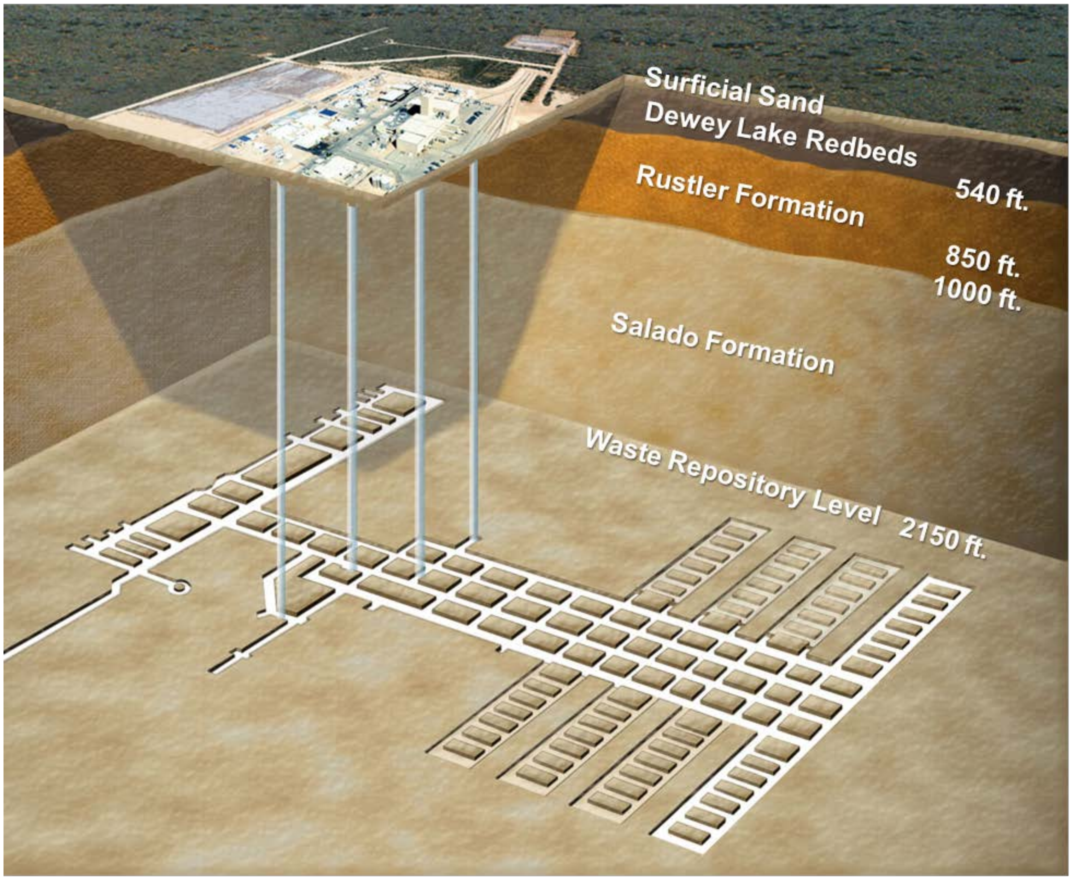
LA-UR-16-28895

THE MICROBIOLOGY OF SUBSURFACE, SALT-BASED NUCLEAR WASTE REPOSITORIES: USING MICROBIAL ECOLOGY, BIOENERGETICS, AND PROJECTED CONDITIONS TO HELP PREDICT MICROBIAL EFFECTS ON REPOSITORY PERFORMANCE



WIPP Facility and Stratigraphic Sequence

The funding for this document is provided by the U.S. Department of Energy, Carlsbad Field Office.

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WIPP TRU Repository

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Issued: November, 2016

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ACKNOWLEDGMENTS

This report was compiled by the Los Alamos National Laboratory-Carlsbad Operations Actinide Chemistry and Repository Science Program in Carlsbad, New Mexico; and by the Helmholtz-Zentrum Dresden-Rossendorf Center for Resource Ecology in Rossendorf, Germany; and by the Gesellschaft für Anlagen und Reaktorsicherheit in Braunschweig, Germany.

The American authors wish to acknowledge the support of Russ Patterson, the Department of Energy (DOE) program manager. LANL research described herein was funded by the Department of Energy-Carlsbad Field Office (DOE-CBFO) as part of the ongoing recertification of the WIPP transuranic repository. HZDR research described herein was funded by the Young Investigators Grant, MicroSALT and by the German Federal Ministry for Economic Affairs and Energy (BMWi).

EXECUTIVE SUMMARY

The evaluation of deep geological settings as sites for nuclear waste disposal is extensive and multidisciplinary, and among the many areas of study is the field of microbiology. The microbiology of granite, basalt, tuff, and clay formations in Europe and the US has been under investigation for decades, and much has been learned about the potential influence of microorganisms on repository performance and about deep subsurface microbiology in general.

In spite of this, there is still uncertainty surrounding the effects of microorganisms on salt-based repository performance. One of the reasons for this is that negative findings (i.e., no growth) cannot be used as performance model input, so as a result, conditions are often manipulated to generate positive findings (i.e., growth). Given the unique microbiology of hypersaline environments, these negative results are both valid and meaningful and should be interpreted from the perspective of feasibility.

The microbial communities present in hypersaline settings are limited in both structural and functional diversity. This is because, in order to survive at high salt concentrations, these organisms must osmotically balance their internal and external environments. This limits their ability to perform certain modes of metabolism, based on the energy required for survival and the energy derived from a given reaction. The field of repository microbiology has assumed that diverse organisms capable of diverse metabolic processes will be present and active in the repository setting; however, this is not likely to be the case at extremely high salt concentrations.

At the highest salt concentrations, extremely halophilic Archaea are dominant members of the microbial population because of their ability to balance osmotic pressure using a low-energy strategy. These organisms are almost all aerobic with limited anaerobic and fermentative capability, thus their role in repository microbiology may be confined to early oxic periods. Still, they are able to survive tens of thousands of years encased in salt, such that they will be present throughout repository history. Some extremely halophilic Bacteria also exist in hypersaline environments. In general, these organisms will have a much more diverse metabolic repertoire, including aerobic, anaerobic, and fermentative capabilities. However, these capabilities narrow as salt concentration increases, due to the high-energy cost strategy utilized by bacteria to maintain osmotic balance. Bacteria present in repository waste or introduced during mining operations are not likely to be halophilic and may not survive long-term. However, the role of microorganisms within drums may be significant.

This report summarizes the potential role of microorganisms in salt-based nuclear waste repositories using available information on the microbial ecology of hypersaline environments, the bioenergetics of survival under high ionic strength conditions, and "repository microbiology" related studies. In areas where microbial activity is in question, there may be a need to shift the research focus toward feasibility studies rather than studies that generate actual input for performance assessments. In areas where activity is not necessary to affect performance (e.g., biocolloid transport), repository-relevant data should be generated. Both approaches will lend a realistic perspective to a safety case/performance scenario that will most likely underscore the conservative value of that case.

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ABBREVIATIONS AND ACRONYMS

ABBREVIATION/ACRONYM	DEFINITION
a_w	water activity
CFU	colony-forming units
CMC	carboxymethylcellulose
CPR	cellulose, plastic and rubber
DGR	deep geologic repository
DNA	deoxyribonucleic acid
DRZ	disturbed rock zone
EC	effective concentration
EDTA	ethylenediamine tetraacetic acid
ERDA-6	Energy Research and Development Administration Well 6
GWB	Generic Weep Brine
HLW	high-level waste
HMW	high-molecular weight (in reference to organic compounds)
ISA	isosaccharinic acid
LLW	low-level waste
L/ILW	low - to intermediate-level waste
LMW	low-molecular weight (in reference to organic compounds)
MgO	magnesium oxide (engineered barrier at the WIPP)
MPa	megapascals
PA	performance assessment
ppm	parts per million
RH	relative humidity
ROS	reactive oxygen species
SRB	sulfate-reducing bacteria
TRU	transuranic
WIPP	Waste Isolation Pilot Plant

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I. INTRODUCTION: REPOSITORY PERFORMANCE AND THE POSSIBLE EFFECTS OF MICROBIAL ACTIVITY

Deep geologic formations are common choices for the disposal of hazardous waste because of their ability to effectively isolate the waste from the environment and the public. Granitic rock, basalt, clay, tuff, and evaporite salt beds have been proposed as sites for nuclear waste repositories. Much research has been conducted on the influence of microorganisms on repository performance (safety cases), ever since it was determined that organisms are active even in such deep geologic settings (Pedersen, 2002; Poulain et al., 2008; Anderson et al., 2011).

Although an abundance of research on halophilic microorganisms exists, few data are available on the microbiology of subterranean salt formations that pertain to proposed and active locations for nuclear waste repositories. Because the biogeochemistry of other deep geologic (e.g., low ionic strength groundwaters) settings differs significantly from subterranean salt, it is not always possible to extrapolate microbial activity from one site type to the other. In lieu of directly applicable data, repository scientists may rely on “indirect” information—such as microbial ecology, genomics, and the thermodynamic feasibility of certain types of metabolism in hypersaline environments—to predict microbial activity and potential impact on performance. Still, supportive research is needed to directly address specific safety case issues.

Because of the lack of data and resulting uncertainty surrounding microbial processes in high ionic strength repository settings, performance assessments/safety cases must be conservative in their predictions of potential microbial impact. While this conservative approach can be defended in a regulatory process, a better understanding of the system might alleviate the need to spend resources engineering around what may only be perceived problems.

The success of a nuclear waste repository is measured as its ability to prevent the release of radionuclides into the surrounding environment or to limit that release to levels deemed acceptable by the appropriate regulatory agencies and public. These mandates have variable lifetimes which may depend upon the repository location and whether the waste has low, intermediate, or high levels of radioactivity.

Microorganisms are predicted to have diverse effects on nuclear waste repository performance, all of which involve their impact on radionuclide migration (McCabe, 1990; Pedersen, 1999; Macaskie and Lloyd, 2002; Pedersen, 2005; Wang and Francis, 2005; Lloyd and Gadd, 2011). These effects are due to any activities that may affect radionuclide speciation and solubility or enhance mobility, and hence the source term used in evaluating repository performance.

Key microbial processes and effects include:

- complexation with carbon dioxide or organic ligands generated from the breakdown of organic waste;
- complexation with microbially-generated ligands;
- creation of a reducing environment through the consumption of oxygen, generation of hydrogen, or formation of reduced metal species;
- alteration of pH;
- redox reactions with metal and/or radionuclide elements;
- any interactions that may indirectly enhance migration, such as surface sorption or internal uptake of radionuclides that may lead to biocolloid transport; and
- any activities that may affect the performance of engineered barriers, such as canister corrosion and alteration of backfill or other barrier materials, such as cement or clay.

The relevance of these processes in subterranean salt formations will be discussed in detail in later sections. In general, microbial considerations for nuclear waste repositories can be summarized as shown in Figure 1.

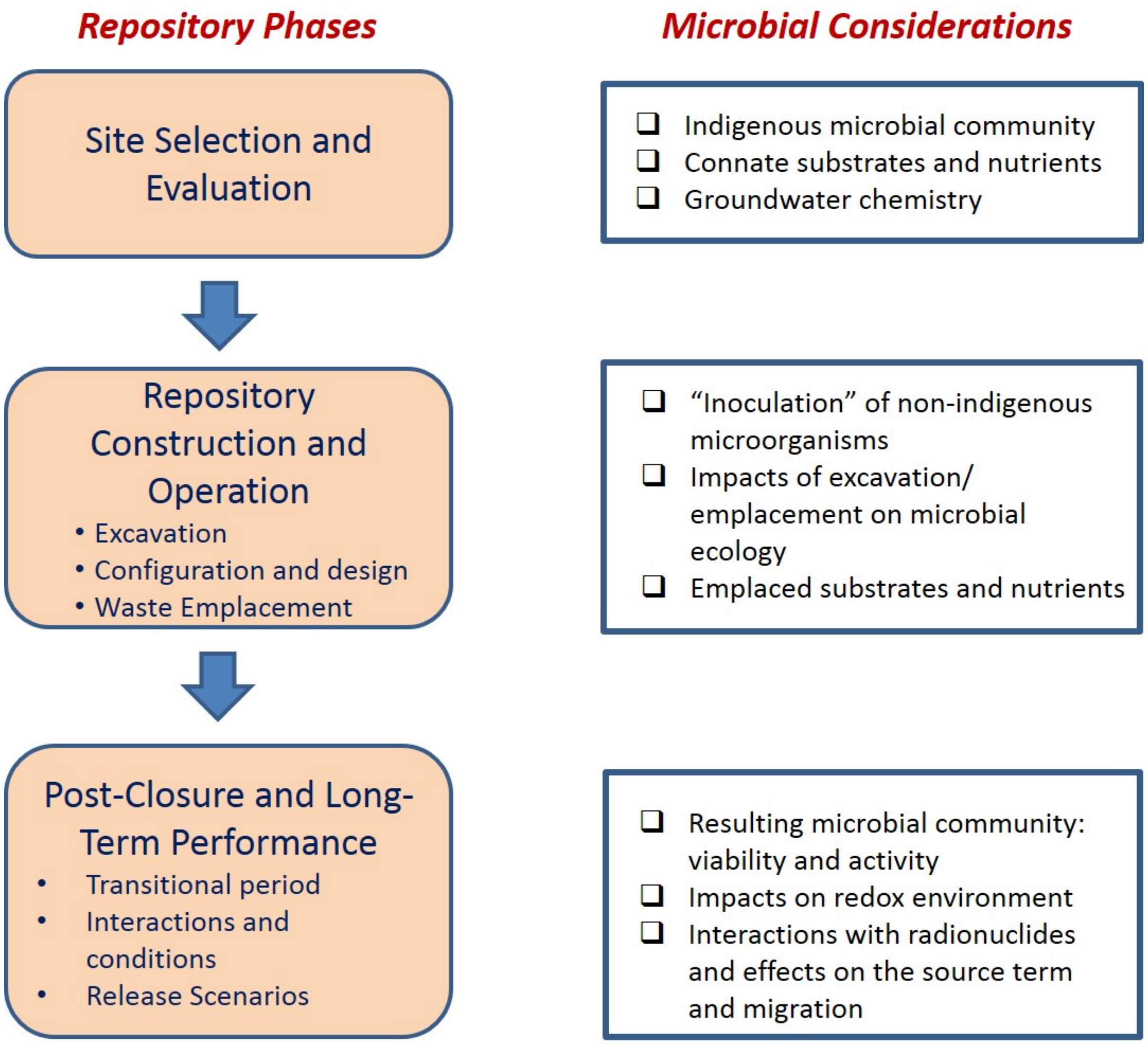


Figure 1: **Overview of key repository phases and the microbial issues associated with each phase. This can be applied generically to all permanent geologic disposal concepts.**

II. BACKGROUND

II.A. The Subterranean Salt Environment and the Potential for Microbial Life

The subterranean salt formations proposed as nuclear waste repository sites exist in different forms—bedded salt and salt domes, or diapirs. Both begin as evaporite beds, of which halite is the dominant component, comprising between 93-97% of the total by weight. Other components include carbonates, anhydrite or gypsum, and chloride or sulfate salts of magnesium and potassium. Impurities, such as sandstones, volcanic materials, and clays may exist due to tectonic movement, wind, and run-off, during and after the deposition period (Harvie et al., 1980; Casas and Lowenstein, 1989). An uprise in the salt layer, due to its lower density in relation to the overlying sedimentary rock and subsidence of the sediment, can lead to dome formation.

In contrast to other geologic formations that may contain deep aquifers, subterranean salt environments are relatively dry. What fluid there is may be external, from a surrounding water-bearing region via intergranular spaces, or internal, as trapped fluid inclusions or bound water in hydrous mineral impurities, e.g. gypsum, and clays (Roedder, 1984). Because of its low permeability, very little fluid or vapor movement occurs within subterranean salt formations. It has been suggested that salt domes contain less intergranular and inclusional fluid due to textural changes during stress deformation, but they may contain more water in hydrated minerals. This appears to vary with site history and mineralogy, such that site-specific testing is warranted (Knauth and Kumar, 1981; De Las Cuevas and Pueyo, 1995).

Most deep geologic environments are dark and oligotrophic. This limits the sources of energy for microbial growth, and although microorganisms may remain viable, they may be inactive or in a state of low activity. Hydrothermal vents in some settings allow a gas-driven biosphere, or deep aquifers may introduce other energy sources. However, like water, this type of external input of potential energy sources for microorganisms is essentially absent in subterranean salt. “Native” substrates and electron donors are limited to syndepositional (e.g. fluid inclusion contents) or potential “migratory” sources (e.g. movement along fractures during past halokinesis, or between salt crystals; Pusch et al., 2014).

Syndepositional substrate sources can include halophilic algae and their associated organic osmolytes trapped within fluid inclusions. These are hypothesized to provide a long-term carbon and energy source to co-located halophilic prokaryotes (Schubert et al., 2010), although others have suggested that those prokaryotes are in a dormant state and not capable of utilizing them or that the algal release of glycerol alone is insufficient for the entombed halophiles to survive (Fendrihan et al., 2012; Winters et al., 2015). Still, the oldest halite in which entrapped algae were found was 150,000 years old (Sankaranarayanan et al., 2011); this is younger than the Permo-Triassic salt beds currently in use or under consideration for nuclear waste repositories (e.g., Salado Formation in US; Zechstein Formation in Europe).

Native hydrocarbons have also been found within inclusions (Roedder, 1984; Pironon et al., 1995) but are more common within intergranular spaces and within anhydrite crystals (Hammer et al., 2012; Pusch et al., 2014) and are especially associated with salt domes (Posey and Kyle, 1988). Other organics may be associated with interstitial impurities, such as clays (Pironon et al., 1995), but whether or not these are available or utilizable substrates for microorganisms remains unknown.

Although there are no hydrothermal vents in subterranean salt to provide gaseous sources of energy, there are trapped gases within fluid inclusions. These may be from the originally trapped atmosphere (e.g., N_2); may have resulted from transient microbial activity (e.g., CO_2, H_2, CH_4, ethane, CS_2, and H_2S) suggestive of the degradation of common marine biological compounds or hydrocarbons (Roedder, 1984; Siemann and Ellendorff, 2001); or from the thermal degradation of entrapped hydrocarbons (Kovalevych et al., 2008). Again, it is unknown whether these are either available or significant energy sources.

II.B. The Microbiology of Hypersaline Systems and the Subterranean Salt Biosphere

Hypersaline conditions result in a unique microbial ecology, due to the thermodynamic constraints imposed upon the organisms inhabiting such environments. Survival depends on an organism’s ability to maintain osmotic balance with its external environment (Oren, 2006). Two strategies exist by which organisms can do this: 1) increasing salt concentrations, usually K^+ or Cl^-, intracellularly (“salting in”) or 2) generating or taking up small, compatible organic solutes to raise the intracellular ionic strength. The former strategy is limited to Archaea (of the order *Halobacteriales*) and a few anaerobic Bacteria (orders *Halanaerobiales* and *Bacteroidetes*); while, the latter strategy is used by all other halophilic and halotolerant Bacteria and all eukaryotes. As a result, haloarchaea generally tend to be the dominant organisms at the highest salt concentrations, while halophilic bacteria generally tend to dominate low-salt environments.

II.B.1. The microbial community in subterranean salt

Although once thought to be sterile environments, it is now accepted that microorganisms inhabit subterranean salt formations. Commonly detected archaeal genera include *Halobacterium*, *Halorubrum*, *Halococcus* and *Natronomonas* spp. (Norton et al., 1993; Stan-Lotter et al., 1999, 2002; Fish et al., 2002; Park et al., 2009; Schubert et al., 2009; Gramain et al., 2011; Swanson et al., 2013a). Less common are organisms belonging to *Haloarcula*, *Halarchaeum*, *Haloterrigena*, and *Halosimplex* spp. (Vreeland et al., 2002; Park et al., 2009; Schubert et al., 2009). Recently described *Halolamina* and *Haloparvum* spp. have also been detected in subterranean salt formations, and many unclassified DNA sequences may yet result in the descriptions of novel genera (Radax et al., 2001; Park et al., 2009; Gramain et al., 2011; Swanson et al., 2013a; Chen et al., 2016).

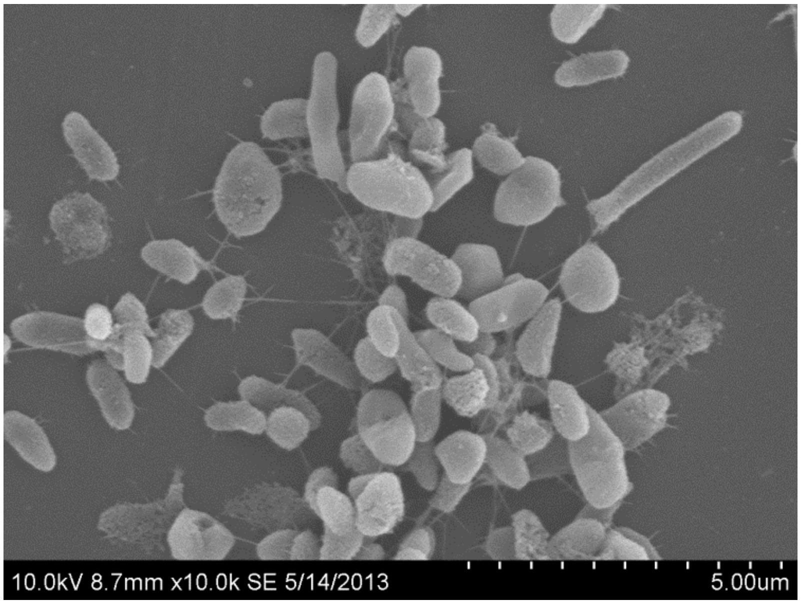


Figure 2: *Halobacterium* sp. (*noricense*) isolated from Salado halite in the WIPP.

Bacteria are less commonly sought from rock salt, and in general, they are the dominant organisms cultivated at lower salt concentrations. Those that have been detected (by DNA signatures) or isolated include members of the phyla *Proteobacteria*, *Firmicutes*, *Actinobacteria*, and *Bacteroidetes* (Vreeland et al., 2000; Fish et al., 2002; Roohi et al., 2012). DNA detection does not translate to cell viability, as high salt concentrations may help preserve nucleic acids (Fish et al., 2002; Borin et al., 2008).

The numbers of organisms that can be cultivated from rock salt vary widely, from 0 to 10^4 colony-forming units per gram of salt (Norton et al., 1993; Vreeland et al., 1998; McGenity et al., 2000; Swanson et al., 2013a). This is likely due to the heterogeneous distribution of microorganisms in rock salt, such that often large quantities must be processed before any growth can be seen (Norton et al., 1993; McGenity et al., 2000). There is little documentation of direct microscopic cell counts in halite, possibly because of the low numbers (Swanson et al., 2013a). Microbial numbers in subterranean salt also appear to be lower than those in surficial hypersaline environments, such as brine lakes or solar salterns (e.g., 10^7 cells/ml in a crystallizer pond of a solar saltern; Anton et al., 2000), most likely due to input of exogenous carbon and energy sources. Brines found in subterranean halite can also range in cell density (none to 10^7 cells/ml) even within the same formation (Norton et al., 1993; Francis and Gillow, 1993;

Vreeland et al., 1998; McGenity et al., 2000; Swanson, unpublished). This may be a function of brine composition, pH, source, or length of exposure to mine air and other mine workings.

II.B.2. The microbial community in subterranean salt-based nuclear waste repositories

The microorganisms present in subterranean salt repositories are either indigenous to the salt formation (i.e. present in fluid inclusions or interstitial brines), indigenous to the surrounding environment (e.g., groundwater infiltrate), introduced in and on the emplaced waste, introduced on mining equipment and personnel, and introduced via air intake shafts. Their true origin is not as relevant as their ability to survive and be active under projected repository conditions.

II.B.3. Thermodynamic feasibility of microbial metabolism at high salt concentrations and documented modes of metabolism in subterranean salt environments

Maintaining osmotic equilibrium with the surrounding high salt environment is energetically costly to organisms, but the organic osmolyte strategy is significantly more expensive than "salting in", especially when the compatible solutes are not available in the surrounding medium and must be synthesized *de novo*. As a result, microbial metabolic processes in hypersaline systems are limited to reaction pathways that result in a high enough energy yield to offset the cost of maintaining this balance.

The following metabolic processes are favorable at salt concentrations greater than 2.5 M NaCl (the cut-off for extremely halophilic microorganisms; see Figure 3; for excellent reviews, see Oren 1999, 2011, 2012):

- oxygenic and anoxygenic photosynthesis (energy from light);
- aerobic respiration;
- nitrate reduction/denitrification;
- fermentation;
- manganese, arsenate, and selenate reduction (anaerobic respiration using oxyanions as terminal electron acceptors);
- dissimilatory sulfate reduction with incomplete organic oxidation;
- reduction of elemental sulfur;
- methanogenesis from methylated amines;
- acetogenesis (generation of acetate from H_2 and CO_2); and
- chemolithotrophic oxidation of sulfur compounds (e.g., S^0, thiosulfate).

All of these processes are either energetically favorable or are performed by organisms that maintain osmotic balance by the less costly strategy of "salting in".

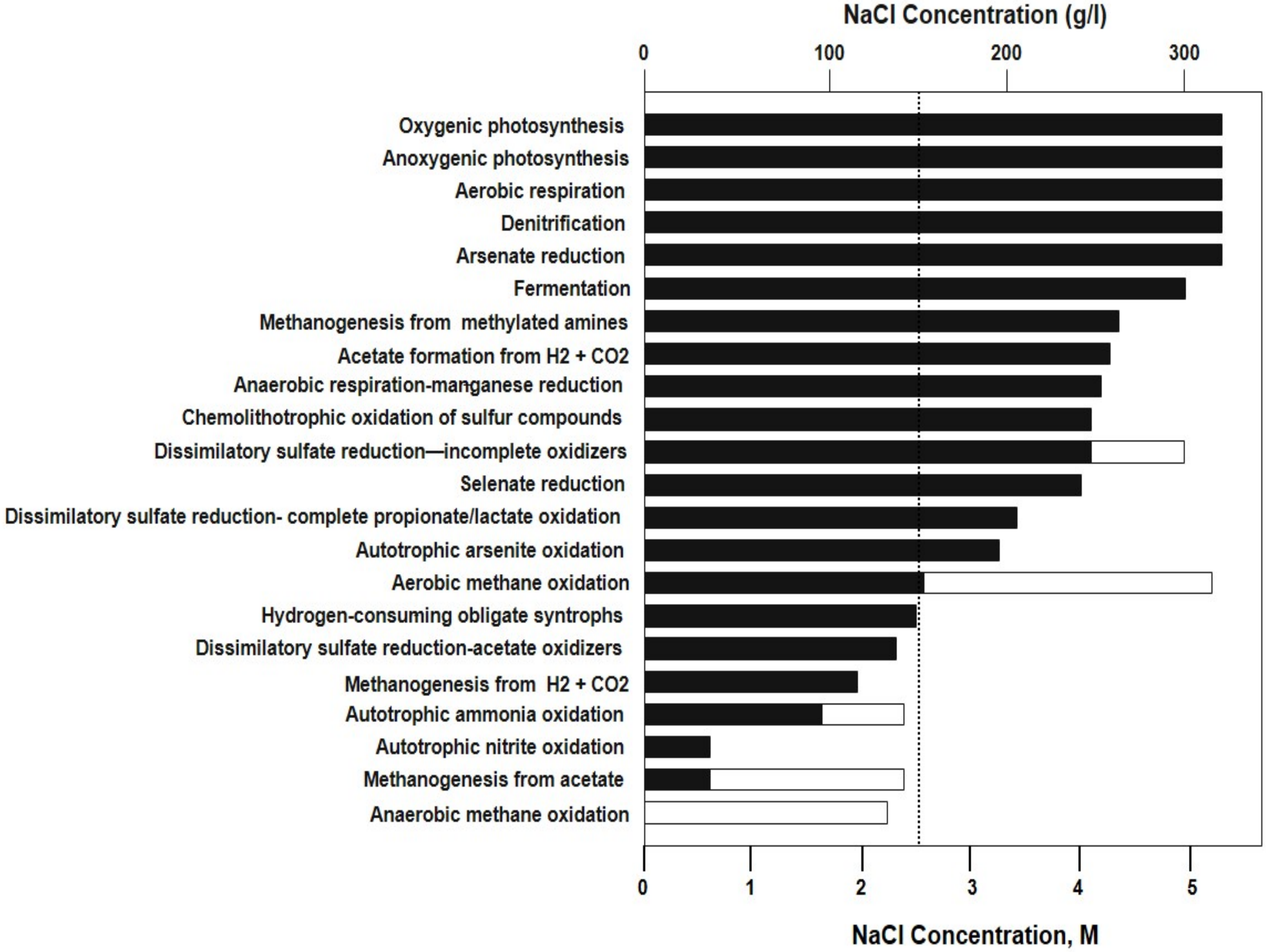


Figure 3: **Approximate upper salt concentration limits for the occurrence of selected microbial processes (from Oren, 2012). Solid bars are derived from laboratory experimental data using pure cultures; open bars are taken from *in situ* measurements of possible microbial activity. Dashed vertical line represents 2.5 M NaCl cut-off for extreme halophiles.**

Of these possible modes of metabolism, some can easily be ruled out for subterranean salt settings: e.g., phototrophy. The rest depend upon the presence of electron donors and acceptors and the appropriate carbon substrates and nutrients. To the authors' knowledge, there are no studies documenting the successful cultivation of halophilic organisms from subterranean halite under anaerobic conditions and only two documented failed attempts to do so (Michaud and van Demark, 1967; Swanson et al., 2013a). However, haloarchaea isolated under aerobic conditions have been found capable of growing anaerobically with the reduction of nitrate, trimethylamine N-oxide (TMAO), dimethyl sulfoxide (DMSO), or fumarate; or are able to ferment amino acids, such as arginine (Oren, 2011; Müller and DasSarma, 2005).

III. THE POTENTIAL FOR MICROBIAL ACTIVITY UNDER PROJECTED REPOSITORY CONDITIONS

What will happen over time to the resident (i.e., indigenous and introduced) microbial population remains a large uncertainty but will depend upon whether or not conditions are favorable for activity or merely survival. In order for microorganisms to influence repository performance (excluding colloid transport), they must not only be viable, they must be active. Microbial activity is dependent upon the presence and availability of moisture, adequate substrates and nutrients, and sources of energy (electron donors and acceptors), and these requirements may vary with organism type. While it may be possible for all these basic requirements to be present in a repository environment, it is also possible that they will never come into contact with microorganisms, or that contact will be limited to microenvironments, due to the uncertainty surrounding humid versus inundation scenarios and the integrity of waste canisters.

III.A. Expected Conditions

Prevailing salt-based repository conditions may vary between sites and/or repository types. For instance, temperatures will differ with waste type (HLW heat-generating versus intermediate/low-level waste); lithostatic pressures may vary with repository depth; and the presence of introduced constituents may affect the physicochemical environment (e.g., clay, cement, magnesium oxide). The only guaranteed shared conditions across all salt-based repositories are high salt content, the presence of radionuclides, and an ultimately closed system due to the self-sealing properties of salt.

After excavation and during waste emplacement, the repository site is essentially an “open reactor”. Air-intake shafts introduce an oxygenated atmosphere, moisture, and microorganisms. Miners and mining equipment provide another source of non-native microorganisms. The emplaced waste may be a source of organics, inorganics, and microorganisms. Additionally, excavation can lead to fluid migration into the disturbed rock zone (DRZ), and this may introduce brine, hydrocarbons, if present, and microorganisms into the repository horizon. Once sealed, the repository is theoretically limited in “reactants” to connate components and the components that were emplaced.

III.A.1. Moisture: water activity, relative humidity, and brine inundation

As mentioned earlier, subterranean salt formations are relatively dry—a desirable attribute for a nuclear waste repository. Still, brine can be present in fluid inclusions or as intergranular solutions. The amount of brine in subterranean salt varies with the salt formation and with its impurities, but in general is very low—e.g., 0.3 weight percent in pure halite; 1.5 % in argillaceous halite (Hansen and Leigh, 2011). The movement of this fluid may depend upon the nature and extent of the DRZ, subsequent salt subsidence and creep, and temperature (especially for HLW repositories). Seeps or sumps of significant volume can result from the redistribution of interstitial brine through the DRZ during excavation activities (Deal et al., 1995).

The availability of water is a key prerequisite for microbial life. Water activity determines its availability and is defined as the ratio of the vapor pressure of a solution (or solid medium) to the vapor pressure of pure water. In dry environments, water becomes unavailable as it is lost to evaporation. In brines, high concentrations of solutes compete for solvent water, making it effectively less available. The lowest documented water activity (a_w) that supports xerophilic life is 0.61 (Grant, 2004). Most bacteria thrive at activities greater than 0.90, but extreme halophiles can thrive under salt (NaCl) saturated conditions, $a_w = 0.75$. Saturated $MgCl_2$ solutions, $a_w = 0.33$, have not been shown to support life, but this may be a result of chaotropicity as much as low water activity.

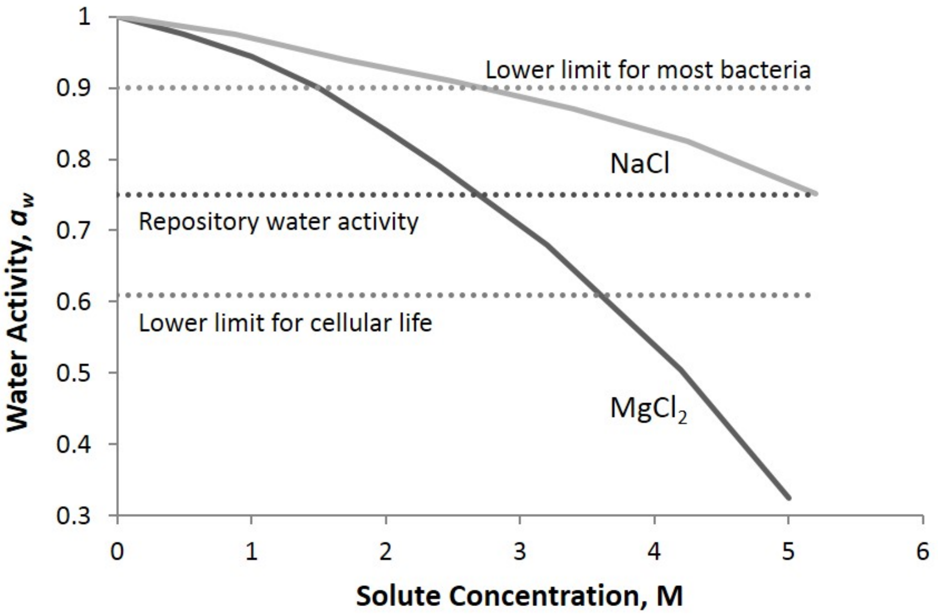


Figure 4: **Graph depicting water activity in pure solutions of sodium chloride and magnesium chloride (modified from Hallsworth et al., 2007).**

Halite deliquescence may occur at a relative humidity (RH) of 75% (25°C), which is equivalent to $a_w = 0.75$. Deliquescence can create a moist environment that, although it is halite-saturated, has been shown to stimulate transient microbial activity (Davila et al., 2008). Condensate may also form and persist in halite pore spaces less than 100 nm in diameter, thus providing a sustained higher RH in the immediate environs (Wierzchos et al., 2012). Thin-layer condensate may support the survival of less halophilic microbial species, since organisms at a liquid/air interface are not exposed to high salt concentrations from all sides. This may explain the isolation of many desiccation-resistant bacteria and fungi from salt that do not otherwise survive at extremely high NaCl concentrations in culture (e.g., *Actinobacteria*, *Firmicutes*; Roohi et al., 2012; Swanson et al., 2013a and unpublished data).

Apart from its availability, "water structure" relationships with solutes and biological macromolecules may also affect microbial survival. For example, $MgCl_2$ is a chaotropic solute that weakens the electrostatic forces that stabilize biomolecules, while NaCl, a kosmotropic solute, counteracts these effects (Hallsworth et al., 2007; Cray et al., 2013). Thus, microbial survival is unlikely at high Mg concentrations unless a sufficient amount of NaCl is also present. The highest Mg concentration that supports microbial life is thought to be 2.3 M in the presence of NaCl, but this upper limit may be pushed to 3 M based on the detection of possibly live cells in deep-sea, Mg-rich brines with a $a_w = 0.631$ (van der Wielen et al., 2005; Hallsworth et al., 2011; Yakimov et al., 2015).

The presence of cementitious waste is predicted to significantly influence brine composition in some concepts. Corrosion of cement in Mg-rich brines can lead to high concentrations of calcium chloride. Calcium, like magnesium, is a chaotropic solute that affects protein structures and water activity. *Chromohalobacter salexigens* was found to tolerate up to 1 M $CaCl_2$ in the presence of NaCl, and *Halomonas* spp. isolated from a $CaCl_2$-rich lake were also found to be Ca-tolerant (Tregoning et al., 2015).

The presence of localized moisture—as relative humidity condensate, seeps, or sumps—may be conducive to microbial survival only if the water activity is sufficient and the chaotropicity is low. If these conditions are met, activity may also be possible, provided that all other components necessary for growth are also present. Because such heterogeneously distributed areas of activity are unlikely to affect global repository performance and are nearly impossible to model, the use of inundation scenarios becomes necessary. Inundation from uncertain anthropogenic causes, such as oil drilling, may lead to the introduction of groundwater from above or below the repository horizon. Should this happen, the fluid that enters the horizon will become the "solvent" for waste dissolution and the resulting solution will become the medium for microbial growth.

Table 1 provides an example of possible brine chemistries that may be encountered by microorganisms in salt-based repository settings. These values are from two brines obtained from the WIPP environs (GWB, ERDA-6), brine from German salt repository investigations (Q), a sample of sump fluid from Asse, and recently sampled brine seepage from the WIPP. These formulations are only examples and, with the exception of GWB and ERDA-6, are not meant to be representative of a given site. Brine composition may vary between and within subterranean salt formations.

Table 1:

Measurements of brine taken from US and German subterranean salt formations.

Component	**Molar Concentrations (except pH)**				
	Generic Weep Brine (GWB)	**ERDA-6 (underlying brine reservoir)**	**Q-brine**	**Sump fluid** (Asse)**	**WIPP brine seep†**
Na^+	3.530	4.870	3.088	0.174	0.522
Mg^{2+}	1.020	0.019	3.781	4.444	3.497
K^+	0.467	0.097	0.813	0.075	0.499
Ca^{2+}	0.014	0.012	---	6E-4	0.001
Cl^-	5.870	4.800	8.386	7.729	7.339
SO_4^{2-}	0.177	0.170	0.150	0.291	0.394
Br^-	0.027	0.011	---	0.045	0.074
$B(OH)_3(aq)$	0.158	0.063	---	---	0.370
I (M)	7.43	5.34	15.4	13.9	12.0
$pH_{measured}$	7	8	4.5	4.7	4-5
pC_{H+}	8.3	8.9	7.9	7.5	6.3-7.3

*Brush and Xiong, 2009; Thies and Schulze, 1996. **Zirnstein et al., 2016, in review. †Swanson et al., unpublished. Ionic strength and pC_{H+} values were derived in EQ3/6 v. 8.0a (Wolery, 2003); pH to be discussed in more detail later.

The chief determinants for survival and community structure in hypersaline systems are sodium, magnesium, and chloride concentrations, but other constituents, such as boron, may also be toxic to some organisms. Thus, the chemistry and water activity of subterranean salt brines alone may be enough to determine which, if any, microorganisms are likely to survive.

III.A.2. Temperature

Repository temperatures may vary significantly, depending upon waste type (LLW vs. HLW). L/ILW repositories will likely maintain ambient temperatures that are conducive to microbial survival. In a HLW repository, temperatures will again depend upon waste type (e.g., defense-related or civilian, spent nuclear fuel) and overall repository configuration. Numerical simulations of HLW waste predict a temperature gradient as the distance increases from a canister, such that organism survival may be possible as little as 2 meters away (Stauffer et al., 2012). However, extreme heat-generating wastes may not permit favorable temperatures until a much greater distance (Bracke and Fischer-Appelt, 2015) or time. Also, computer simulations predict fluid movement toward the heat source, which may be detrimental to organisms entrained within the fluid. Lithology and water content may also be influenced by heat generation and, in turn, influence organism viability.

The highest documented temperature for haloarchaeal survival is 61°C (Bowers and Wiegel, 2011), and DNA of non-thermophilic organisms will degrade near 94°C.

III.A.3. Pressure

Once a repository has been sealed, pressures are expected to reach as high as 10 MPa or greater, depending upon repository depth (e.g., lithostatic pressure in WIPP is 15 MPa; Gorleben, 18 MPa).

The same attributes that make halophilic organisms resistant to osmotic stress also make them resistant to pressure, although the exact mechanisms are not known for all. For example, the low intracellular water activity and high concentrations of K^+ and Cl^- resulting from the “salting-in” strategy help stabilize proteins in both high-salt and high-pressure environments. For bacteria that use organic compatible solutes instead of ions, these molecules may serve as piezolytes as well, although not always (Martin et al., 2002). These solutes also help maintain membrane fluidity, and hence stability, in bacteria; while the presence of ether lipids and S-layers maintain membrane fluidity in archaea. High pressure has been shown to upregulate responses to oxidative stress, such as redox proteins and reactive oxygen species (ROS) scavengers. Although long-term survival under high-pressure conditions has not previously been addressed for halophilic organisms, many remain viable and even active after short-term exposures to pressures up to 400 MPa (Kish et al., 2012).

The effect of increased pressure on non-halophilic (e.g. introduced) organisms is less certain. Bacterial spores present in the waste can easily survive expected repository pressures, and their survival under pressure might be enhanced by low water activity (Sale et al., 1970). An increase in medium ionic strength permitted the survival, but not necessarily activity, of a non-spore-forming member of the *Firmicutes* at increased pressures (Molina-Höppner et al., 2004). Thus, although these organisms may survive, they may not necessarily be active. Still, directed evolution experiments have resulted in adaptation to pressure over time in organisms not known to be either halotolerant or piezophilic (Vanlint et al., 2011).

III.A.4. pH

The term pH, as used in this document, refers to the pH of a solution as measured with standard pH electrodes but uncorrected for ionic strength. This usage allows a more direct comparison with microbial pH tolerances reported in the microbiology literature. However, corrections for ionic strength are needed to express the pH in terms of the hydrogen ion concentration (pC_{H+}; Rai et al., 1995; Borkowski et al., 2009). The corrected pC_{H+} value can be significantly higher at the ionic strengths that are considered during the assessment of salt-based repository performance (see Table 1). This distinction has not been incorporated into the microbial literature but must be considered when applying this literature to a salt-based repository concept.

The examples, provided in Table 1, of measured and projected pH for repository brines range widely and may be repository-specific. For example, brines high in magnesium chloride tend to be mildly acidic; whereas, an increase in pH is expected in any repository with a significant cement inventory or with added oxides of magnesium. pH optima for extremely halophilic archaea vary with their source environment—thalassohaline (pH 7-8) versus athalassohaline (≥ 8.5)—and seem to correlate with the sodium concentrations in those respective environments (Oren, 2006; Bowers and Wiegel, 2011).

Microbial signatures have been detected in acid lakes using cultivation-independent analyses (pH ~ 2.3; Mormile et al., 2009), but no organisms have been isolated yet from such settings. Diverse haloalkaliphilic microorganisms can be found in environments where sodium carbonate or bicarbonate contributes to the high ionic strength and where divalent cation concentrations are low (e.g. soda lakes, pH 9.5-11; Sorokin et al., 2014). In general, haloalkaliphilic organisms are not found in thalassohaline settings, such as subterranean salt; however, *Halomonas* spp. have been isolated from soda lakes as well as subterranean halite (Boltyanskaya et al., 2007).

III.A.5. Atmosphere

During excavation and waste emplacement, the repository atmosphere will contain ambient air that is forced through the air intake shafts. Once sealed, canister corrosion and microbial activity will consume this residual oxygen to create a reducing atmosphere. The effects of sub- to anoxia on microbial metabolism will be discussed in a later section.

III.B. Expected Repository Contents

III.B.1. Actinides, fission products, activation products and other radionuclides

Depending upon the waste source, the radionuclide content of parent isotopes (e.g., U, Pu, Am, Np), fission products (e.g., 129-I, 137-Cs, 244-Cm, 90-Sr, 79-Se, 99-Tc), and activated elements (e.g., 36-Cl, 14-C, 3-H, 59-Ni) may differ, as will their relative activities over time (Keesmann et al., 2005; Umeki, 2007; Grambow, 2008; Schwartz, 2012). In considering microbial interactions with these elements, toxicity and biological relevance must also be addressed, and few studies have done this at higher ionic strengths.

Of the numerous elements present as parent radionuclides, fission products, and activation products in nuclear waste, the following can be considered biologically relevant in their non-radioactive forms: a) C, H, Cl, S, Ca, Na, K, as major elements in biological systems; b) Fe, Mn, Co, Ni, Zn, Mo, as major biological transition metals; c) Se, as an essential trace element; and d) As, Cd, and Ba, as elements with specialized uses. Of these, 14-C, 36-Cl, and 79-Se have been listed as concerns for long-term exposure in nuclear repository concepts (NEA/OECD 2007). In addition, oxides of Mn, As, Tc, Te, Se, U, and I and chelates of Co can serve as electron acceptors for cell respiration for some organisms (Lovley et al., 1991; Ahmann et al., 1994; Gorby et al., 1998; Lloyd et al., 2000; Maltman et al., 2015). These and other radionuclides (e.g., Np, Pu) can also be reduced as a means of resistance or detoxification by microorganisms (Lloyd, 2003). Pertechnetate, manganese oxides, and arsenate respiration have been documented for halophilic organisms, the latter two only under alkaline conditions (Oremland et al., 2000; Khijniak et al., 2003; Sorokin and Muyzer, 2010). The genes for selenite reductase were found in the genome of WIPP isolate, *Halobacterium noricense*, and aerobic selenite reduction as a resistance strategy has been shown in a *Halorubrum* sp. (Güven et al., 2013; Swanson, unpublished). *Halomonas* spp. were hypothesized to reduce pertechnetate at cell surfaces under aerobic conditions (Fujimoto and Morita, 2006).

Additional problems may arise from the ability of some fission products to substitute for biologically essential elements. For example, Cs can replace K, and Sr can substitute for Ca (Bossemeyer et al., 1989). Because extreme halophiles maintain molar concentrations of potassium and chloride intracellularly, these radioisotopes may be taken up into cells. It is unknown what effect such substitutions or other interactions with radioactive, biologically relevant metals may have on active repository organisms. Furthermore, little is known about what effect mere exposure to any radioactive element may have on repository indigenous organisms.

In the early repository phase, these radionuclides and products will not come into physical contact with indigenous microorganisms until the containers are breached. However, in the case of HLW, gamma radiation may exert an adverse effect. Once a container has been breached, exposure may occur, if solvent (brine) is present to enhance bioavailability. Bioavailability is usually a function of the dissolved concentration of a substance and thus will depend upon the radionuclide content of the waste and the nature and extent of intruding brine. (The speciation of radionuclides in repository brine is beyond the scope of this review and will depend upon many factors, such as redox conditions, pH, and soluble concentrations of waste ligands.)

Radionuclide toxicity may be either chemically or radiolytically induced. Chemical toxicity requires that radionuclides be soluble in the inundating liquid, such that microbial exposure occurs, at a concentration that causes a given deleterious effect (i.e., effective concentration, or EC). Thus, chemical toxicity is more dependent upon concentration, oxidation state, and the presence or absence of a complexant, and the effects on microorganisms are presumably similar to heavy-metal induced effects. Radiation effects are a result of oxidative damage to DNA and proteins and are therefore more dependent upon isotope and activity but do not require that a radionuclide be present in solution. Both radiation and chemical effects may vary with the microorganism tested.

Haloarchaea have numerous unique attributes making them more radiation tolerant than other organisms, including protective carotenoid pigmentation, bias against amino acids prone to reactive oxygen species, high intracellular Mn/Fe ratios, redundancy of genes encoding antioxidants, polyploidy, and the ability to differentially regulate genes needed for ROS repair functions (Sharma et al., 2012). Moreover, extracellular and intracellular chloride will help confer resistance by scavenging free radicals (Shahmohammadi et al., 1998; Kish et al., 2009). Radiotolerant mutants of *Halobacterium salinarum*, able to survive doses as high as 25 kGy, are thought to be the most radiation resistant organisms known (DeVeaux et al., 2007). Because the mechanisms involved in desiccation resistance are similar to those involved in radiation resistance, organisms present in LLW/ILW waste drums may also be radiation resistant.

The chemical toxicity of radionuclides to halophiles is not well studied. The growth of *Halobacterium* sp. (*noricense*), isolated from WIPP halite, was significantly inhibited by exposure to 10^{-6} M neptunium(V) and 10^{-4} M plutonium(V/VI), but the organism remained viable (Swanson et al., unpublished). Earlier studies of WIPP-relevant halophiles showed an inhibition of growth at 10^{-5} M Pu, 10^{-6} M Am, and 10^{-4} M of U and Np when all were complexed with EDTA (Francis et al., 1998; Strietelmeier et al., 1999). These effective concentrations approach the solubilities of some actinides in simplified and complex brines (for example, ~10^{-6} M calculated for Am in reacted WIPP brines at pC_{H+} 8.7-9 in the presence of organics, per Brush and Xiong, 2009; ~10^{-5} M calculated for Am in Gorleben, per Schwartz, 2012; ~10^{-5} M for Nd measured in 5M NaCl at a molal-based pH of 9, per Kienzler et al., 2012). Thus, a toxic effect may be observed.

The solubilities of fission and activation products in brines tend to be somewhat higher; for example, Ni, Tc, and Se were determined to be soluble at 0.1 mM concentrations in brines from the Zechstein formation (Keesmann et al., 2005; Schwartz, 2012). It is possible that an inhibitory effect might be observed for some organisms at these concentrations. However, salinity has been found to enhance metal resistance in some halotolerant bacteria (Amoozegar et al., 2005), and haloarchaea have been shown to resist levels of selenite up to 30 mM (Güven et al., 2013).

III.B.2. Carbon substrates and energy sources

Prior to the loss of container integrity, the sources of carbon within the repository will be limited to connate compounds. As mentioned earlier, organics may be present in the form of native, heterogeneously distributed hydrocarbons. For example in the Gorleben salt dome, these range from light gases to C40 alkanes to aromatics and from as low as 1 ppm to over 400 ppm (Hammer et al., 2011; Pusch et al., 2014; Bracke and Fischer-Appelt, 2015). In general, increasing salinity leads to a decrease in hydrocarbon solubility, due to a salting out effect. However, many LMW aromatics (e.g. benzene, toluene) are highly soluble in brine, as are some aliphatics; while, HMW polycyclic aromatics have limited solubility in water, let alone brines.

In many cases, waste drums have been stored for decades, such that within-drum degradation of waste organics may have already occurred and may continue after emplacement but prior to brine exposure. Moisture and radioactivity would be the chief limiting factors to microbial activity within the canister. The possibility or extent of “pre-degradation” of waste organics has never been measured and is not considered in performance assessments. However, it may yield lower molecular weight compounds, such as organic acids and alcohols, which are more soluble in brine and possibly more readily degradable. “Pre-degradation” may also have generated CO_2 or methane within the drum that could be utilized by some organisms; although not likely once brine inundation has occurred and conditions become anoxic.

Once a container has been breached, repository organisms may enter the compromised container and/or contents may be released. Many low molecular weight organics (LMW: e.g., EDTA, citrate, oxalate, acetate, tartrate) were used as ligands during weapons cleanup and are included in waste inventories. Other LMW organics may include surfactants, such as tenside, or chlorinated solvents; while, HMW organics also include bitumen, graphite-containing materials, and sludges. Organic inventories in HLW may contain radiocarbon compounds generated from the spent fuel matrix and cladding (Nübel et al., 2013).

While the organic inventories of L/ILW waste canisters may be very high, their actual solubilities in brine may be much lower, and solubility is a prerequisite for bioavailability. For example, oxalate solubility in high-magnesium WIPP brine was lower than inventory-calculated concentrations, and it influenced the solubility of other LMW ligands (Swanson et al., 2013b). Solubility is a significant issue for the various HMW organics that can be found in LLW repositories (i.e. CPR). These compounds will be discussed in greater detail later.

While many microorganisms have the ability to use a broad range of substrates, not all organic carbon compounds are appropriate substrates or energy sources for all organisms. Thus, the organics present in nuclear waste may not be deemed palatable by resident organisms. Bacteria are better suited for a broader range of organic substrates, but their activity may be limited in high salt. In both cases, there are limits to degradative capability. Many haloarchaea require complex media for growth; that is, they rely on undefined components—such as amino acids, yeast extract, or peptone—that cannot be quantified easily. In natural hypersaline environments, these components may derive from the breakdown of other microorganisms or may be synthesized *in situ*. These supplements will eventually be limited by a closed repository system.

III.B.3. Inorganic nutrients and energy sources

Nitrate and phosphate are generally present in nuclear wastes from clean-up operations, and sulfate is a natural component of subterranean salt brines, given the close association of anhydrite. Phosphate will precipitate in high-magnesium brines, thus lowering its effective availability as a nutrient. High levels of certain minerals considered essential for microbial growth (e.g., Mn, Fe, Co, Ca, Zn, Cu, W, Ni) have been measured in vitrified HLW (Meleshyn and Noseck, 2012), but whether or not these will leach into the brine and become bioavailable is uncertain.

Inorganic electron donors may also be present in, or generated from, the repository waste. These include H_2 and Fe(II) from waste canister corrosion or CO_2 from microbial processes. Sulfur exists mostly as sulfate in the surrounding geology and brines; the authors are not aware if other forms of sulfur (e.g., S^0, $S_2O_3^{2-}$) have been measured in subterranean salt formations. CO_2 may be utilized by acetogens and sulfur oxidizers, and CO can be utilized aerobically by some haloarchaea.

III.B.4. Electron acceptors

Oxygen will be present immediately after repository closure until levels decrease from the corrosion of iron canisters and microbial activity. Nitrate and organic acids in the waste, and sulfate from the surrounding geology, will be present as potential electron acceptors for any nitrate-reducing or sulfate-reducing bacteria that are capable of surviving in repository brines. If canister iron is oxidized to its +3 valence state, it too may serve as an electron acceptor.

Oxidized radionuclides, such as uranium, pertechnetate, or iodate (if present in the near-field), may also serve as electron acceptors (Lovley et al., 1991; Lloyd et al., 2000; Amachi et al., 2007). Although manganese oxide is a well-documented electron acceptor, its radioactive form (present as an activation product) has not been tested in this capacity. The use of other radionuclides as terminal electron acceptors for cell respiration has not been definitively proven although it has been postulated. Only manganese and pertechnetate respiration have been shown in halophilic organisms, but both cases were under alkaline conditions (i.e., high carbonate, low sodium chloride; Khijniak et al., 2003; Sorokin and Muyzer, 2010).

IV. PREDICTED MODES OF METABOLISM IN THE REPOSITORY SETTING

Apart from the thermodynamic constraints imposed by high salt and the limitations of subsurface existence, the repertoire of potential microbial metabolic pathways within subterranean salt-based repositories may be limited even further by 1) physical confinement of the repository without input of exogenous electron acceptors (especially oxygen) and moisture (i.e., brine); 2) high ionic strength; 3) high pH (cement, MgO); and 4) inventory (e.g., nonideal substrates, lack of usable electron acceptors). These factors may restrict or effectively eliminate many capabilities. High ionic strength alone will limit activity to halophiles in an inundation scenario; therefore, only these organisms will be discussed (i.e., processes occurring between 200-300 g/L, or 3.4 to 5 M, of sodium, as depicted in Figure 3).

Each type of metabolism is reviewed below for its feasibility in a salt-based repository. **This section assumes that all other needs of the organism are being met** (e.g., nutrients, carbon/energy source) and only addresses the mode of energy conservation. It must also assume that the waste drums have been breached and have come into contact with either salt or brine. Unlike soil or sediment environments, there may not be a clear-cut order of succession after the depletion of oxygen. The modes of metabolism below are listed in one possible order of importance. Within-drum activity prior to emplacement or breach is not discussed here.

IV.A. Aerobic Respiration (dissimilatory metabolism involving oxidation of organic carbon using molecular oxygen as the terminal electron acceptor)

After repository closure and prior to inundation, aerobic respiration by haloarchaea, halophilic bacteria and fungi may occur and persist until oxygen is depleted. This will likely occur prior to brine inundation. If oxygen is still present after inundation, it will lead to a predominance of aerobic haloarchaea. Although this oxic period is projected to be the shortest in repository lifetime, it is likely to be when the majority of organic degradation takes place. Still, only those organics that are soluble or utilizable by the resident population will be degraded.

IV.B. Nitrate Reduction/Denitrification (anaerobic respiration in which nitrate serves as the terminal electron acceptor, often resulting in the formation of nitrogen gas)

Once oxygen is depleted, nitrate if present in the waste may serve as an electron acceptor. While most haloarchaea are obligate aerobes, two genera are capable of nitrate respiration (*Haloarcula* and *Haloferax*; Mancinelli and Hochstein, 1986). Only the former has been detected in subterranean halite (Norton et al., 1993). Halophilic bacteria that are able to survive in brine may also be capable of this type of metabolism, using either organics or inorganics (e.g., thiosulfate) as electron donors. Examples include *Halomonas* and *Arhodomonas* spp. (Vreeland et al., 1980; Francis and Gillow, 1993; Sorokin et al., 2011), the former having been isolated from numerous subterranean halites.

IV.C. Fermentation

Fermentation refers to the conservation of energy at the substrate level where the electron donor and acceptor are organics and may even be the same compound. This is the most probable means of energy conservation for extremely halophilic microorganisms within an anoxic, salt-based repository. LMW organics in the waste (e.g., citrate and tartrate) may serve as both electron donors and/or acceptors for fermenters. Organic waste constituents, such as cellulose, may have already undergone initial hydrolysis reactions in the drums that would yield fermentable by-products, such as glucose. Other indigenous sources of fermentable substrates are the small organics produced by halophilic bacteria for maintaining their osmotic balance in high salt (e.g., glycine betaine, glycerol, trehalose). These can serve as substrates for both haloarchaea and extremely halophilic, anaerobic bacteria (*Halanaerobiales*). This latter group is capable of fermenting a broader range of substrates, including LMW organic acids and sugars, and may be a key player in the repository, if present.

IV.D. Sulfate Reduction with Incomplete Organic Oxidation (anaerobic respiration using sulfate as electron acceptor, usually resulting in acetate build-up):

e.g., $2C_3H_5O_3^- + SO_4^{2-} \rightarrow 2CH_3COO^- + 2HCO_3^- + HS^- + H^+$

Data on bacterial sulfate reduction at high salt concentrations are somewhat conflicting. High *in situ* rates of sulfate reduction have been measured at salinities as high as 25% NaCl (Porter et al., 2007); while, most laboratory studies show a decrease in rates as salinity increases (Sorokin et al., 2011; Brandt et al., 2001; Kulp et al., 2007). Additionally, fewer substrates can be oxidized at the higher salinities, such that incomplete organic oxidation and the generation of acetate as a by-product may occur (Oren, 2011). Finally, the organisms isolated from these environments seem unable to grow when transferred back into media with the same salinity as their source. It is unknown whether this discrepancy is a function of culture bias, the presence of lower-salinity niches within the source environment, or true differences between *in situ* and *in vitro* tolerance to salt (Kjeldsen et al., 2007; Oren, 2011). It is also important to note that the above-mentioned studies investigated sediments (from brine lakes, solar salterns, salt pans). Sediment samples are extremely different from rock salt, in that they are generally richer in organics and other nutrients and are a good source of microbial inocula, especially of anaerobes.

Nevertheless, sulfate-reducing bacteria (SRB) are associated with hydrocarbon pools in salt dome caprocks (Saunders and Thomas, 1996), which emphasizes the need for further work in this area. Another matter of interest is that boron has been shown to have an inhibitory effect on SRB, such that subterranean brines (e.g., GWB from WIPP, ~158 mM borate) may be detrimental to their growth (Kulp et al., 2007).

IV.E. Methanogenesis from Methylated Amines:

e.g., $4(CH_3)NH_3^+ + 2H_2O \rightarrow 4NH_4^+ + 3CH_4 + CO_2$

Methanogenesis has drawn much attention from deep geologic repository scientists and engineers. Methane is produced via three pathways: 1) hydrogenotrophy (H_2/CO_2); 2) acetoclasticism (acetate); and 3) methylotrophy (methylated compounds, such as methanol and methylamines). Only the latter process, using methylamines, has been found to occur in hypersaline systems; while, the first two processes are thermodynamically unfavorable and have not been shown at salt concentrations above 120g/L, ~ 2M (Ollivier et al., 1998; Waldron et al., 2007; Oren, 2011).

In surficial hypersaline systems, such as salterns and brine lakes, there should be a ready source of methylamines, as they can be formed from the degradation of certain organic osmolytes generated by indigenous microbiota (bacteria, algae, etc.) for their survival in high salt. However, the abundance of methylated compounds may vary in different environments with different salt concentrations (Potter et al., 2009). Studies have shown that substrate limitation is a chief cause of low rates of methanogenesis in hypersaline settings, suggesting that concentrations of methylamines are low (Kelley et al., 2012; Kelley et al., 2015). TMA and other methylated amines are volatile and can be rapidly broken down by microorganisms under both aerobic and anaerobic conditions, which may be a reason for low concentrations in nature.

Methylated amines should provide a non-competitive substrate for methanogens in hypersaline environments, given that they are not utilized by sulfate reducers. To the authors' knowledge, no studies have looked for methylamines in subterranean salt formations.

IV.F. Homoacetogenesis

Acetate formation from $H_2 + CO_2$ or from lactate in the repository setting would be limited to a small group of anaerobic, halophilic bacteria (*Halanaerobiales*). As noted previously, these organisms are also capable of fermenting betaine, an osmolyte, to methylamines; thereby, generating a possible energy source for methanogens.

IV.G. Other Sulfidogenic Reactions

e.g., $CH_3COO^- + 4S^0 + 4H_2O \rightarrow 4HS^- + 2HCO_3^- + 5H^+$

The generation of sulfides from elemental sulfur can occur at extremely high ionic strengths. Both bacteria (*Halanaerobiales*) and haloarchaea are capable of sulfur reduction, with acetate or pyruvate as the electron donor (Sorokin et al., 2011). A novel genus, and the first and only known obligately anaerobic haloarchaeon, was recently isolated; it is capable of S^0 reduction as a low-energy form of respiration (Sorokin et al., 2016). Although mineral sulfates may be plentiful in subterranean salt settings, the existence of elemental sulfur or its polysulfide derivatives in these settings is unknown. However, this recent study suggests that there are repository-relevant archaeal metabolic processes yet to be discovered, even though they may be of low-energy yield.

IV.H. Reduction of Other Oxyanions

To the authors' knowledge, the presence of other potential electron-accepting oxyanions (e.g., selenate, arsenate, manganese oxides) in subterranean salt settings has not been investigated. Perchlorate may be a co-contaminant in nuclear wastes because of its use as a solubilizing agent. Several halotolerant bacteria and even a haloarchaeon have been shown to reduce perchlorate, but rates of reduction decrease with increasing salt concentration, and nitrate is often required (Okeke et al., 2002; Chung et al., 2009; Ryu et al., 2012).

IV.I. Metabolism Variation in Space

The variation of microbial communities in space concerns the near-field versus far-field and reflects the possible variation in ionic strength in these spaces. "Far-field" is an operational definition; for example, it may describe overlying, water-bearing formations or any area outside of the containment rock zone.

With differing ionic strength comes differing community compositions and, hence, different metabolic potential. Additionally, the far-field may contain other, natural, substrates and nutrients that could stimulate microbial activity. Depending upon the ionic strength of the far-field environment and the hypothetical release scenarios for a particular repository concept, these organisms may have a significant impact on released waste and radionuclide migration. At lower ionic strengths, the community predominance will switch from Archaea to Bacteria, although some haloarchaea may survive in ionic strengths as low as 5% NaCl, due to the ability of some to switch between coping mechanisms—i.e., "salting in" versus osmolyte synthesis or uptake (Purdy et al., 2004; Youssef et al., 2014).

As outlined earlier, the haloarchaea are restricted metabolically, such that their role in waste transformation will be limited. However, bacteria are more metabolically diverse and their dominance in the far-field may lead to significant transformation of waste in that space, should a release occur.

While degradation in the far-field will play no role in gas generation scenarios for the repositories, it may generate or further degrade ligands. Metal-reducing organisms may directly reduce radionuclides as well, leading to a less soluble oxidation state. Indirect radionuclide reduction may also occur as a result of reduced iron or sulfide formation or the creation of a reducing environment by fermenters.

IV.J. Metabolism Variation in Time and the Ability to Adapt and Evolve

As mentioned earlier, there may be no well-defined succession of modes of metabolism within a salt-based nuclear waste repository. It is presumed that, once oxygen has been depleted, haloarchaeal numbers will decrease and cells will become dormant. If bacteria survive the expected high ionic strength conditions, then anaerobic respiration may occur. Fermentation will likely proceed throughout sub- to anoxic periods. The longevity of haloarchaea entrapped in fluid inclusions or in interstitial brines is well documented; thus, they may be present long into repository history (depending upon mandated lifetime) but are not likely to be active because of unfavorable growth conditions (Norton and Grant, 1988; Mormile et al., 2003; Schubert et al., 2009, 2010).

Many have questioned whether indigenous or emplaced microorganisms will adapt over the long time frames proposed for nuclear waste repositories (10^4-10^6 years), eventually evolving the ability to survive under projected conditions. Directed evolution experiments (i.e., cell manipulation to mimic natural selection) have shown the ability of some organisms to adapt to specific stressors over time, and this may be the case for some repository organisms (Harris et al., 2009). However, these experiments rarely progress to extreme states of the stressor (e.g., adaptation to increases in salinity ≠ adaptation to hypersaline conditions). Secondly, in order to adapt and evolve, some proportion of the population must initially survive. In many cases, cells only survive because they have become dormant, not because they have mutated; such that if regrown and re-exposed to the stressor, they will exhibit the same response as their parent population. That being said, horizontal gene transfer or genetic mutation may provide the theoretical ability for organisms to evolve specific enzymatic capabilities (e.g., evolution of citrate degradation capability by *E. coli* under aerobic conditions; Bount et al., 2012). However, this is not equivalent to evolving modes of respiration (e.g., aerobic versus anaerobic respiration). Interestingly, haloarchaea are thought to have evolved from primitive methanogens via significant lateral gene transfer events from bacteria throughout geologic time (Forterre et al., 2002; Matte-Tailliez et al., 2002; Nelson-Sathi et al., 2015; Groussin et al., 2016). Still, this type of evolution occurred over a time frame much longer than a typical repository's lifetime (hundreds of millions of years), and the anaerobic capability that a haloarchaeal ancestor may have once possessed has long been lost from its genome.

V. EFFECTS OF MICROBIAL ACTIVITY ON SALT-BASED REPOSITORY PERFORMANCE

As mentioned in the introduction, microorganisms may influence radionuclide migration via: 1) complexation of radionuclides with carbon dioxide generated from the complete oxidation of waste organic matter; 2) complexation of radionuclides with other microbially-generated ligands; 3) creation of a reducing environment; 4) alteration of pH; 5) redox reactions that affect radionuclide speciation; and 6) serving as colloid vectors after taking up radionuclides. Canister corrosion is also deemed deleterious in some safety cases. Many aspects discussed in this section are specific to a particular repository concept and/or nature of the waste form and may not apply to all cases.

V.A. Oxidation of Repository Organic Matter

A wide range of organic content is possible depending upon the repository concept and nature of the waste. In LLW and TRU concepts (e.g., WIPP), very high and diverse organic content is present and is critical in modelling the impact of microorganisms on repository performance. Organic inventories in HLW may contain radiocarbon compounds generated from the spent fuel matrix and cladding (Nübel et al., 2013). These may include LMW alcohols and organic acids (Kaneko et al., 2003).

When organic waste is present, complete oxidation of these organics will result in the generation of carbon dioxide. Dissolved CO_2 may lower pH, or the resultant carbonate species can act as complexants in the pC_{H+} range of 8-10, both of which may enhance radionuclide solubility. Additives, such as MgO, may be used to sequester CO_2, thereby controlling its fugacity and buffering pH.

In order for carbon dioxide to be problematic, the microbes must be able to metabolize waste organics. As reviewed, there are few substrates present in waste that are palatable to the haloarchaea found in subterranean salt (acetate, oxalate, citrate), and this has been shown only under aerobic conditions. Halophilic bacteria are more competent degraders; however, their survival at the high salt concentrations and low water activity expected in salt-based repositories is questionable. Of the possible HMW waste forms (cellulose, plastic, rubber, and bitumen), cellulose and some LMW constituents of bitumen are the most degradable.

It is probable that waste degradation has already occurred within organic-containing canisters during storage and may continue after emplacement but prior to brine exposure. As mentioned earlier, moisture and radiation may limit the activity of emplaced organisms. The extent of within-drum “pre-degradation”, if any, is not known.

V.A.1. Cellulose degradation

Cellulose degradation is a complex process requiring the concerted efforts of many different groups of organisms, few of which are either found or would survive in hypersaline systems. Additionally, the degradation process differs between aerobic and anaerobic environments, as the organisms within those spaces utilize different mechanisms for hydrolysis (Lynd et al., 2002; Wilson, 2011).

Initial hydrolysis of cellulose is carried out by cellulolytic organisms; following this process, saccharolytic organisms and other opportunists catabolize soluble by-products. Most Fungi possess aerobic cellulolytic and lignolytic capability, but their only known anaerobic capability is in animal rumen (Lynd et al., 2002). Both aerobic (e.g. *Actinobacteria*, members of CFB) and anaerobic (*Firmicutes*, *Acidobacteria*) cellulolytic Bacteria exist.

In general, laboratory-derived cellulosics found in repository waste have been processed (e.g. paper, paper towels, Kimwipes, cardboard), although some natural and pretreated wood may also be present. In contrast, cellulose sources in nature are derived from plant matter, contain both crystalline and amorphous regions, and are likely to be more amenable to microbial attack than treated products.

Many halophilic microorganisms possessing cellulase activity or capable of growth on cellulosic substrates have been reported. For example, two fungi isolated from subterranean halite, one with documented ligninolytic capability (*Cladosporium*; Gunde-Cimerman et al., 2009), were capable of growth on Kimwipes and carboxymethylcellulose (CMC) as the sole carbon sources (Swanson et al., 2013a).

Still, of the *Bacteria* and *Fungi* capable of cellulose breakdown, most are not halophilic and are unlikely to be active in high ionic strength brines, and all are obligately aerobic. Only one anaerobic, cellulolytic microorganism has been isolated from a hypersaline environment—*Halocella halocellulolytica* (Simankova and Zavarzin, 1992; Simankova et al., 1993). This organism was able to degrade cellulose (filter paper) in concentrations of NaCl up to 20% (3.4 M). It is unknown whether any significant anaerobic cellulose degradation will occur in a salt repository near-field; however, lower salinities may permit utilization in the far-field.

In early cellulose degradation experiments carried out for the WIPP, Kim-wipes underwent a significant change in appearance, and organic acids were produced during long-term, aerobic (Vreeland et al., 1998) and anaerobic incubations (Gillow and Francis, 2006) at high salt concentrations. Interestingly, the organism that appeared across all inoculated incubations in the Gillow and Francis study, *Halorhabdus utahense*, is capable of fermenting glucose with the concomitant production of sulfide in the presence of elemental sulfur and also reduces nitrate, although not as a respiratory process (Wainø et al., 2000). Samples incubated with excess nitrate were found to generate more gas. (The presence of nitrate is often necessary for anaerobic growth of haloarchaea to occur, although it may not be used for respiration.) The only genus identified in those samples that has been found capable of denitrification was *Haloarcula* (Ichiki et al., 2001). It should be noted that these studies used a combined inoculum of salt lake water, salt lake sediment, WIPP brine, and WIPP muck pile salt. None of these haloarchaea has been detected thus far in WIPP brine or halite alone, but *Haloarcula* spp. have been found in other subterranean halites (e.g., in UK and Spain; Norton et al., 1993; Park et al., 2009).

The presence of exogenous, cellulolytic bacteria introduced in waste drums themselves cannot be ruled out and, in fact, these organisms have been detected in simulated waste pits (Field et al., 2010). If any moisture were present in the drums and if the organisms are radiation-resistant, they may have a chance to cause initial cellulose breakdown to products more easily metabolized by cellulase-producing bacteria should they come into contact with these by-products during early oxic periods. Again, these emplaced organisms are unlikely to be active in brine, although some may be halotolerant.

Cellulase-producing haloarchaea, including *Haloarcula*, *Halobacterium*, and *Halorubrum* spp., have been isolated previously from hypersaline salt lakes and salterns (Birbir et al., 2007). While these organisms are likely to thrive at high ionic strength, their use of cellulose by-products will be limited, once again, to early oxic periods. Polysaccharide-degrading capability (e.g., xylan) among haloarchaea appears to be associated with those organisms isolated from terrestrial environments, i.e. soil (Anderson et al., 2011), although not all genera have been screened.

The possibility of dramatic increases in pH from cementitious waste will likely have a greater effect on cellulose degradation than indigenous microbiota. Chemical “peeling” reactions of cellulose under alkaline conditions have been found to generate isosaccharinic acid among other organics, only described as “dissolved organic carbon”, or DOC (Pavasars et al., 2003; Glaus and van Loon, 2008). It is possible that such DOC might comprise adequate substrates for microorganisms, but the organisms would still need to be able to survive in haloalkaline conditions.

V.A.2. Plastic and rubber degradation

Plastics and rubber are even less soluble and less degradable than cellulose and are specifically designed to be resistant to degradation. Some organisms (members of the obligately aerobic Fungi and bacteria mostly of the order *Actinomycetales* with some few of the phylum *Proteobacteria*) are capable of degrading natural and even vulcanized rubber compounds (Yikmis and Steinbuchel, 2012). It is very possible that these organisms, like cellulose degraders, are present in the emplaced waste and may have already acted on these compounds. Again, this may depend upon moisture (Fungi will require less than Bacteria), radiation effects, and in the case of Fungi, oxygen. Still, it is unlikely that these organisms will be active at high ionic strengths.

V.A.3. Other carbon sources in waste

Although CPR are the predominant, potentially gas-generating organics listed in most safety cases, it is clear that other organics can be present in much of the LLW/ILW waste. These may include sludges containing chlorinated solvents used as degreasers during weapons clean-up (e.g., CCl_4) and LMW complexants, such as EDTA, tartrate, or citrate. The degradation of these compounds under hypersaline, anaerobic conditions has not been shown.

V.A.4. Native hydrocarbons

The degradation of hydrocarbons by haloarchaea has gained recent attention (Al-Mailem et al., 2010; Tapilatu et al., 2010) and should be investigated further for settings in which hydrocarbons are found. Members of the *Halobacteriaceae* (*Haloferax, Haloarcula, Halococcus, Halobacterium* spp.) were found to utilize crude oil vapor, short and medium length *n*-alkanes, and even 2-3-ring aromatics as sole sources of carbon. Additionally, specific genes encoding for enzymes involved in aromatic degradation have been found in some haloarchaea (Erdoğmus et al., 2013).

Halotolerant, hydrocarbon-degrading bacteria are well known and are frequently enriched after marine oil spills. A halophilic *Arhodomonas* sp. degraded monoaromatic hydrocarbons in salt concentrations up to 5 M (292 g/L), and other *γ-Proteobacteria* were reported to degrade LMW aromatics at salt concentrations up to 2.6 M (152 g/L). In both cases, degradation rates and extent were higher at lower salt concentrations (Sei and Fathepure, 2009; Zhao et al., 2009).

It is important to note that some of these studies were set up using hydrocarbon concentrations exceeding their solubility, were incubated in the light, were incubated at elevated temperatures, and almost always required supplementation with a complex nutrient mix. This makes it difficult to rule out precipitation, photodegradation, volatilization, or even co-oxidation in these cases. Additionally, some haloarchaea were reported to be capable of degrading HMW polycyclic aromatics that even known PAH-degraders isolated from PAH-contaminated soils are incapable of degrading (Bonfa et al., 2011). Clearly, more work is needed to elucidate hydrocarbon degradation processes by haloarchaea, especially as they might relate to expected repository conditions.

The production of surfactants may aid microorganisms in accessing hydrocarbons in brine and overcome the need to maintain a hydrophilic exterior for survival in salt, when a hydrophobic exterior is needed for hydrocarbon uptake. Biosurfactant production by a *Bacillus* sp. was found to dramatically reduce surface tension as salt concentrations increased until it stabilized between 10-15% NaCl, (1.7-2.6 M) (DaSilva et al., 2015). Two species of haloarchaea were shown to produce biosurfactants during their lag phase of growth, in response to diesel fuel exposure (Kebbouche-Gana et al., 2009).

To the authors' knowledge, no anaerobic hydrocarbon degradation studies by haloarchaea or halophilic bacteria have been documented. However, other *Arhodomonas* spp. are known to be facultatively anaerobic with nitrate as an electron acceptor, although the BTEX-degrading *Arhodomonas* was not tested for this capability (Saralov et al., 2012; Swanson et al., 2013a).

V.A.5. Bitumen degradation

Some HLW scenarios entail encasement of the waste in a bitumen matrix or using bitumen as a sealant. Additionally, bitumen has been detected in subterranean halite fluid inclusions (Siemann and Ellendorff, 2001; Kovalevych et al., 2008). Constituents of bitumen—saturated hydrocarbons, aromatics, resins, and asphaltenes (Hansen et al., 2013)—will have extremely limited solubility in high ionic strength media. Studies have shown some bitumen fractions to be degradable and to support biofilm formation (Roffey and Norqvist, 1991; Wolf and Bachofen, 1991). In one study, rates of degradation were positively correlated to the surface area of bitumen, suggesting degradation of components that were surface-accessible and/or enhanced degradation by biofilm formation (Wolf and Bachofen, 1991). It is possible that hydrocarbon-degrading, halophilic organisms present in subterranean salt (see above) may be able to degrade certain bitumen fractions. However, it is also clear that once the easily-degraded and accessible fraction is oxidized, that rates decrease significantly. The presence of ancient bitumen deposits (and ancient petroleum deposits near salt domes) and bitumen-containing fluid inclusions suggests that, clearly, not all organic carbon is mineralized.

V.A.6. Incomplete oxidation of organic carbon

Organics that are soluble (e.g., LMW ligands) may be consumed and subsequently replaced by other LMW organics going into solution. This suggests that degradation is simply an issue of time. However, assuming that all organics are soluble and degradable, not all carbon is mineralized. Some must go toward the synthesis of biomolecules and subsequent cell growth, and some may be in the form of unusable metabolites. These metabolites may serve as ligands themselves, or their buildup in a closed repository system may be detrimental to further microbial growth.

Most of the organic carbon in waste will be recalcitrant to degradation, and possibly even more so in anaerobic, hypersaline systems. For example, cellulose fibers have been preserved in reportedly ancient fluid inclusions extracted from halite. The authors suggest this recalcitrance to degradation was due to the lack of ionizing radiation, water available for hydrolysis, and microbial activity (Griffith et al., 2008).

V.B. Complexation of Radionuclides with other Microbially Generated Ligands

Apart from the ligands produced as waste metabolites, microorganisms can generate other organic and inorganic ligands. Bacterial mineralization of uranium via phosphate, as a detoxification mechanism, may result in precipitation and impede migration (Plummer and Macaskie, 1990; Tolley et al., 1992). Phosphate may also serve as a "nucleation focus" on cell surfaces (Lloyd and Macaskie, 2002). A possible association of uranium with phosphate produced by a haloarchaeon has been suggested (Francis et al., 2004).

Sulfides are also potential radionuclide ligands. Sulfidogenesis (from sulfate, thiosulfate, or elemental sulfur) decreases with increasing salinity; however, it has been shown in incubations of hypersaline sediments, resulting in enrichment of known SRB from the *δ-Proteobacteria*, as well as *Halanaerobiaceae* (Sorokin et al., 2011). Sulfide from elemental sulfur was generated at the highest salinities by a novel haloarchaeon (Sorokin et al., 2015).

Most bacteria can produce siderophores in response to iron-limiting conditions. In contrast, five of seven tested haloarchaeal genera produced only carboxylate siderophores (Davé et al., 2006); while the genus *Halobacterium* was only able to use exogenous siderophores generated by other organisms (Hubmacher et al., 2002). Three haloarchaea isolated from saline soils were found to contain genes for siderophore synthesis, although the ability was not tested (Anderson et al., 2011). Presumably, the waste-emplaced organisms originating from soil can produce siderophores, but again, their activity is uncertain.

V.C. Creation of a Reducing Environment

Creating a reducing environment may lower the oxidation state of certain multivalent radionuclides, thereby altering their solubility. Microorganisms can do this by consuming oxygen or generating hydrogen or other reductants (e.g., Fe^{2+}, sulfides). Canister corrosion is also expected to be a main contributor to hydrogen generation.

Again, in order for microorganisms to change the redox environment, they must be actively respiring or fermenting. Haloarchaea are, for the most part, obligate aerobes; thus, utilization of oxygen will occur, if all other conditions for growth are met. Once conditions become sub- to anoxic, fermentation reactions will drive the production of hydrogen in this setting. In nature, hydrogen generation is usually coupled with H_2-utilizing organisms. Of those, only acetogens (producing acetate from $H_2 + CO_2$) have been shown to survive at extremely high salt concentrations. There are no documented hydrogen gas-metabolizing or generating haloarchaea (Schwartz et al., 2012). Fermentation is likely to be a dominant form of metabolism occurring in salt-based waste repositories after oxygen is utilized, and it is also likely that fermentation of waste components will occur within L/ILW canisters, if all other conditions for survival are met.

V.D. Alteration of pH

Changes in pH due to microbial activity can affect radionuclide solubility. The generation of CO_2 and organic acids from waste degradation will lower pH and also complex radionuclides. The addition of MgO will buffer the pH of inundating brine by inducing precipitation. In general however, the solubility of CO_2 in brine decreases with increasing salinity. Changes in pH may also result from hydrogen generation via fermentation, if no concomitant microbial consumption of hydrogen occurs.

V.E. Redox Reactions with Iron and Radionuclides

Microorganisms may influence radionuclide oxidation state (and hence, solubility) indirectly or directly by controlling the redox potential in their immediate environs, generating iron(II), hydrogen, or hydrogen sulfide, or via enzymatic reduction. There are very few data concerning metal reduction in hypersaline environments (Sorokin and Muyzer, 2010; Emmerich et al., 2012). This is likely due to the low solubility of oxidized metal species in these systems; thus, the data are generally limited to lower ionic strength systems, insoluble metal oxides in sediments, or metals associated with particulate organic matter or microbial mats.

An association has been suggested between a haloarchaeon (*Halobaculum gomorrense*) and bacteria (*Virgibacillus* and *Pontibacillus* spp.) in hypersaline sediments showing iron reduction, but this has not been elucidated (Emmerich et al., 2012). Iron reduction by SRB and other fermenters was also shown, and fermentation was presented as a possible mechanism used by the bacterial-archaeal enrichment. In a separate study, incubations of hypersaline groundwater under iron-reducing conditions also yielded cultures of *Virgibacillus* and *Pontibacillus* spp., along with a haloarchaeon, *Haloferax* (Swanson et al., 2013a).

The reduction of metals occurs primarily as a result of bacterial activity; haloarchaea have not been shown to directly reduce metals. However, metal-reducing bacteria may or may not be active in the repository near-field due to the high ionic strength. The potential for redox reactions to occur within L/ILW waste drums is unknown but is, as mentioned previously, most likely limited by the absence of water.

If abiotic oxidation of zero-valent canisters occurs, then iron-oxidizing microorganisms may contribute further to corrosion by generating reactive ferric oxides. Biotic ferrous iron oxidation occurs under aerobic, microaerophilic, and anaerobic conditions, often concomitantly with nitrate, perchlorate, or chlorate reduction (Weber et al., 2006). Nitrate-dependent iron oxidation occurs in a variety of environments, including marine sediments, soils, and sludges, and is thought to lead to transient redox fluctuations that might also affect radionuclide solubility (Benz et al., 1998). Iron oxidizers have been detected in hypersaline lake sediments, but their level of activity could not be established (Emmerich et al., 2012); thus it is unknown whether they will play a role in salt-based repositories.

As mentioned earlier, bioreduction of radionuclides as an anaerobic respiratory process has only been shown for uranium and technetium, but reduction of other radionuclides occurs nonetheless, either advertently as a detoxification mechanism or inadvertently in response to normal cellular processes. Pertechnetate was reduced by a *Halomonas* sp. isolated from a hypersaline soda lake (Khijniak et al., 2003), but this is the only documented case of direct radionuclide reduction by a halophilic bacterium.

V.F. Biocolloid Vectors

V.F.1. Radionuclide uptake

Uptake of radionuclides may be extracellular—as in surface sorption or nucleation and precipitation—or intracellular, via active or passive transport. If mobilized, microorganisms may contribute to radionuclide migration; if immobilized, microorganisms may become a radionuclide “sink”.

Although few surface sorption studies have been conducted at high ionic strength, significant progress has been made in this area (Ams et al., 2013; Reed et al., 2013; Bader et al., submitted). Surface sorption of metals occurs through their interaction with anionic functional groups in cell walls. This phenomenon has been shown to be dependent upon many factors, including pH; radionuclide speciation; organism type (bacterium versus archaeon) and biomass concentration; ionic strength of the test matrix, especially magnesium or calcium concentrations; and the presence or absence of strong complexants, such as EDTA. In general, increases in pH initially result in increased metal sorption, when controlled for precipitation and carbonate complexation, presumably from the increased availability of negatively charged sites on the surface of the cell coupled with a decrease in possible competition by cations, such as magnesium. However, once radionuclide speciation becomes predominantly anionic (e.g., due to complexation or hydrolysis), a decrease in sorption may be observed. Thus, cell surface-radionuclide interactions are not always straightforward.

Bacteria have been shown, in some studies, to adsorb more than Archaea (Reitz et al., 2011), presumably due to differences in cell wall characteristics, especially S-layer structures. However, this may not hold true for all Archaea. Neptunium sorption onto a halophilic bacterium in sodium perchlorate media increased with increasing ionic strength, thought to be due to increased Np(V) ion activity at higher ionic strength (Ams et al., 2013). This ionic strength dependence did not hold for complex, magnesium-containing brines, due to competition with Mg for cell surface sorption sites and potential changes in Np speciation due to inorganic complexation (e.g., with sulfate, carbonate, or borate).

By convention, sorption experiments are generally conducted with resting cells rather than actively growing cells. In hypersaline settings, movement of ions across cell membranes occurs regardless of cell growth (especially for organisms that “salt-in”), in order for the organisms to maintain osmotic balance. It is unknown whether this phenomenon has any effect on “uptake”.

The presence of strong complexants, such as EDTA, or other organic ligands may significantly decrease the extent of adsorption across a broad pH range. EDTA also has a “rounding up” effect on microorganisms that can lead to “shedding” of external layers (e.g., EPS) and alter surface area (and surface area/volume ratios), all of which can affect the extent of uptake.

V.F.2. Biomass dependence

Once again, biomass concentrations will be constrained by repository conditions and thermodynamics. Additionally, biomass concentrations do not increase in perpetuity in closed systems. They will reach a maximum, based on substrate and nutrient supply, in addition to the formation of inhibitory by-products. Haloarchaeal cultures growing under optimum aerobic conditions can reach ~10^9 cells/mL. The reality in a salt-based repository setting is likely to be significantly fewer numbers, given a poor starting inoculum, less than ideal conditions, and an anaerobic atmosphere.

Adsorption of metals onto bacterial surfaces does not necessarily depend upon cell viability. Studies using dead archaeal biomass have not been conducted. The constant flow of osmotic-stabilizing ions across the membranes of live haloarchaeal cells may lead to a difference between uptake into or onto dead cells.

V.F.3. Biomass mobility

Motility can be used by organisms to move toward an attractive substance or away from a noxious one. Many haloarchaea possess flagella for motility and are capable of swarming. In addition, gas vesicle formation is used appreciably by these organisms in order to move vertically through the water column, presumably to areas of higher oxygen concentration or nutrient load. Still, a sessile existence as a biofilm is of more benefit to microorganisms in that it allows them to retain resources while offering protection from the external environment.

Although cells are predominantly found attached to surfaces in nature, very little research has been conducted on biofilm formation by halophilic microorganisms, especially haloarchaea (Fröls et al., 2012). That being said, over half of the strains tested by this group were able to adhere to surfaces using pili and to form biofilms. A tested *Halobacterium* sp. formed biofilms up to 80 µm thick but was outcompeted for surface area by an introduced *Halorubrum* sp. (DiMeglio et al., 2014).

Given the self-sealing nature of salt and the assumption that organisms remain near a substrate source (i.e., waste), significant movement away from a salt-based repository can theoretically only take place during a pressurized release of brine and repository contents, such as during an intrusion scenario. In this case, inherent motility is not important, as the organisms will be entrained in a pressurized brine flow. However, lysis becomes a realistic possibility, as many haloarchaea will lyse with decreasing ionic strength or as pH's reach extremes. For instance, the Austrian *Halobacterium noricense* isolate cannot survive below 2.1 M NaCl (Gruber et al., 2004); while, the lower limit for the US (WIPP) isolate is lower (~1.4 M). However, there was a significant lag period (8 weeks) before lysis occurred, suggesting that this organism has potential as a vector for radionuclide transport. These values should be determined for salt-indigenous isolates, in relation to the ionic strength of the surrounding groundwaters. If lysis occurs, the sorbed actinide may or may not precipitate with cell walls; this needs to be verified experimentally. Moreover, this lysis phenomenon may not occur with halophilic bacteria if the change in salt concentration is gradual, although it may occur at pH extremes.

V.G. Emplaced Organisms

It is probable that radionuclides have already been taken up (either internally or externally) by emplaced organisms within waste drums. The fate of these organisms is unknown during brine inundation. However, preliminary studies on a WIPP TRU waste isolate, *Arthrobacter* sp., have shown that it can survive intact in both GWB and ERDA brines for at least one month and can recover when subcultured back into lower ionic strength media (Swanson, unpublished). This suggests that any radionuclides attached to, or taken up by, this organism may remain so and migrate during a pressurized brine release. Furthermore, it is highly likely that there are spores within waste drums; these may also remain intact during inundation. It is unknown how many such cells are present in TRU waste.

V.H. Microbially Induced Canister Corrosion

The relative importance of canister corrosion in repository performance may be viewed differently by different regulatory agencies and safety cases. In some concepts (e.g., WIPP), the corrosion process is expected to be self-limiting as available water is completely consumed, and the generation of hydrogen, as well as reduced iron phases or species, is viewed as a benefit, since it establishes a reducing environment in which radionuclide solubility is generally not favored (SOTERM, 2014). Additionally, canisters are expected to be crushed by salt over time, but the self-sealing salt will prevent radionuclide release. In other regulatory concepts, canister integrity is a significant aspect of performance assessments. For example, corrosion may lead to the release of gaseous (e.g., ^{14}C as $^{14}CO_2$) and long-lived radioactive activation products, especially from high level irradiated fuel assemblies and vitrified waste (Meleshyn and Noseck, 2012).

In either case, the chemical contribution to canister corrosion is predicted to greatly outweigh the microbial contribution, and there is still uncertainty surrounding the latter in high ionic strength systems. In abiotic, humid experiments with CO_2 added to simulate microbial activity in TRU waste, the corrosion rates of iron and steel coupons were negligible (Roselle, 2013). Hydrogen generation by fermenters and hydrogen sulfide generation by SRB and other sulfidogens may play a role in canister corrosion, but the presence and activity of these organisms, as discussed previously, is uncertain (Telander, 1993). For HLW scenarios, the cycling of heated brine/water vapor may accelerate corrosion (Stauffer et al., 2012), but microbial activity may be inhibited by temperature until later in repository history. It is unknown whether the radiation field at the canister surface will be inhibitory; microbial biofilms have been observed on irradiated spent nuclear fuel cladding (Bruhn et al., 2009), although not in brine.

VI. INCORPORATING MICROBIAL PARAMETERS INTO A REPOSITORY PERFORMANCE ASSESSMENT/SAFETY CASE: CASE STUDY OF THE WASTE ISOLATION PILOT PLANT

VI.A. Model Assumptions

Along with geochemical and geophysical parameters, the potential for microorganisms to influence repository performance must be incorporated into a safety case model (e.g., gas generation, biocolloids). Generally, several assumptions must be made about repository conditions that allow for model incorporation. These assumptions can generate simplified, yet "worst-case", scenarios, thereby building significant conservativism into the model. They may also vary depending upon the intended purpose of the repository (e.g., low versus high-level waste, mandated lifetime).

In the WIPP case, a first assumption is that the repository horizon will eventually become a homogeneous "soup", where all waste components are equally accessible to all organisms. This assumption must be made for purposes of modeling solution chemistry. In reality, unless a repository becomes inundated with brine, there will more likely be localized areas of brine seepage and most waste components will be inaccessible to organisms. Microbial activity, if any in these pockets, presents a large uncertainty and is likely to be much lower than conservatively predicted.

A second assumption is that all basic requirements for microbial survival are optimally met, such that all organisms are viable and active, all types of metabolism will occur, all waste organic matter, and all indigenous carbon sources will be mineralized to carbon dioxide with no apparent contribution to biomass. This assumption has the greatest impact of all on the projected influence of microorganisms in salt-based nuclear waste repositories. Repository conditions, microbial ecology, and energy conservation in high salt have been reviewed here and suggest, again, that model assumptions of activity and capability are extremely optimistic.

A third assumption is that all organisms will take up actinides (either internally or externally; whether alive or dead), and all organisms are mobile. Again, this assumption lends conservativism to a safety case/performance model. Unlike gas generation, however, more data can easily be generated to address this supposition.

VI.B. Incorporated Model/Safety Case Parameters at the WIPP

VI.B.1. Gas generation

It is unknown whether microbial gas generation under realistic, near-field repository conditions can ever be shown. Numerous attempts to do so have failed, but input for performance assessments is necessary. In order to generate input, experiments must be manipulated beyond realistic repository conditions, thus resulting in optimistic and conservative estimates of gas generation. WIPP currently uses gas generation data obtained from experiments using a rich inoculum containing brine lake sediments (Gillow and Francis, 2006).

VI.B.2. Biocolloid contribution

Biocolloid formation is a highly variable process and, therefore, difficult to model. If models are based on toxicity data, then uptake by dead organisms must be ruled out. If models are based on actual uptake, then biomass concentration becomes the chief influencing factor. Biomass concentrations measured under optimum growth conditions will, once again, provide a conservative value for the model. The values used for biocolloid transport in the WIPP model are based on the concentration of actinide at which no cell growth was observed during toxicity testing of a *Halomonas* sp. exposed to various actinide-ligand complexes. Such values should be acceptable if the test organisms are the most relevant to the tested space (e.g., near- versus far-field), and the testing is conducted under expected repository conditions.

VI.B.3. Near- versus far-field modeling

Hydrology testing in the overlying Culebra has been ongoing since the inception of the WIPP, with the justification that those water-bearing formations “could potentially transport wastes to the biosphere if the proposed facility were breached” (Mercer, 1983).

Given the certainty of microbial activity in the far-field environment, safety cases should consider the far-field space as a unique “transformation zone”. This requires knowledge of the microbial communities in surrounding groundwaters or water-bearing formations. While gas generation will be difficult and probably unnecessary to model under these conditions, organic transformation, radionuclide reduction and other redox changes, and biocolloid transport can be tested.

VII. MICROBIAL ISSUES LACKING SUFFICIENT DATA

VII.A. Presence of Sulfate Reducers and other Sulfidogens

The generation of sulfide and the precipitation of dolomite are properties of sulfate-reducing bacteria that could be beneficial to salt-based nuclear waste repositories, in that they can lead to carbon dioxide or radionuclide sequestration. While sulfate reduction has been deemed thermodynamically feasible at high salt concentrations, sulfate-reducing bacteria (SRB) have yet to be isolated or detected in subterranean halites, despite their frequent association with anhydrite and gypsum. However, the presence of oil deposits and high concentrations of hydrocarbons in domal salt formations (e.g., Gorleben) suggests that the growth of SRB may be supported. DNA sequences belonging to SRB were detected in groundwater overlying the WIPP, but none have been isolated. Sulfate reduction has also been reported for the aquifers overlying Gorleben, with up to 200 g/L of total dissolved solids (Klinge et al., 2007).

Sulfidogenesis, unrelated to SRB, may still occur. Fermentation of organics by anaerobic, halophilic bacteria (*Halanaerobiales*) has been shown in the WIPP setting to cause enough change in redox potential to yield sulfide formation via iron reduction (Swanson et al., 2013a). Precipitation of dolomite has been shown at high salt concentrations by both SRB and a haloarchaeon (Deng et al., 2010; Kenward et al., 2013). Whether or not radionuclides can be sequestered in these minerals remains to be tested, but this type of biomineralization has been shown for calcite (Lauchner et al., 2013).

VII.B. Presence of Methylated Amines and Methanogenesis

Methanogenesis from methylated amines has been measured at concentrations of 250 g NaCl/L, 4.3 M (Zhilina, 1986). These compounds are omnipresent in marine settings and are often derived from the solutes utilized by marine organisms, such as cyanobacteria and algae, to maintain their osmotic balance. Methylated amines provide a noncompetitive substrate for methanogens, i.e. one that is not routinely utilized by sulfate-reducing bacteria, allowing for the coexistence of both groups. Methylated amines tend to be volatile and may have disappeared during the formation of evaporite salt beds. Still, it would be prudent to test for the presence of these compounds in subterranean brines and in overlying groundwaters.

VII.C. LLW/TRU versus HLW

The majority of US research to date has focused on TRU waste. The European safety case approaches salt-based repository design from the perspective of both high and low level waste, and the US is now considering salt for defense HLW and commercial spent fuel waste disposal.

The major factors leading to the inhibition of microbial activity in HLW-salt repositories (apart from those of LLW) are higher temperatures and radioactivity. Both of these areas require further research to determine whether microbial effects on HLW will occur. Repository concepts in which both waste types are present but not segregated should also be addressed for microbial impact.

VII.D. Radionuclide Toxicity

Since most microbial impacts are dependent upon cell viability, further investigation is warranted into the toxic or radiative effects of radionuclides on salt-indigenous microorganisms. Additionally, studies are warranted on available waste organisms and far-field organisms, as they may play a role in waste transformation prior to repository emplacement and in the far-field, respectively.

VII.E. Microbial Interactions with Barrier Components

Many repositories use clay and cement as backfill, and salt-based repositories may also use crushed salt or salt/clay mixes. To our knowledge, no work has been done to investigate the impact of salt-indigenous microorganisms on such barrier materials or the impact of the materials on the microorganisms, or the influence of clay-associated microorganisms under high ionic strength conditions.

VII.E.1. Clay

Clay mixed with crushed salt has been investigated as a potential backfill material for waste repositories (Popp et al., 2013). Additionally, clay is found naturally in seams in subterranean salt formations. The heterogeneity, lack of pore space and interconnectivity, moisture, and aeration within clays constrain possible microbial activity and survival. Still, microorganisms have been isolated from and biosignatures have been detected in several subsurface clay formations (Mauclaire et al., 2006; Stroes-Gascoyne et al., 2007; Poulain et al., 2008; Lopez-Fernandez et al., 2015). These include clays being considered for, or intended for use in, deep geologic repositories (e.g. Opalinus and Boom clays). The organisms isolated and detected are dominated by *Firmicutes* and *Actinobacteria*, but also include *Proteobacteria*. It is hypothesized that these organisms are mostly in a dormant state but that perturbations, such as excavation and transfer to a repository, could revive them (Stroes-Gascoyne et al., 2007). Most of the cultivated organisms were derived from areas exposed to moisture (walls and faults; Boivin-Jahns et al., 1996; Urios et al., 2012).

Sulfate-reducing organisms (*Desulfovibrio* sp.) have been isolated from incubations of MX-80 bentonite, a proposed backfill material (Masurat et al., 2010). The survival of clay-derived organisms at high-salt concentrations remains to be investigated, but the *Desulfovibrio* in this study were only capable of survival at 4% NaCl (0.7 M). Aerobic incubations of argillaceous halite samples from the WIPP resulted in less diverse cultures than those of near-pure halite, possibly due to effects of clay-associated impurities, but whether or not this will be the case when large amounts of exogenous clay are introduced is unknown.

Investigations of porosity and compactibility of wetted salt/clay (85%/15%) mixtures showed enhanced compactibility with a dramatic decrease in permeability (Popp et al., 2013). This may serve to confine microbial activity to any remaining pore space, provided all conditions are optimal.

Clays have the potential to adsorb radionuclides, even in hypersaline conditions, (Schnurr et al., 2015). It is unknown whether this leads to an enhanced toxic effect on any surviving microorganisms in the vicinity or whether the sorbed radionuclides become biologically unavailable. Studies at low ionic strength have found that Pu and U sorb preferentially to *Bacillus subtilis* cells rather than clay particles (Ohnuki et al., 2005, 2007).

VII.E.2. Cement

The presence of cement may raise the pH of any inundating brine to extremely alkaline levels that may not support microbial life. This will alter the brine chemistry as well as waste organics, specifically cellulose. Cement inventories vary throughout repository concepts.

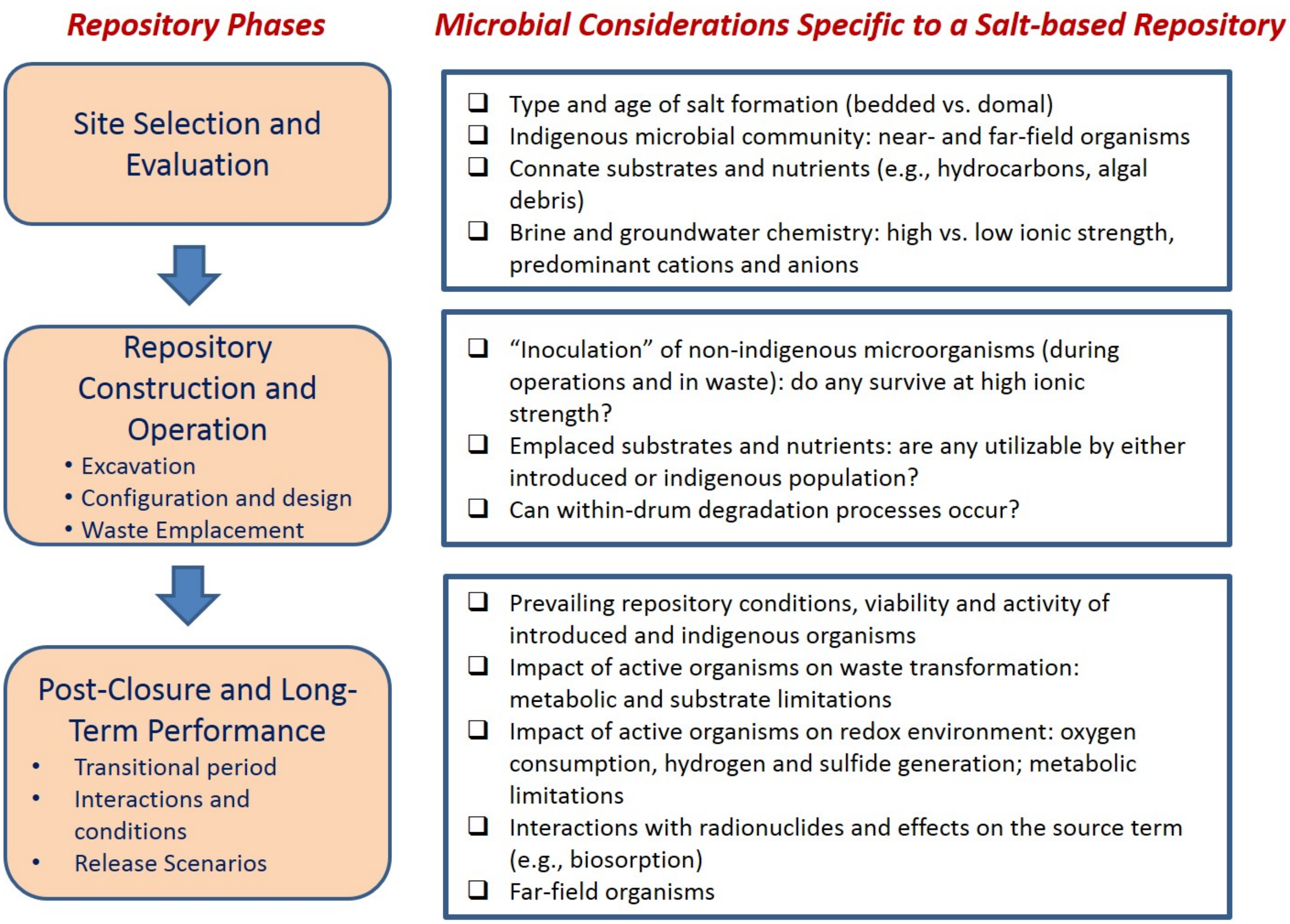
As discussed earlier, at high enough pH (~12-13), cellulose can undergo abiotic degradation to utilizable glucose monomers and subsequently to lower molecular weight ligands, such as isosaccharinic acid (Pavasars et al., 2003). The presence of glucose at neutral pH could encourage the growth of fermenters (e.g. *Halanaerobiaceae*), but it is unknown whether these organisms will be viable under alkaline conditions this extreme. *Halanaerobium hydrogeniformans*, isolated from a soda lake, is capable of cellobiose degradation at pH 11, but other *Halanaerobium* spp. have circumneutral pH optima (Begemann et al., 2012). Whether or not haloalkaliphilic ISA-degrading organisms exist is unknown, but alkaliphilic degraders have been isolated from contaminated alkaline sediments (Bassil et al., 2014).

VIII. CONCLUSIONS

In order to affect repository performance, microorganisms must be present and, in most cases, active. Subterranean salt settings contain a unique community of microorganisms with limited metabolic capacity. The additional constraints of repository conditions and waste and barrier constituents suggest that the overall effect of such microorganisms may be severely limited in the near-field.

In contrast to other deep geologic settings, the usual assumptions about microbial processes cannot always be made. The chief questions for a salt-based repository concept are: will anything survive and, if it does, will it do anything? In light of this difference, Figure 1 can be revised as shown in Figure 5.

Although they cannot be incorporated into a performance model, the negative results obtained when trying to grow salt-indigenous organisms under repository conditions should be viewed as meaningful. These negative findings can be supported by community characterization studies and genome sequencing to determine the feasibility of microbial activity under given conditions. Areas in which data can be generated include any microbe-radionuclide interaction studies. Due diligence in all these areas can help mitigate the remaining uncertainty surrounding the effects of microorganisms on salt-based nuclear waste repositories.



Repository Phases

Microbial Considerations Specific to a Salt-based Repository

Site Selection and Evaluation

- ☐ Type and age of salt formation (bedded vs. domal)
- ☐ Indigenous microbial community: near- and far-field organisms
- ☐ Connate substrates and nutrients (e.g., hydrocarbons, algal debris)
- ☐ Brine and groundwater chemistry: high vs. low ionic strength, predominant cations and anions

Repository Construction and Operation

- Excavation
- Configuration and design
- Waste Emplacement

- ☐ "Inoculation" of non-indigenous microorganisms (during operations and in waste): do any survive at high ionic strength?
- ☐ Emplaced substrates and nutrients: are any utilizable by either introduced or indigenous population?
- ☐ Can within-drum degradation processes occur?

Post-Closure and Long-Term Performance

- Transitional period
- Interactions and conditions
- Release Scenarios

- ☐ Prevailing repository conditions, viability and activity of introduced and indigenous organisms
- ☐ Impact of active organisms on waste transformation: metabolic and substrate limitations
- ☐ Impact of active organisms on redox environment: oxygen consumption, hydrogen and sulfide generation; metabolic limitations
- ☐ Interactions with radionuclides and effects on the source term (e.g., biosorption)
- ☐ Far-field organisms

Figure 5: Repository phases and microbial issues to be considered for salt-based repositories.

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