 2.1.4); and ii) microorganisms are common even in igneous/metamorphic rock (Section 3).

2. The review of international programs shows that microbiological characterization programs can be integrated with the geologic/hydrologic/geochemical investigations to ensure feedback between these approaches (Humphreys et al., 2010).

3. The presence, diversity and activity of indigenous microbial populations in the far-field is controlled by a number of factors, including principally: geologic (physical) and chemical (including mineralogical) properties of the host rock; transport properties of the host rock; geochemistry of the associated groundwater (and rock-water interactions), particularly its role in controlling the rate of supply of nutrients, energy sources and metabolites in and out of the system; rock properties such as porosity, permeability, hydraulic conductivity and the presence and degree of inter-connections between fractures (especially for crystalline rock); and, both the geologic and geochemical history of the site, including burial, uplift, tectonic history, depth and degree of groundwater circulation and fluid/gas migration through the system.

4. Hydrology, geochemistry, and resident microbial populations may be sensitive to changes or perturbations in the system. Perturbations related to the near-field of the DGR are not in the scope of this report. However, it should be noted that perturbations related to the far-field investigation program itself should be considered, such as perturbation of the subsurface via drillholes and associated drilling fluids. These can change redox and other geochemical characteristics of the groundwater and may add materials, which can temporarily increase nutrient and energy sources for the local microbiology.

5. As discussed more extensively in Section 2.2, microbiological far-field investigations should incorporate techniques that eliminate, to the highest degree possible, any contamination due to sampling/drilling. However, contamination due to drilling is unlikely to be able to be eliminated completely. Contamination can be addressed by characterizing not only the indigenous geochemistry and microbiology but also the geochemical and microbiological properties of any potential contaminant end-members introduced during the investigation (e.g., drilling fluids or muds, service water, surface volatiles). The literature reviewed herein demonstrates how comparison and contrast between potential contaminant end-members and other samples permit evaluation of the degree of any remaining contamination.

5.2 MICROBIAL EFFECTS IN THE FAR-FIELD

Notwithstanding the site-specific nature of far-field microbiology, international programs and research have identified potential ranges of microorganisms likely to be detected in particular geological environments, both sedimentary and crystalline, as reviewed in Section 3 (and references therein). Similarly, while the specific effects of the far-field microbiology should be investigated on a case by case basis, as reviewed in Section 3, certain general observations can be made:

1. Given the likely (but not exclusive) predominance of anaerobic microbial processes, and recent findings of the importance of H_2 metabolism in the subsurface (Section 3), it is likely that H_2 oxidation will be an important process to consider in far-field investigations. This finding is consistent with Humphreys et al. (2010) who suggested that, depending on the local nutrient and energy supply, H_2 oxidation would likely be coupled with iron reduction, sulfate reduction, methanogenesis or acetogenesis. Anaerobic corrosion of waste containers in the near-field may produce H_2 (King, 2007; Kwong, 2011), which, if migrating to the far-field may enhance H_2 metabolism. A second important set of gaseous reactions involves production of reduced gases, such as H_2S, methane and higher hydrocarbons, either during H_2 oxidation reactions (such as methanogenesis or sulfate reduction) or in sedimentary systems (in particular, through natural gas production related to microbial transformation of sedimentary organic matter) (Section 3). Further, either aerobic oxidation or anaerobic oxidation of methane by microbial populations in the subsurface should be considered to fully assess the role of the far-field microbiology in gas generation (Beadle et al., 2001).

2. While the influence of far-field microbiology on fluid geochemistry and pH is complex and specific to a given geological setting, current international findings suggest that the overall effects of indigenous microbes will be to drive the system to more anaerobic conditions, partly due to the consumption of available oxidants and energy sources (such as organic substances) faster than the rate of resupply (Section 3). Redox changes can significantly impact the solubility, mobility and transport of solutes and radionuclides (Section 3). As noted, effects on pH are difficult to predict and, as with specific effects on the geochemistry, require additional investigation particular to the site. The large buffering capacity of the geosphere to microbial perturbations has been demonstrated, for instance, in the case of temporary increase in the activity of sulfate-reducing bacteria. An experiment in the OPA clay borehole at the Mont Terri Rock Laboratory showed geochemical conditions returning to pre-disturbance conditions over a 5-year period (Stroes-Gascoyne et al., 2010).

3. While still an area of active research, international programs recognize that microbial communities in the subsurface may impact the transformation and transport of solutes in general, and radionuclides specifically, by a number of means. While the role of planktonic microorganisms should not be neglected (Bass et al., 2002), the role of microbial communities attached to surfaces (biofilm) may also be important, both chemically and physically. Bacteria may influence the overall transport properties by clogging pores and affecting permeability and hydraulic conductivity. They do this not only by cell/biofilm growth and proliferation, but also by concentrating elements involved in mineral precipitation in localized micro-environments; these processes can accelerate either mineral formation or dissolution (Humphreys et al., 2010). Biofilms may further retard radionuclides by sorption to the biofilm or cells themselves, or in some cases enhance transport if they cover and, thereby, reduce the number of sites for sorption on the mineral surfaces (Bass et al., 2002). The detailed effects should be assessed as a function of the local far-field geological, hydrogeological and geochemical settings, and the nature of the specific solute or radionuclide.

5.3 RECOMMENDATIONS

The review of international programs, literature and best practices relevant to a DGR for used nuclear fuel provided in this report has highlighted the importance of investigations of the far-field microbiology, has outlined important processes and parameters influenced by subsurface microorganisms, and has provided examples of the results of far-field investigations in many international programs. Consistent with other international programs, the detailed effects of far-field microbiology should be investigated on a site-specific basis. Humphreys et al. (2010) demonstrated that integrated microbiological/geochemical/hydrogeologic approaches are key to international strategies designed to guide far-field microbiological investigations. Those investigations should be planned and implemented early in the site characterization process, and integrated directly with investigations of the geochemistry and hydrogeology, following a model that has become the best practice in the investigation of near surface hydrogeology and microbial biogeochemistry (Weiss and Cozzarelli, 2008; Humphreys et al., 2010).

Specifically, investigation of the geologic setting, including the hydrogeology, is essential to define the scale and scope of the system including: the depth and volume of the geologic units, the rate and dominant transport mechanism of groundwater movement, and the degree of transport and interconnection of groundwater with other geologic units, including the surface hydrology on a local- or regional-scale. Geochemical investigations are important not only to define the hydrogeology, but also to provide measurements of the inorganic and organic solutes in the aqueous, gaseous and solid phases in the subsurface that assist in a first assessment of the nature and viability of any microbial populations. Such analyses may include conventional hydrogeological techniques (such as stable isotopes, tritium content, and groundwater dating techniques) that are not the focus of this report, as well as measurements of solutes, metabolites, nutrients and redox-sensitive species (including gases), and also may include isotopic characterization of reactants or products. This information can be used to screen or estimate potential microbial processes relevant to the geologic/hydrogeologic/geochemical setting. Such information is essential to effectively characterize the spatial and temporal variability of microbial activity in the system, given that, as noted in Sections 2 and 3, many microbial techniques can provide information on “who is there”, but that geochemical indicators

are a critical component of determining what fraction of an enumerated population is active (i.e., “what do they do?”).

Both culture-based and molecular microbiological approaches discussed in Section 2 can be carried out effectively and simultaneously with the above techniques - however the results must be integrated in real-time because the combined results from the hydrogeology, geochemistry and microbiological approaches will provide feedback that is critical to designing the next phase of investigations. Humphreys et al. (2010) provide a conceptual model for this approach, adapted from Weiss and Cozzarelli (2008) (Figure 2). As discussed, culture-based techniques require assumptions/choices about what microbial populations might be present. Similarly, molecular techniques may require choices of specific primers. In both cases, simultaneous characterization of the geochemical environment is required to select appropriate methodologies.

Logistically, such an approach, which systematically builds up a knowledge base from the hydrogeological to geochemical to microbiological investigations, can be undertaken by collecting core, groundwater and volatiles, even from the first reconnaissance boreholes at a site. Ideally, however, enough prior knowledge of the geology and hydrogeology would be obtained to allow a well designed series of boreholes for geochemical and microbiological investigations (i.e., targeted geologic units as well as units with which they might be hydrogeologically connected). As recommended by Nirex (2006), establishing the size and nature of the *in situ* population is the first step in designing the appropriate additional techniques and research program to assess the relevance of far-field microbiological processes to the site. The number and levels of the techniques depicted in Figure 2 that would eventually be carried out would be determined by the results at each stage - but the shaded elements are typically those used by the international programs that are reviewed in this report.

Emerging literature should continue to be reviewed to fine-tune the sampling approaches to deal with the different challenges associated with: the hydrogeology of low conductivity sediments or sedimentary rock; the more challenging extraction of pore fluids from low permeability clays (Stroes-Gascoyne et al., 2007); and the fracture-controlled hydrogeology frequently encountered in granitic terrains. To advance this goal, the preparation of a site characterization document that outlines “best practices” for drilling, sampling, and sample storage for microbiological and hydrogeochemical investigations is recommended. In addition, periodic review of emerging developments in microbiological sampling, analysis, and interpretation are recommended as the field is rapidly evolving.

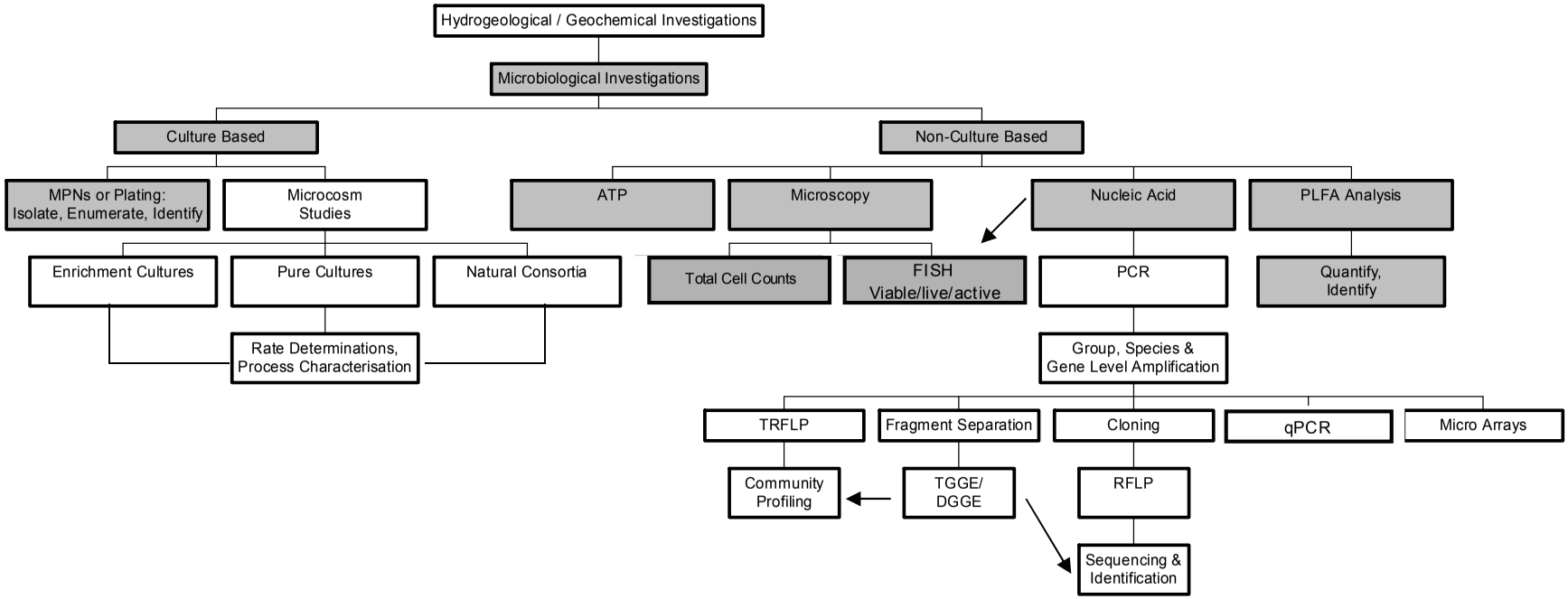

Figure 2: Microbiological Methods (Modified from Weiss and Cozzarelli, 2008)
Information feed-through in integrated hydrogeological/geochemical/microbiological far-field investigation flow chart originally proposed by Weiss and Cozzarelli (2008) and modified after Humphreys et al. (2010).

6. SUMMARY

This report presents a state of science review of international literature and knowledge on the role of microorganisms in relation to the key issues affecting the design and performance of a used nuclear fuel repository, with a focus on far-field microbial processes. The 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. The report is aimed at those who are familiar with the area of long-term nuclear waste management but who are not experts in microbiology and the geochemistry of the subsurface.

The report addresses the relationships between environmental conditions of the deep subsurface (salinity, porosity, water activity, temperature, geochemistry) and microbial activity. The review considers the rock types that are potential host rocks for a DGR for used nuclear fuel in Canada, and highlights differences between crystalline and sedimentary rocks, where relevant. Specifically, with regard to the far-field microbiological considerations relevant to a DGR for used nuclear fuel, the following is included: i) the diversity and activity of microbial life at depth in low permeability rocks with high total dissolved solids and ionic strength; ii) the immediate impact of active microbial populations and the potential impacts of inactive cells that may become active as a result of changing conditions; iii) the effects of microorganisms on the geochemistry of the far-field, with particular emphasis on the role of microbes in developing and maintaining reducing conditions; iv) microbial effects on radionuclide migration, including effects from colloids or biofilms; and v) the effects of microbial gas production and consumption (e.g., H_2 metabolism).

The literature for international programs on far-field microbiology, as reviewed in this report, typically reported several general findings:

1. It is recognized that indigenous microbes exist in a broad range of geologic environments and it cannot *a priori* be assumed that a subsurface geologic environment is sterile.

2. The review of international programs shows that microbiological characterization programs can be integrated with geologic/hydrologic/geochemical investigations to ensure feedback between these approaches.

3. The presence, diversity and activity of indigenous microbial populations in the far-field is controlled by a number of factors, including principally: geologic (physical) and chemical (including mineralogical) properties of the host rock; transport properties of the host rock; geochemistry of the associated groundwater (including TIC, TOC and electron donors and acceptors); hydrogeologic properties; and both geologic and geochemical history of the site.

4. Hydrology, geochemistry and resident microbial populations may be sensitive to changes or perturbations in the system. Many systems possess geochemical buffering capacity to drive site conditions to pre-disturbance background.

5. Microbiological far-field investigations should incorporate techniques that eliminate, to the degree possible, any contamination due to sampling/drilling. The techniques should

also control for contamination by the characterization of not only the indigenous geochemistry and microbiology, but also the geochemical and microbiological properties of any potential contaminant end-members.

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